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Cell Metabolism
Review

Role of Endothelial Cell Metabolism in Vessel Sprouting

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Endothelial cells (ECs) are quiescent for years but can plastically switch to angiogenesis. Vascular sprouting relies on the coordinated activity of migrating tip cells at the forefront and proliferating stalk cells that elongate the sprout. Past studies have identified genetic signals that control vascular branching. Prominent are VEGF, activating tip cells, and Notch, which stimulates stalk cells. After the branch is formed and perfused, ECs become quiescent phalanx cells. Now, emerging evidence has accumulated indicating that ECs not only adapt their metabolism when switching from quiescence to sprouting but also that metabolism regulates vascular sprouting in parallel to the control by genetic signals.

Blood vessels arose in evolution for various reasons. First and foremost, they supply oxygen, nutrients, and growth factors to tissues while draining toxic metabolic waste. They also ensure immune surveillance, thus allowing immune cells to patrol the organism for foreign antigens or invaders. Interestingly, vessels are evolutionarily closely associated with organismal metabolism. Indeed, in primitive invertebrates, blood vessels were initially hollow matrix tubes that were not lined by endothelial cells (ECs), thus allowing only slow, sluggish, turbulent blood flow and limited tissue perfusion (Muñoz-Chápuli et al., 2005). Only when organisms required a more rapid metabolism (for instance, to predate) did vessels become lined by ECs in order to establish faster laminar blood flow and more efficient perfusion (Muñoz-Chápuli et al., 2005). However, how ECs rewire their own metabolism when switching from quiescence to vascular branching and whether such metabolic adaptations affect vascular branching remain much less studied.

ECs are highly plastic cells and can rapidly switch from a long-term quiescent state to active growth upon stimulation by hypoxia or growth factors. According to the prevalent model of vascular sprouting (Potente et al., 2011), an endothelial tip cell takes the lead by navigating at the vascular forefront. Following the tip cell, endothelial stalk cells elongate the branch by proliferating, whereas endothelial phalanx cells line quiescent perfused vessels. The process of tip and stalk cell differentiation is under the tight control of VEGF and Notch signaling and other genetic signals (Potente et al., 2011). VEGF promotes tip cell induction and filopodia formation and induces the expression of the Notch ligand Delta-like 4 (DLL4), which activates Notch signaling in neighboring cells and thereby suppresses VEGF receptor 2 (VEGFR-2) expression and tip cell behavior (Figure 1A). Tip and stalk cells do not exhibit permanently fixed cell fates but dynamically switch between tip and stalk cell phenotypes (Jakobsson et al., 2010). In a matter of hours, a tip cell that lacks the fitness to compete for the leading position can be overtaken by a stalk cell, which then acquires a tip position. This mechanism may ensure that vessel branching relies on the fittest cells. However, little is known about the different metabolic characteristics and requirements of these various EC subtypes and whether Notch controls metabolism in ECs. First, we will overview our current understanding of the various metabolic pathways in ECs, and then we will discuss how these pathways regulate vessel sprouting, illustrating a major role for glycolysis in this process.

Metabolic Pathways in Endothelial Cells

Glucose Uptake and Transport

Glucose delivery to peripheral organs occurs via paracellular transport as well as a transcellular route. In fact, only a small fraction of the glucose that is taken up by ECs is phosphorylated for further internal metabolization. ECs take up glucose through facilitated diffusion, an energy-independent process facilitated by glucose transporters (GLUT), mainly by GLUT-1. VEGF increases GLUT-1 expression in ECs through the activation of PI3K-AKT signaling (Yeh et al., 2008). Reduced GLUT-1 levels in ECs decrease glucose uptake in peripheral organs (Huang et al., 2012). In humans, impaired glucose transport across the blood-brain barrier due to GLUT-1 mutations causes the glucose transporter protein syndrome, which is characterized by infantile seizures, developmental delay, and microcephaly (Klepper et al., 1999). GLUT-1 mutations have also been linked to learning disability and Alzheimer's disease (Guo et al., 2005; Shulman et al., 2011). In ECs of intact coronary arteries, glucose is taken up at the periphery of the cell and accumulates close to cell-to-cell junctions, where the majority of glucose transporters are anchored. This compartmentalization of glucose produces a concentration gradient between the cytosol and the interstitial space that might facilitate transcellular transport of glucose (Gaudreault et al., 2008).

Divergent effects of insulin on glucose uptake and metabolism in ECs have been reported (Artwohl et al., 2007; Gaudreault et al., 2008; Gerritsen et al., 1988; Wu et al., 1994). Insulin signaling and insulin-induced phosphorylation of endothelial nitric-oxide synthase (eNOS) in ECs control glucose uptake via skeletal muscle cells (Kubota et al., 2011). The vascular effects of insulin rely on the production of nitric oxide (NO), which promotes capillary

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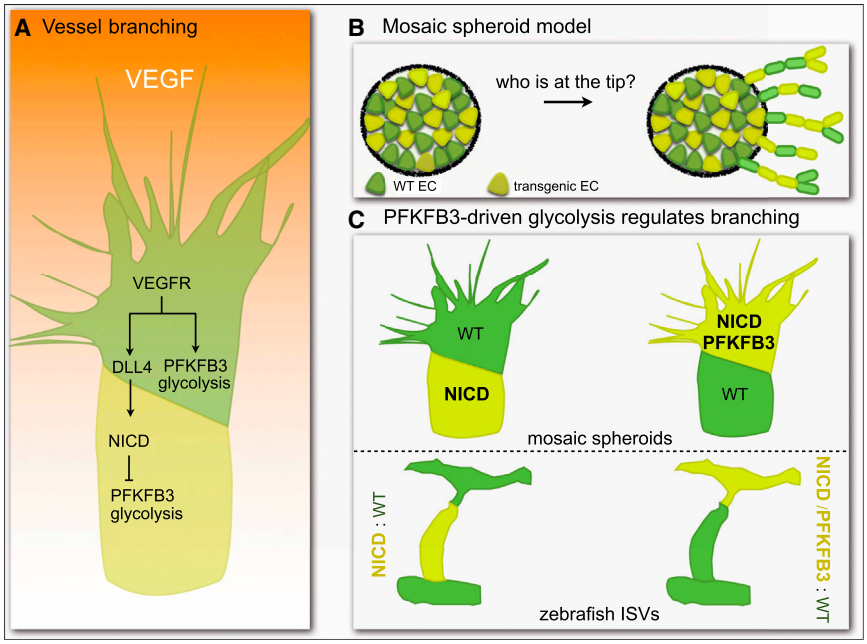

Figure 1. Metabolic Fitness Influences Vessel Branching

(A) A schematic of a tip (green) and a stalk (yellow) cell in a vascular sprout. The tip cell extends numerous filopodia in order to sense the environment. In response to the VEGF gradient (orange), the tip cell upregulates Notch ligand Delta-like 4 (DLL4), which activates Notch signaling in the stalk cell and promotes stalk cell formation by downregulating VEGFR-2. The activation of VEGFR signaling by VEGF upregulates PFKFB3 levels and glycolysis, whereas Notch intracellular domain (NCID, active Notch) lowers PFKFB3 expression and glycolytic flux.

(B) A schematic of mosaic spheroid assay, in which ECs compete for the tip cell position when forming vascular sprouts. By using spheroids containing ECs of two different genotypes, it is possible to determine which EC is at the tip cell position.

(C) A schematic illustrating a tip and stalk cell in a vascular branch of a mosaic spheroid (top) and zebrafish intersomitic vessels (ISVs, bottom) showing that, in comparison to wild-type (WT) cells (green), cells overexpressing NICD (yellow cell, left) are more frequently present in the stalk. However, when NICD-overexpressing cells also overexpress PFKFB3, these cells can compete again for the tip position (yellow cell, right). Thus, PFKFB3 overexpression overrules the stalk-cell-inducing activity of NICD.

recruitment, vasodilation, and perfusion, altogether enhancing glucose disposal in skeletal muscle (Muniyappa and Quon, 2007). Insulin also signals in ECs in order to facilitate its own transendothelial transport to perivascular organs (Barrett and Liu, 2013; Kubota et al., 2011).

Endothelial Cells Are Addicted to Glycolysis

After glucose is taken up inside the cell, it is metabolized to pyruvate in the glycolytic pathway (Figure 2). ECs line blood vessels and have immediate access to oxygen in the blood, which could promote mitochondrial respiration. Nonetheless, most studies report that ECs do not rely on oxidative metabolism but are highly glycolytic, generating more than 80% of their ATP in this pathway (Culic et al., 1997; De Bock et al., 2013; Krützfeldt et al., 1990). In the presence of physiological glucose concentrations, only <1% of pyruvate generated in glycolysis is oxidized in the tricarboxylic acid (TCA) cycle (De Bock et al., 2013). However, when glucose and glycolysis levels drop, the oxidation of glucose (as well as of palmitate and amino acids) is enhanced, indicating that ECs switch to oxidative metabolism when anaerobic glycolysis is impaired (known as the Crabtree effect) (Krützfeldt et al., 1990).

ECs increase their glycolytic flux when switching from quiescence to proliferation and migration (De Bock et al., 2013). In pathological conditions, such as pulmonary hypertension or latent infection with Kaposi's sarcoma-associated herpesvirus, glycolysis is increased while oxygen consumption is reduced in ECs (Delgado et al., 2010; Fijalkowska et al., 2010). Thus, ECs metabolically resemble other rapidly proliferating healthy and malignant cell types (Dang, 2012; Marelli-Berg et al., 2012; Mullen and DeBerardinis, 2012; Vander Heiden et al., 2011). Consequently, reducing glycolysis by silencing phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3), which generates fructose-2,6-bisphosphate, a potent allosteric activator of phosphofructokinase-1 (PFK1), impairs EC proliferation, migration, and vascular sprouting in vitro (De Bock et al., 2013). Also, the genetic deficiency of PFKFB3 in ECs causes vascular hypobranching in mice (De Bock et al., 2013).

Similar to fibroblasts (Lemons et al., 2010; Valcourt et al., 2012), ECs have substantial baseline glycolysis levels when they are quiescent and only double their glycolysis flux when they are activated to divide and migrate (De Bock et al., 2013). Accordingly, when vessel sprouting is stimulated in hypoxic conditions, ECs enhance glycolysis by no more than 50% (Dobrina and Rossi, 1983). Thus, ECs differ from immune cells, which have negligible glycolysis in their quiescent nonactivated state and upregulate glycolysis by 20- to 30-fold upon activation (Frauwirth et al., 2002; Wang et al., 2011b). Rather, like quiescent fibroblasts (Lemons et al., 2010; Valcourt et al., 2012), ECs need a high baseline glycolysis level for homeostatic maintenance, and blocking glycolysis by 80% by 2-deoxy-D-glucose is toxic for these cells (Merchan et al., 2010; Wang et al., 2011a).

Glycolysis levels are subject to environmental conditions and molecular signals. Arterial, venous, microvascular, and lymphatic ECs are all glycolytic (De Bock et al., 2013), but, in comparison to rapidly proliferating, highly glycolytic microvascular ECs, arterial ECs that grow more slowly are less glycolytic but consume more oxygen, though it remains to be determined to what extent adaptation to cell culture conditions influences these results (Parra-Bonilla et al., 2010). Hemodynamic forces such as blood flow also stimulate glycolysis through shear forces acting on the EC glycocalyx (Suárez and Rubio, 1991). The proangiogenic molecules VEGF and FGF2 increase PFKFB3-driven glycolysis, whereas DLL4, which activates Notch signaling and decreases branching, reduces glycolysis in ECs (De Bock et al., 2013).

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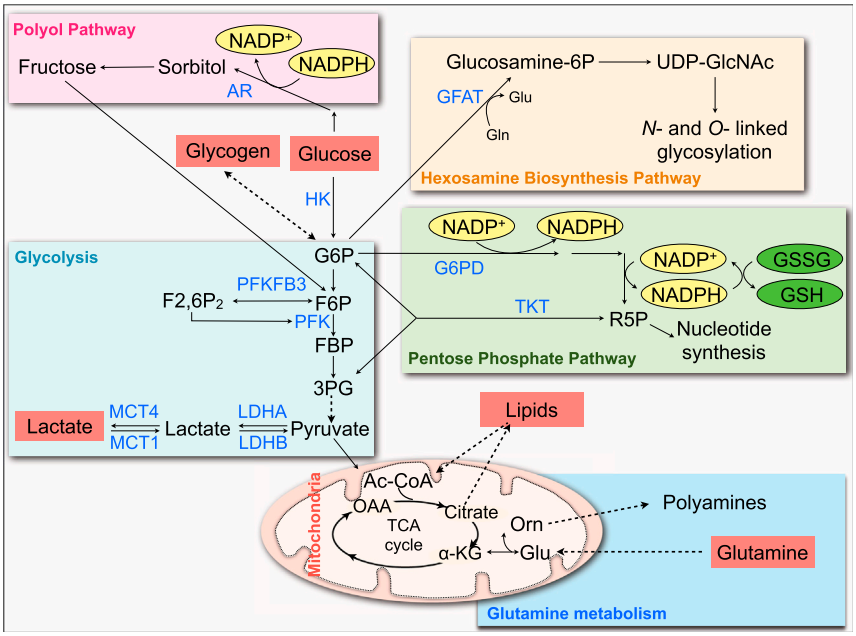

Figure 2. Schematic of Endothelial Cell Metabolism

A simplified schematic of EC metabolism showing the known metabolic pathways in ECs and their rate-limiting metabolic enzymes. α-KG, α-ketoglutarate; Ac-CoA, acetyl coenzyme A; AR, aldolase reductase; $F2,6P_2$, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GFAT, glutamine fructose-6-phosphate amino-transferase; Gln, glutamine; GLS, glutaminase; Glu, glutamate; GSH, glutathione; GSSG, glutathione disulfite; 3PG, glyceraldehyde-3-phosphate; HK, hexokinase; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; OAA, oxaloacetate; Orn, ornithine; PFK, phosphofructokinase; PFKFB3, phosphofructokinase-2/fructose-2,6-bisphosphatase isoform 3; R5P, ribose-5-phosphate; TKT, transketolase; TCA, tricarboxylic acid; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine.

At first sight, it may seem paradoxical that quiescent ECs rely on glycolysis, given that they could take advantage of the available oxygen in their immediate environment in the blood to more efficiently generate ATP via oxidative phosphorylation. Indeed, per glucose molecule, glycolysis produces a net total of only two molecules of ATP, whereas glucose oxidation yields up to 36 molecules of ATP. Nonetheless, one of the prime tasks of ECs is to vascularize avascular tissues through sprouting. If they relied primarily (or solely) on oxidative metabolism, then ECs would be unable to generate ATP in oxygen-depleted areas. In fact, given that interstitial oxygen levels drop faster than glucose levels over a distance away from a blood vessel, ECs can continue to rely on anaerobic glycolysis in such conditions (Buchwald, 2011; Gatenby and Gillies, 2004). Indeed, ECs are resistant to hypoxia as long as glucose is available but become sensitive to oxygen deprivation when glucose is limiting (Mertens et al., 1990). Another reason is that glycolysis rapidly generates ATP, which ECs need in order to form highly motile and rapidly moving lamellipodia and filopodia. Moreover, as long as glucose is not limiting in the extracellular milieu, glycolysis can generate similar amounts of ATP as glucose oxidation (Locasale and Cantley, 2011). Another advantage of glycolytic metabolism is that glycolysis and its side pathways generate the necessary precursors for macromolecules needed in order for ECs to grow, divide, and migrate (see below). Also, a low-oxidative metabolism generates fewer reactive oxygen species (ROS) and less oxidative stress in the high-oxygen environment that quiescent ECs are exposed to. Finally, by consuming less oxygen, they can transfer more oxygen to perivascular cells, thereby improving tissue oxygenation.

ECs also store intracellular glucose reserves as glycogen (Amemiya, 1983; Numano et al., 1974; Vizán et al., 2009). However, glycogen breakdown only becomes significant in glucose-deprived conditions and not in hypoxia, although it is not clear whether glycogenolysis is used for bioenergetic purposes alone (Krützfeldt et al., 1990; Vizán et al., 2009). In cerebral microvascular ECs, norepinephrine induces glycogenolysis, whereas 5-hydroxytryptamine stimulates glycogenesis (Spatz et al., 1986). Overall, little is known about the role and importance of glycogen in ECs, but the inhibition of glycogen phosphorylase impairs EC viability and migration (Vizán et al., 2009). This raises the question of whether ECs use this endogenous glucose storage to sprout into avascular glucose-deprived areas.

Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) is a side branch of glycolysis that cells use for divergent purposes (Figure 2). In this pathway, glucose-6-phosphate is oxidized to pentose sugars and reduces equivalents in two phases. The irreversible oxidative branch (oxPPP) generates NADPH and ribose-5-phosphate (R5P), whereas the reversible nonoxidative arm (non-oxPPP) produces only R5P. The latter is used for the synthesis of nucleotides, whereas NADPH is used for the reductive biosynthesis of lipids, production of NO, or reconversion of oxidized glutathione (GSSG) to reduced glutathione (GSH), a major cellular redox buffer. Depending on the cellular needs and context, the PPP can serve to promote cellular growth and division by increasing the biosynthesis of macromolecules (Cairns et al., 2011; Lunt and Vander Heiden, 2011; Vander Heiden et al., 2009), or the oxPPP can ensure redox homeostasis (Anastasiou et al., 2011). As rate-limiting enzymes, glucose-6-phosphate dehydrogenase (G6PD) controls the oxPPP arm, whereas transketolase (TKT) regulates the non-oxPPP branch. A recent study highlighted that ECs possess additional mechanisms for maintaining the redox balance. Indeed, ECs express UBIAD1, a nonmitochondrial prenyltransferase that synthesizes the electron carrier CoQ10 in the Golgi membrane compartment in order to prevent lipid peroxidation and protect membranes from oxidative damage (Mugoni et al., 2013).

Of all the glucose utilized by ECs, only 1%–3% normally enters the PPP in physiological conditions (Dobrina and Rossi, 1983; Jongkind et al., 1989; Krützfeldt et al., 1990; Spolarics and Spitzer, 1993; Vizán et al., 2009). However, in conditions of increased

oxidative stress, such as after treatment with lipopolysaccharide in vivo (Spolarics and Spitzer, 1993) or methylene blue in vitro (Dixit et al., 2008; Krützfeldt et al., 1990), up to 80% of glucose can enter the PPP, allowing cells to sustain GSH levels in order to reduce possibly harmful ROS (Spolarics and Wu, 1997). G6PD overexpression in ECs also increases NADPH and NO production and maintains intracellular glutathione stores when exposed to oxidants (Leopold et al., 2003b). The ability to activate the oxPPP enables quiescent ECs to better survive and tolerate oxidative stress during hypoxia reoxygenation events (Buderus et al., 1989). By activating protein kinase A, high glucose levels impair G6PD activity in ECs, thereby decreasing cell survival because of insufficient redox control (Zhang et al., 2000). A reduction of G6PD expression in vivo increases ROS levels and decreases eNOS activity in the aorta, thereby reducing vascular reactivity (Leopold et al., 2007).

The PPP might also influence vascular sprouting via other mechanisms. First, G6PD can modulate VEGF signaling, as revealed by findings that inhibition of G6PD impairs and G6PD overexpression promotes angiogenesis in vitro by regulating NO production via VEGF and tyrosine phosphorylation of VEGFR-2 (Leopold et al., 2003a; Vizán et al., 2009). In a positive feedback, the proangiogenic factor VEGF increases the oxPPP flux (Vizán et al., 2009) and enhances G6PD activity and localization at the plasma membrane (Pan et al., 2009). Second, the non-oxPPP can promote angiogenesis via the production of macromolecules, explaining why the inhibition of TKT reduces EC viability and migration (Vizán et al., 2009). Third, because low amounts of ROS can be proangiogenic (Okuno et al., 2012), hereditary G6PD deficiency in diabetes patients promotes the development of ocular neovascularization, presumably by increasing ROS levels as a result of the decreased production of NADPH (Cappai et al., 2011). Fourth, insulin may regulate NO generation in ECs by stimulating oxPPP and NADPH production, which is required for NO synthesis (Wu et al., 1994). Overall, the role of the oxPPP in vessel sprouting and maintenance is contextual, and understanding its role and regulation requires further study.

Hexosamine Biosynthesis Pathway

A fraction of glucose can also flux through the hexosamine biosynthesis pathway (HBP), where it is used for protein glycosylation (Figure 2). The rate-limiting step is catalyzed by glutamine:fructose-6-phosphate amidotransferase, which regulates the HBP in order to produce UDP-N-acetylglucosamine (UDP-GlcNAc), a substrate used for N-linked and O-linked glycosylation (Hart et al., 2007; Helenius, 1994; Love and Hanover, 2005). Because the HBP depends on the availability of glucose, glutamine, acetyl-CoA, and ATP, it is considered to be a "nutrient sensor" (Zachara and Hart, 2004a, b). Despite its presumed importance in regulating glycosylation, only a few reports studied the role of the HBP or glycosylation in angiogenesis. For instance, 2-deoxy-D-glucose inhibits angiogenesis by interfering with N-linked glycosylation (Merchan et al., 2010), whereas elevated protein O-GlcNAc modification in ECs impairs angiogenesis, possibly by inhibiting AKT signaling (Luo et al., 2008). Furthermore, by interacting with N-glycans of VEGFR-2, galectin-3 facilitates VEGFR-2 plasma membrane retention and phosphorylation and thereby stimulates VEGF-mediated angiogenesis (Markowska et al., 2011). Glycoproteins bearing N-linked oligosaccharides are essential for capillary tube formation (Nguyen et al., 1992) and the formation of the glycocalyx layer, which acts as a mechanosensor and controls EC permeability (Curry and Adamson, 2012). Also, glycosylation of the Notch receptor determines its responsiveness toward its ligands DLL4 and Jagged1, thereby playing a crucial role in tip-stalk cell differentiation during vessel branching (Benedito et al., 2009). Whether glycosylation of the angiogenic receptor is dynamically controlled through the HBP and whether those changes are dependent on nutrient availability and sensing awaits further insight.

Polyol Pathway

Hyperglycemia promotes vascular complications in diabetes. When glucose is present in excess of what the glycolytic pathway can handle, glucose enters the polyol pathway, a two-step pathway in which aldose reductase reduces glucose to sorbitol, which is then converted to fructose (Lorenzi, 2007; Tang et al., 2012) (Figure 2). Because the aldose reductase reaction converts NADPH to $NADP^+$, the activation of the polyol pathway can deplete stores of NADPH, which are necessary for maintaining reduced GSH levels for redox homeostasis, thus leading to the accumulation of ROS. It is unknown to what extent the accumulation of polyols themselves can also be toxic. In humans, high levels of aldose reductase are associated with toxicity. Human aldose reductase transgene expression in ECs in low-density lipoprotein receptor knockout mice (a model of atherosclerosis) aggravated vascular disease in diabetic conditions (Vedantham et al., 2011). Conversely, pharmacologic inhibition of the aldose reductase reduced EC oxidative damage and apoptosis in vitro and retinal vascular overgrowth through the upregulation of VEGF in diabetic rats and mice in vivo (Obrosova and Kador, 2011; Oyama et al., 2006), whereas aldose reductase deficiency diminished retinal vascular changes in a model of oxygen-induced retinopathy (Fu et al., 2012). Whether aldose reductase is a target in humans remains debated. Normal polyol pathway activity is also required for physiological angiogenesis by affecting VEGFR-2 and FGF signaling (Tammali et al., 2011; Yadav et al., 2012).

Mitochondria and Respiration in ECs

Unlike other glycolysis-addicted cell types such as red blood cells (lacking mitochondria) or embryonic stem cells (containing few inactive mitochondria) (Kondoh et al., 2007), ECs have active mitochondria, but they contain fewer mitochondria than oxidative cell types. Indeed, mitochondria make up only 5% of the cellular volume, in contrast to 30% in hepatocytes (Blouin et al., 1977). With gestational age during embryonic development, respiratory chain complexes and oxidative phosphorylation are reduced, whereas glycolytic activity is increased in ECs, presumably to cope with the metabolic stress during birth and render ECs less susceptible to peripartum hypoxic damage (Illsinger et al., 2011). Vascular branching signals also regulate mitochondrial biogenesis. Indeed, VEGF stimulates mitochondrial biogenesis via AKT-dependent signaling (Wright et al., 2008), whereas the silencing of SIRT1, a negative regulator of the Notch pathway (Guarani et al., 2011) (thus leading to enhanced Notch signaling and reduced vessel branching), inhibits mitochondrial biogenesis in ECs (Csiszar et al., 2009). However, whether mitochondrial biogenesis influences vessel sprouting and how it may do so remain to be assessed. Notably,

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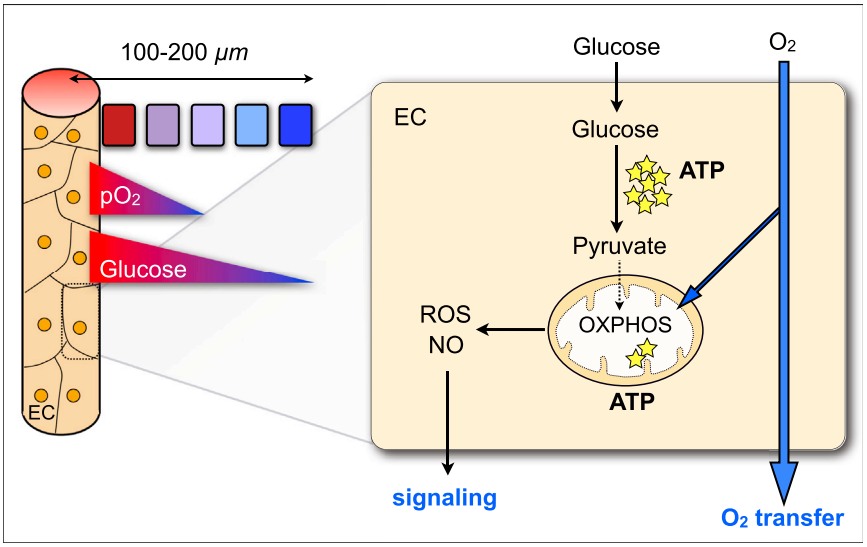

Figure 3. Mitochondria and Respiration in Endothelial Cells

Despite having immediate access to oxygen in the blood, ECs rely on glycolysis to generate ATP. Mitochondria in ECs are not considered important bioenergetic powerhouses but, rather, act primarily as signaling organelles by generating proangiogenic reactive oxygen species (ROS) and nitric oxide (NO). Moreover, by utilizing low amounts of oxygen, they ensure oxygen diffusion across the endothelial barrier to the perivascular tissues.

ATP derived from glycolysis is essential for maintaining the mitochondrial network (Giedt et al., 2012).

In vitro, ECs consume relatively low amounts of oxygen, which allows them to transfer most of the oxygen that enters these cells to perivascular cells in vivo (Figure 3) (Helmlinger et al., 2000). However, in vivo measurements documented a drop of oxygen levels across the arteriolar vessel wall (Tsai et al., 1998; Tsai et al., 2003). Though the cellular source consuming oxygen (ECs versus smooth muscle cells) was not identified, these studies postulated that the vessel wall can act as an "oxygen sink" to prevent the exposure of perivascular tissues to high oxygen levels and oxidative damage, though these observations are debated (Golub et al., 2011).

Primarily, mitochondria in ECs are generally considered to have a signaling function (via the production of proangiogenic ROS levels and NO) rather than serving as a bioenergetic powerhouse (Davidson, 2010; Quintero et al., 2006) (Figure 3). For instance, ECs detect mechanical stress by transmitting force via the cytoskeleton to the mitochondria and triggering mitochondrial ROS signaling, leading to an increase in the expression of NF-κB and VCAM-1 (Ali et al., 2004). Mitochondria have also been implicated as regulators of ion (H^+ and Ca^{2+}) homeostasis and apoptosis in ECs. Indeed, exposure of ECs to high levels of glucose (as occurs in diabetes) enhances mitochondrial fission and/or reduces mitochondrial fusion, resulting in mitochondrial fragmentation, ROS production, and Ca^{2+} overload, altogether leading to EC dysfunction and death (Pangare and Makino, 2012). Prohibitin 1 (PHB1), a protein localized to the inner mitochondrial membrane, inhibits mitochondrial complex I and thereby prevents ROS-induced senescence as well as AKT-dependent Rac1 hyperactivation, which leads to cytoskeletal rearrangements, decreased EC motility, and impaired capillary tube formation. Thus, PHB1 is important for proper mitochondrial function and maintaining the angiogenic capacity of ECs (Schleicher et al., 2008). Because of their role in signaling rather than in bioenergetics, mitochondria in ECs have been considered targets for angiogenesis inhibition (Park and Dilda, 2010).

In line with findings that mitochondria in ECs are not bioenergetic powerhouses, oxidative pathways only account for 15% of the total amount of ATP generated in ECs (De Bock et al., 2013). Also, mitochondrial respiration poisons that reduce oxygen consumption do not impair vessel branching, whereas the supplementation of NADH or pyruvate, which increase oxygen consumption, do not stimulate vascular sprouting (De Bock et al., 2013). Nonetheless, mitochondria in ECs have a high bioenergetic reserve capacity and can increase respiration substantially in stress conditions of glucose deprivation or oxidative stress (Dranka et al., 2010; Mertens et al., 1990). An exception may be the quiescent ECs of the blood-brain barrier, which have up to 5-fold more mitochondria than ECs in peripheral organs, presumably to provide large amounts of energy for transport of nutrients and ions across the blood-brain barrier in order to ensure brain homeostasis (Oldendorf and Brown, 1975).

Fatty Acid Metabolism

The role of other metabolic pathways in ECs remains poorly characterized. Some studies report that fatty acid oxidation (FAO) is a critical bioenergetic supply pathway for ECs (Figure 2), especially when carnitine is supplemented (Dagher et al., 1999, 2001; Hülsmann and Dubelaar, 1988, 1992) or glucose is removed from the medium (Dagher et al., 2001; Krützfeldt et al., 1990). In the latter condition, an increase in FAO compensates for the lack of glycolytic ATP production. Carnitine palmitoyl transferase 1 is a rate-limiting enzyme of FAO whose activity is inhibited by malonyl-CoA, itself produced by acetyl-CoA carboxylase (ACC). By inactivating ACC, AMPK stimulates FAO in ECs (Dagher et al., 2001; Fisslthaler and Fleming, 2009). ECs can oxidize both extra- and intra-cellular fatty acids, though the relative contribution of FAO versus glycolysis to ATP generation in ECs is debated (Dagher et al., 1999, 2001; Delgado et al., 2010; Dobrina and Rossi, 1983; Polet and Feron, 2013; Spolarics et al., 1991). In glucose-deprived or matrix-detached tumor cells, FAO produces metabolites for the TCA cycle, and these metabolites generate citrate and malate; i.e., substrates of the NAPDH-producing isocitrate dehydrogenase and malic enzyme, respectively (Jeon et al., 2012; Pike et al., 2011). Hence, by indirectly generating NADPH needed to convert GSSG to its reduced form (GSH), FAO regulates redox homeostasis and prevents excessive oxidative stress (which is antiangiogenic) (Jeon et al., 2012; Pike et al., 2011), but such a role has not been

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described in ECs. The bioenergetic importance of FAO for vessel branching remains unknown.

Fatty-acid-binding protein 4 (FABP4), an intracellular lipid chaperone, is a target of VEGF and stimulates EC proliferation in vitro (Elmasri et al., 2009), whereas the loss of FABP4 impairs VEGF transgene-induced neovascularization in airways, in part by decreasing VEGF-induced EC proliferation and lowering eNOS and stem cell factor expression (Ghelfi et al., 2013). Also, more FABP4-immunoreactive vessels are detected in bronchial biopsies of patients with asthma (Ghelfi et al., 2013). Notably, VEGF-B promotes endothelial lipid uptake and transport to peripheral tissues (heart and skeletal muscle) through the upregulation of the expression of fatty acid transporters FATP3 and FATP4 (Hagberg et al., 2010). In addition, VEGF-B stimulates AMPK required for aortic EC proliferation in vitro independently of an increase in FAO (Reihill et al., 2011). In agreement with findings that ectopic lipid deposition is associated with the pathogenesis of type II diabetes, neutralizing VEGF-B antibody restored insulin sensitivity and glucose tolerance by reducing endothelial-to-tissue lipid transport, thereby creating a novel option for diabetes therapy (Carmeliet et al., 2012; Hagberg et al., 2012).

Lipids are also required for the formation of membranes and act as intracellular signaling molecules. The upregulation of lipid metabolism is a hallmark of multiple cancer types (Biswas et al., 2012; Santos and Schulze, 2012; Schug et al., 2012). The expression of fatty acid synthase (FAS), which catalyzes de novo lipid synthesis, is generally low or undetectable in adult healthy tissues, given that the majority of fatty acids are taken up from dietary sources. In contrast, even with an adequate nutritional lipid supply, FAS is highly upregulated in cancer cells to provide these rapidly proliferating cells with sufficient amounts of lipids for membrane biogenesis and to confer them a growth and survival advantage (Pandey et al., 2012; Santos and Schulze, 2012). The role of lipogenesis in vascular branching remains poorly studied. Pharmacological inhibition of FAS inhibits tumor angiogenesis (Seguin et al., 2012), whereas the genetic loss of endothelial FAS impairs pathological angiogenesis by decreasing plasma membrane targeting of eNOS (via reduced palmitoylation) and VEGFR-2 (Wei et al., 2011).

A recent study showed that maintaining efficient cholesterol efflux from ECs is essential for angiogenesis (Fang et al., 2013). In order to prevent cholesterol overload in cells, ATP-binding cassette transporters mediate cholesterol efflux from cells to apolipoprotein A-I (apoA-I) and the apoA-I-containing high-density lipoprotein. In ECs, cholesterol efflux reduces membrane lipid rafts, which interferes with VEGFR-2 membrane localization, dimerization, and endocytosis and impairs VEGF-induced angiogenesis (Fang et al., 2013). Another apolipoprotein (e.g., apoB), impairs angiogenesis by upregulating the antiangiogenic VEGF trap VEGFR-1 (Avraham-Davidi et al., 2012). The effects of apoB-containing lipoproteins on vessel growth were not induced by a decreased delivery of fatty acids to tissues or due to global lipid starvation, given that apoC-II deficiency did not phenocopy the vascular defects resulting from the deficiency of microsomal triglyceride transfer protein, which is involved in the biosynthesis of apoB-containing lipoproteins. It remains to be determined whether this mechanism underlies the EC dysfunction that precedes the formation of atherosclerotic plaques or impairs collateral vessel growth in hypercholesterolemic patients.

Amino Acid Metabolism

Besides glucose, rapidly growing cancer cells and embryonic stem cells also metabolize various types of amino acids, such as glutamine, proline, and serine (Dang, 2012; DeBerardinis and Thompson, 2012; Kalhan and Hanson, 2012; Phang and Liu, 2012; Shyh-Chang et al., 2013) (Figure 2). Of these amino acids, glutamine is a key metabolic fuel for proliferating cells. The role of glutamine metabolism has not been studied extensively in ECs, and, therefore, its role in angiogenesis remains unclear. ECs take up glutamine via Na^+-dependent transport mechanisms but also have the capacity to produce this amino acid via glutamine synthetase, though the physiological relevance of this process for vessel branching remains unknown (Lohmann et al., 1999). Glutamine taken up by ECs can be converted to glutamate and ammonia (Wu et al., 2000) (Figure 2). The activity of glutaminase 1, the first step in the glutaminolysis pathway, is higher in ECs than it is in lymphocytes (Leighton et al., 1987). Pharmacological inhibition of glutaminolysis impairs the proliferative capacity of ECs and induces a senescent-like phenotype in ECs (Unterluggauer et al., 2008). Nonetheless, it is still debated whether glutamine oxidation contributes substantially to ATP production in ECs (De Bock et al., 2013; Krützfeldt et al., 1990; Spolarics et al., 1991; Wu et al., 2000). However, glutamine contributes more significantly to ATP production and promotes survival when oxidative stress impairs glucose-dependent pathways of ATP production (Hinshaw and Burger, 1990), indicating that the contribution of glutamine metabolism to ATP production in ECs is contextual (De Bock et al., 2013; Krützfeldt et al., 1990; Spolarics et al., 1991; Wu et al., 2000).

Glutamine also serves as a carbon source for the biosynthesis of macromolecules, but stable isotope-tracer-based metabolomic flux analysis of glutamine in ECS has not been performed yet. Glutamine metabolism by ECs could also be important for the provision of nitrogen for biosynthetic purposes. Indeed, the synthesis of the polyamine precursor from glutamine sustains EC growth (Wu et al., 2000) (Figure 2). Furthermore, glutamine inhibits the endothelial production of NO, in part via the conversion of glutamine to glucosamine in the HBP, which inhibits the oxPPP activity and thereby reduces the availability of NADPH, an essential cofactor for eNOS (Wu et al., 2001). Glutamine also impairs NO production, inhibiting the formation of arginine from citrulline through reducing citrulline transport (Kawaguchi et al., 2005; Meininger and Wu, 1997; Sessa et al., 1990). Also, arginine controls angiogenesis by regulating the levels of ROS in ECs (Park et al., 2003; Zhuo et al., 2011). No studies have been reported on the possible role of proline, serine, and threonine metabolism in ECs.

Metabolic Changes during Vascular Sprouting

Migrating Tip and Proliferating Stalk Cells

In ECs, the tip-cell-activating signal VEGF increases glycolysis by upregulating PFKFB3 levels, suggesting that tip cells require elevated levels of PFKFB3-driven glycolysis (De Bock et al., 2013) (Figure 1A). Accordingly, PFKFB3 silencing and/or deletion impairs the formation of distal sprouts with tip cell filopodia and the number of filopodia in retinal sprouting vessels and reduces the lamellipodia area of cultured ECs (De Bock et al., 2013).

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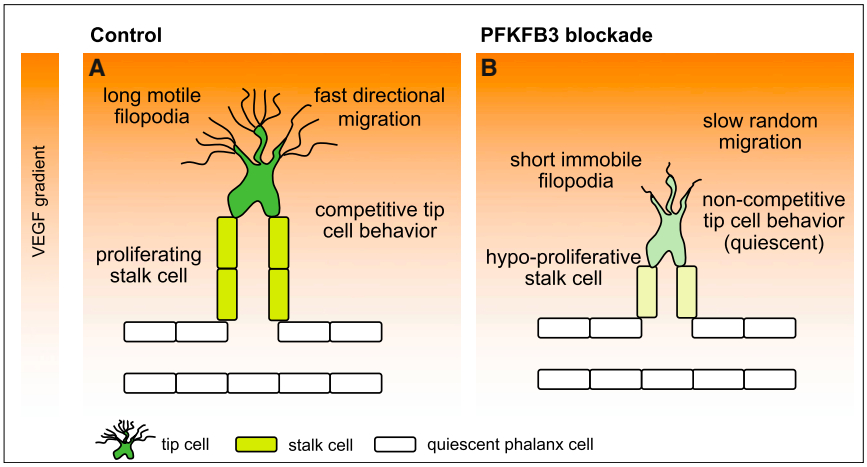

Figure 4. PFKFB3 Blockade Impairs Tip and Stalk Cell Behavior

(A) A schematic model demonstrating that, in control WT conditions, stalk cells proliferate, and tip cells migrate directionally, extend long motile filopodia, and compete for the tip.

(B) Upon PFKFB3 blockade, stalk cells are hypo-proliferative, whereas tip cells have impaired migration and directional movement with short immobile filopodia so that tip cells lose their competitive advantage for the tip position.

However, aside from a role for PFKFB3 in tip cells, PFKFB3 also regulates stalk cell functions. Consistent with a reported increase in glycolysis when cells enter S-phase (Almeida et al., 2010), PFKFB3 silencing and/or deletion reduced EC proliferation in various angiogenesis assays in vitro and sprouting retinal vessels in vivo (De Bock et al., 2013). DLL4-mediated Notch activation also lowered PFKFB3 levels and glycolysis (De Bock et al., 2013) (Figure 1A). Yet, stalk cells proliferate in order to elongate the stalk, and cell proliferation is known to require increased levels of glycolysis (Vander Heiden et al., 2011). This paradox is resolved by findings that the growth-inhibitory activity of Notch is overruled by other genetic (Wnt) signals (Phng et al., 2009). Notably, in mosaic sprouting assays (Figure 1B), the overexpression of PFKFB3 is able to overcome the pro-stalk-cell activity of Notch and favors the tip localization of ECs overexpressing both PFKFB3 and the transcriptionally active Notch domain NICD when using an endothelial spheroid sprouting model in vitro or analyzing vascular branching in zebrafish embryos in vivo (De Bock et al., 2013) (Figure 1C). This is remarkable, given that no other genetic signal has been shown to be able to overrule the prostalk activity of Notch. Conversely, PFKFB3-silenced ECs are less capable of competing for the tip position in mosaic sprouting assays (De Bock et al., 2013). The consequences of PFKFB3 blockade on tip and stalk cells is illustrated in Figure 4. Overall, in parallel to genetic signals, PFKFB3-driven glycolysis also regulates vascular branching.

Quiescent Phalanx Cells

Little is known about the metabolic changes that accompany, promote, or are necessary for inducing EC quiescence. Glycolysis is decreased in quiescent ECs, which might serve several purposes. First, given that ECs rely on glycolysis in order to divide, thus lowering glycolysis reduces proliferation and promotes quiescence. Second, quiescent ECs are exposed to high oxygen levels in the blood, which may cause oxidative damage. Thus, similar to erythrocytes, quiescent ECs must protect themselves against oxidative damage. By maintaining a low oxidative metabolism, ECs minimize ROS production, thereby providing protection against their high-oxygen milieu. Third, of the total amount of glycolysis, ECs use 40% to proliferate and migrate, whereas they use the remaining 60% for maintenance homeostasis (De Bock et al., 2013), which is in line with findings that proliferation only requires a 30% increase in ATP production (Kilburn et al., 1969; Locasale and Cantley, 2011). Quiescent ECs need this residual 60% of glycolysis in order to perform energy-demanding homeostatic maintenance functions such as transendothelial transport, barrier formation, glycocalyx deposition, and matrix production (Curry and Adamson, 2012; Potente et al., 2011)—resembling quiescent fibroblasts, which also have an active metabolism in baseline conditions (Lemons et al., 2010). It has been postulated that cellular quiescence requires a higher baseline metabolism than previously anticipated to ensure maintenance of ion gradients, protein and RNA synthesis, and other processes (Locasale and Cantley, 2011). Overall, because of their particular milieu and cellular activities, quiescent ECs adapt their metabolism to optimally accommodate the need for reduced proliferation on one side with the homeostatic needs for redox control and baseline maintenance activities on the other side.

Compartmentalization of Metabolism

Tip cells extend lamellipodia and filopodia in order to migrate. The formation of these motile structures relies on the remodeling of the actin cytoskeleton, a process that requires the rapid production of high amounts of ATP. In motile ECs, a large fraction of the total amount of glycolytic ATP generated is utilized by the actomyosin ATPase (Culic et al., 1997). In quiescent contact-inhibited ECs, enzymes of the glycolytic cascade are primarily present in the perinuclear cytosol. However, once they become motile and start migrating, these glycolytic enzymes also become translocated to lamellipodia, where they are highly concentrated along with F-actin in membrane ruffles at the leading front to generate high levels of ATP in lamellipodial "ATP hot spots"—mitochondria are excluded from lamellipodia and filopodia (De Bock et al., 2013) (Figures 5A–5D). Furthermore, biochemical analysis indicates that PFKFB3 is present in F-actin-enriched fractions and to higher levels in proliferating and migrating ECs (De Bock et al., 2013).

Various glycolytic enzymes are inactive as dimers but become more active in a tetrameric configuration. Through actin binding sites, these enzymes bind to actin, which stabilizes their tetrameric configuration and enhances their enzymatic activity (Real-Hohn et al., 2010) (Figure 5E). Such compartmentalization of glycolysis with the actin cytoskeleton offers various advantages. High levels of ATP are rapidly generated in lamellipodia and filopodia at the site where energy is needed during EC migration (Figures 5E and 5F). In addition, the rapid

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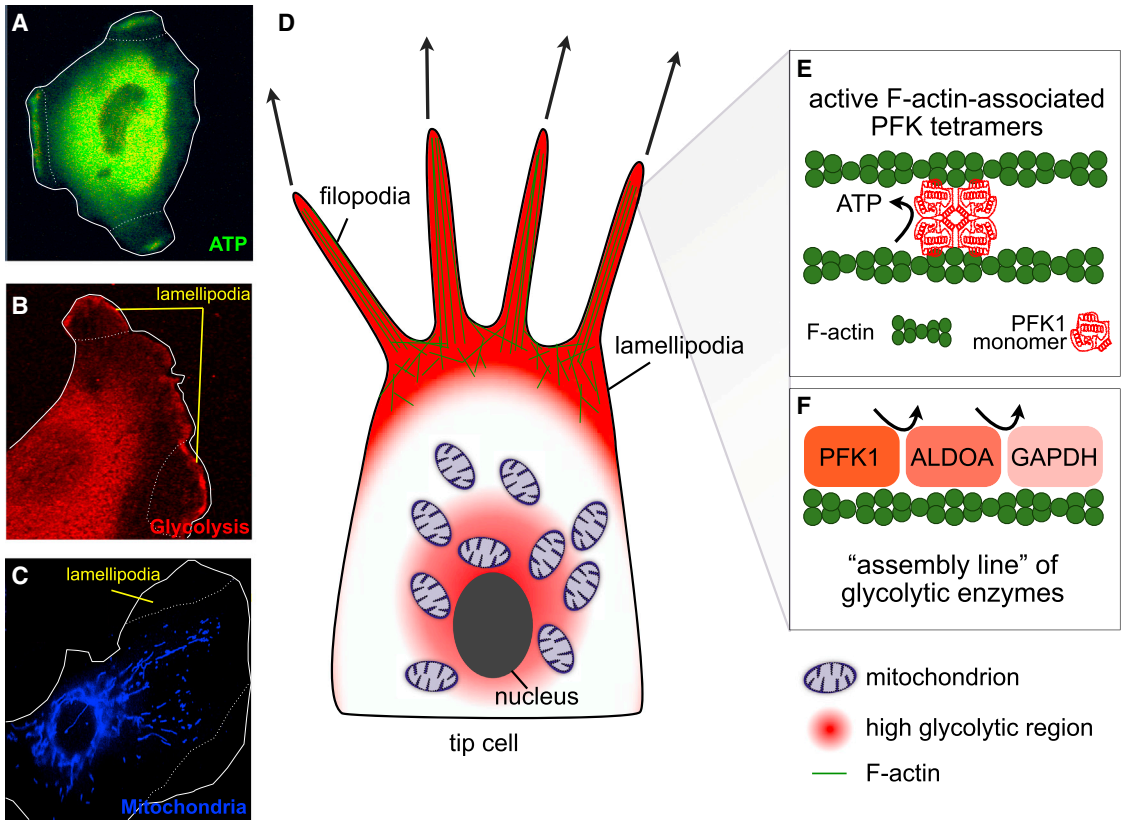

Figure 5. Compartmentalization of Metabolism

(A–C) Representative images of ECs expressing the ATP biosensor GO-ATeam (A), the glycolytic enzyme phosphoglycerate kinase (B), and the mitochondrial marker TOMM20 (C), revealing high ATP levels and glycolytic enzymes in the perinuclear cytosol as well as at the membrane ruffles of lamellipodia, whereas mitochondria are present around the nucleus but excluded from lamellipodia. Lamellipodia are denoted with a white dotted line.

(D) A schematic showing the localization of glycolytic enzymes in the perinuclear cytosol and the compartmentalization in lamellipodia and filopodia of the tip cell. The mitochondria are excluded from the filopodia because they are too large to fit into the thin cytosolic protrusions.

(E) A schematic illustrating the compartmentalization of the glycolytic regulator PFK1 with F-actin. Actin binding stabilizes PFK1 in its tetrameric active configuration.

(F) A schematic illustrating that the glycolytic enzymes are arranged in an assembly line, creating a "glycolytic hub."

extension-retraction of filopodia and lamellipodia may create an ATP drain for the cell body. Localizing ATP supply to compartments where ATP is consumed can prevent catastrophic ATP depletion for the cell. Moreover, through binding to actin, an assembly line of the glycolytic machinery is generated wherein the product of one glycolytic enzyme becomes the substrate of its neighboring glycolytic enzyme because of their close proximity (al-Habori, 1995; Fulgenzi et al., 2001; Lagana et al., 2000; Real-Hohn et al., 2010). Motile structures in other organisms, such as flagella in sperm and predatory tentacles in hydra, also concentrate glycolytic enzymes (Baquer et al., 1975; Hereng et al., 2011; Mitchell et al., 2005; Pavlova, 2010). The functional relevance of this link is underscored by findings that a mutant fruit fly expressing an aldolase variant that cannot bind actin is unable to fly (Wojtas et al., 1997). In vascular smooth muscle cells, glycolysis and gluconeogenesis occur in separate "compartments" because of the spatial separation of glycolytic and gluconeogenic enzymes in distinct plasma membrane microdomains (Lloyd and Hardin, 2001). Whether a comparable compartmentalization of glycolysis versus gluconeogenesis occurs in ECs is unknown.

Feedback Regulation of Vascular Branching by Metabolism

An intriguing question is whether metabolism provides a feedback for the genetic signals that control vascular branching. One example is how Notch is subject to regulation by metabolic signals in ECs. Indeed, Notch is a direct target of the NAD^+-dependent deacetylase SIRT1, a key regulator of cellular metabolism that is activated by nutrient deprivation (Chalkiadaki and Guarente, 2012; Guarani and Potente, 2010). By deacetylating NICD and thereby reducing its protein stability, SIRT1 reduces the amplitude and duration of the Notch response in a negative feedback loop (Guarani et al., 2011). This promotes the vascularization of the nutrient-deprived tissue. Accordingly, in the absence of SIRT1 (a condition mimicking nutrient abundance), ECs are sensitized to Notch signaling, resulting in a stalk-cell-like phenotype with impaired vessel outgrowth (Potente et al., 2007).

Another mechanism by which SIRT1 controls vascular sprouting is via deacetylating the transcription factor FOXO1, which controls cell growth and metabolism (Eijkelenboom and Burgering, 2013). FOXO1 is activated by nutrient stress (and is a target

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of SIRT1 and AMPK), but, unlike SIRT1, this transcription factor inhibits vessel branching (Oellerich and Potente, 2012). Indeed, loss of FOXO1 results in embryonic lethality because of vascular abnormalities (Furuyama et al., 2004), and the inducible postnatal deletion of FOXO1, FOXO2, and FOXO3 together induces hemangioma formation (Paik et al., 2007). These data suggest that a finely tuned balance between SIRT1 and FOXO1 is required in order to orchestrate the vessel-branching response to nutrient deprivation.

Metabolism has been shown to control growth factor signaling in cancer cells (DeBerardinis and Thompson, 2012). Interestingly, the response of Notch to its ligands DLL4 and Jagged1 is determined by the glycosylation state of its extracellular domain in a tip-versus-stalk cell manner (Benedito et al., 2009), thus providing another example of how metabolism impacts on angiogenic signal transduction. In particular, glycosylation by FRINGE glycosyltransferases favors the activation of Notch by DLL4 over Jagged1 (Eilken and Adams, 2010). Even though stalk cells primarily express Jagged1 (and tip cells predominantly express DLL4), Jagged1 is only able to weakly activate Notch1 in comparison to DLL4 (Eilken and Adams, 2010). As a result, DLL4 causes hypobranching, whereas Jagged1 induces opposite effects. An outstanding question is whether the glycosylation of other key angiogenic receptors (such as VEGFR-2) provides another level of metabolic control of angiogenesis.

Metabolites as Angiogenic Signals

In the brain, muscle, and tumors, lactate is a fuel for neighboring cells (Bergersen, 2007; Brooks, 2009; Draoui and Feron, 2011; Whitaker-Menezes et al., 2011). This metabolite is generated through the conversion of pyruvate, itself produced in the glycolytic pathway (or other pathways) by lactate dehydrogenase A (LDH-A). However, more than being a waste product, lactate also serves as a fuel for oxidative metabolism after conversion to pyruvate by LDH-B (Figure 2). For instance, in tumors, lactate is oxidized by oxygenated tumor cells, thereby sparing glucose for more hypoxic glycolytic cancer cells (Draoui and Feron, 2011). Cancer-associated fibroblasts also have aerobic glycolysis and extrude lactate to "feed" adjacent tumor cells (Whitaker-Menezes et al., 2011). In the brain, a cell-to-cell lactate shuttle between astrocytes and neurons is linked to glutamatergic signaling (Brooks, 2007). However, in ECs, only <1% of glucose is oxidized in the TCA cycle, and glucose oxidation generates only 6% of the total amount of ATP in ECs (De Bock et al., 2013). Also, lactate is only a significant substrate for oxidation in the absence of glucose in ECs (Krützfeldt et al., 1990).

Lactate in ECs can act as a signaling molecule rather than a metabolic substrate. Indeed, lactate inhibits the activity of the oxygen-sensing prolyl-hydroxylase domain protein PHD2, thereby activating the hypoxia-inducible transcription factor HIF-1α in normoxic oxidative tumor cells and triggering tumor angiogenesis by upregulating VEGF and other proangiogenic cytokines (De Saedeleer et al., 2012; Hunt et al., 2007). By inhibiting PHD2, lactate also triggers the phosphorylation and degradation of the inhibitory subunit IκBα, thus stimulating an autocrine proangiogenic NF-κB-IL-8 pathway (Végran et al., 2011). Lactate also accelerates EC progenitor recruitment and differentiation via the release of HIF-1α-dependent angiogenic factors (Milovanova et al., 2008). Blockade of lactate influx in ECs by monocarboxylate transporter 1 blockers, inhibiting lactate uptake in tumor ECs, impedes HIF-1α-dependent angiogenesis (Sonveaux et al., 2012).

Another paracrine metabolic signal is glutaminolysis-derived ammonia, which induces autophagy and enhances tumor cell survival at the expense of cell growth and proliferation (Eng et al., 2010). Glutamine metabolism not only enhances the proliferation and survival of oxygenated nutrient-rich tumor cells via the anabolic replenishment of TCA cycle intermediates, but it also helps nutritionally stressed neighbor tumor cells cope with nutrient deprivation by inducing autophagy in order to preserve cellular functions (Eng et al., 2010). The importance of autophagy in EC biology remains poorly studied, though haplodeficiency of the autophagy mediator Beclin-1 increases hypoxia-induced angiogenesis associated with an increase in HIF-2α expression and erythropoietin production (Lee et al., 2011). Moreover, during retinal vascular development, the inhibition of autophagy reduces hyaloid regression (Kim et al., 2010).

Another metabolic interaction between the tumor and its stroma was recently described. ECs and tumor cells make direct cell-cell contact and can exchange cellular components by generating tunneling nanotubes. The transfer of mitochondria from ECs to tumor cells via these nanotubes conferred tumor resistance against chemotherapy (Pasquier et al., 2013). Thus, besides providing the tumor with nutrients and oxygen, ECs might also feed the tumor with their own metabolic machinery, though further studies will be required to validate these findings.

Conclusions and Perspectives

In a field where >44,000 papers have been published on a single molecule such as VEGF, it is surprising that <100 papers on how ECs rewire their metabolism during vascular branching have been reported. In order to grasp the importance of how metabolism might influence EC behavior and vascular sprouting, it will be necessary to first establish a metabolic roadmap of the different metabolic pathways in the different EC subtypes involved in vascular branching and to characterize how these various metabolic pathways adapt during the various steps in vessel sprouting. This will require state-of-the-art metabolic flux analytic methods with stable isotope tracers in combination with measurements of radioactive tracer flux analyses and steady-state metabolite levels. It will also be intriguing to characterize the metabolism of transformed hemangiomas and angiosarcomas or study the effects of diabetes and hypercholesterolemia on EC metabolism and sprouting. Another appealing question is how ECs can regulate organismal metabolism by differentiating into metabolically active adipocytes (Gupta et al., 2012; Tran et al., 2012). Yet another unexplored field is the metabolism-epigenome interaction. Does metabolism epigenetically regulate vascular branching, similar to cancer cells (Mazzarelli et al., 2007; Teperino et al., 2010; Yun et al., 2012), or can the epigenome influence EC metabolism? Finding an answer to these questions promises to be an exciting endeavor.

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Review

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Control of vessel sprouting by genetic and metabolic determinants

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Vessel sprouting by endothelial cells (ECs) during angiogenesis relies on a navigating tip cell and on proliferating stalk cells that elongate the shaft. To date, only genetic signals have been shown to regulate vessel sprouting. However, emerging evidence indicates that the angiogenic switch also requires a metabolic switch. Indeed, angiogenic signals not only induce a change in EC metabolism but this metabolic adaptation also co-determines vessel sprouting. The glycolytic activator PFKFB3 regulates stalk cell proliferation and renders ECs more competitive to reach the tip. We discuss the emerging link between angiogenesis and EC metabolism during the various stages of vessel sprouting, focusing only on genetic signals for which an effect on EC metabolism has been documented.

Model of vessel sprouting

Blood vessels rapidly switch from a state of quiescence to active sprouting in response to pro-angiogenic stimuli. Specific EC subtypes orchestrate vessel sprouting. Navigating tip cells (see Glossary) lead the vessel branch at the vascular forefront, while proliferating stalk cells elongate the shaft in the new sprout [1]. Neighboring sprouts anastomose through interactions between filopodia on their respective tip cells to form a new and perfused vessel branch. ECs then regain their quiescent state (phalanx cells) and are stabilized by the coverage of perivascular mural pericytes [2]. Instead of providing a complete survey of all known genetic molecules known to regulate vessel sprouting (for which we refer to more historical overviews [3–6]), we limit our overview here to molecules with known effects on EC metabolism. We first describe the metabolic pathways that ECs use to generate energy.

Energy generation in ECs

Glucose metabolism

In contrast to other healthy cells [7,8], ECs rely heavily on glycolysis to generate energy (Figure 1) [8–10]. The glycolysis rate in ECs is comparable to the rate measured in various tumor cells [8]. Glycolysis is essential for ECs to maintain homeostasis because nearly complete inhibition of glycolysis with the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) induces cell death [8]. Notably, ECs rely minimally on glucose oxidation and mitochondrial respiration for ATP production [8,10]. Nonetheless, mitochondria represent a bioenergetic reserve to which ECs appeal under stressed conditions [11].

Upon induction of sprouting by growth factors such as vascular endothelial growth factor (VEGF), quiescent

Glossary

Anastomosis: the process in which a tip cell meets and fuses with another tip cell or a pre-existing blood vessel to form a lumenized loop that allows blood flow.
Filopodia: thin (0.1–0.3 μm) long (10 μm) finger-like actin-rich plasma membrane protrusions composed of parallel bundles of filamentous actin important in cell migration, adhesion to the extracellular matrix, guidance towards chemoattractants, cell–cell signaling, embryonic development, and wound healing.
Glycocalyx: an interconnected gel-like network of membrane-bound proteoglycans and glycoproteins coating the luminal surface of ECs. This layer is involved in the mechano-transduction of shear stress, the adherence of blood cells, vascular permeability, and vessel wall homeostasis.
Glycolysis: the breakdown of glucose to pyruvate in 10 consecutive cytoplasmic reactions to generate energy (ATP) and reducing power (NADH).
Hexosamine biosynthesis pathway (HBP): the metabolic pathway of which the rate-limiting step is the transfer of the glutamine amido group to fructose-6-phosphate. Ultimately, glucosamine-6-phosphate, the key precursor for *O*- and *N*-linked protein glycosylation, is generated.
Lamellipodia: thin (0.1–0.2 μm) sheet-like protrusions behind the protruding cell edge consisting of a densely branched filamentous actin network and that are important in cell migration, substrate adhesion, macropinocytosis, and phagocytosis.
Pentose phosphate pathway (PPP): the metabolic pathway that converts glucose-6-phosphate to ribose-5-phosphate, a key intermediate of nucleotide synthesis, with production of NADPH, and is important for several anabolic processes and redox homeostasis.
Pericytes: (peri, around; cyte, cell) mural cells that cover blood microvessels and share the vascular basement membrane. They provide support, ensure vessel stability, and regulate permeability by direct physical contact and paracrine signaling with ECs.
Phalanx cells: specialized ECs that are quiescent, lack filopodia, and are aligned in a smooth tight cobblestone monolayer to ensure optimal blood flow and stable perfusion of the surrounding tissues.
6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3): a key regulatory bifunctional enzyme of the glycolytic flux. It has a high kinase:phosphatase ratio (~740:1) and as such generates high amounts of fructose-2,6-bisphosphate, the most potent allosteric activator of the rate-limiting glycolytic enzyme phosphofructokinase-1 (PFK-1).
Spheroid assay: the use of *in vitro* established 3D EC aggregates to study EC sprouting.
Stalk cells: specialized ECs that form the stalk of vascular sprouts. Stalk cells trail behind tip cells, proliferate to elongate the stalk, and form a lumen. They also prevent retraction of ECs.
Tip cells: specialized ECs located at the tip of the vascular sprout. These cells are highly polarized, extend numerous filo- and lamellipodia with which they probe the environment, and are key to both anastomosis and migration because they direct the sprout.

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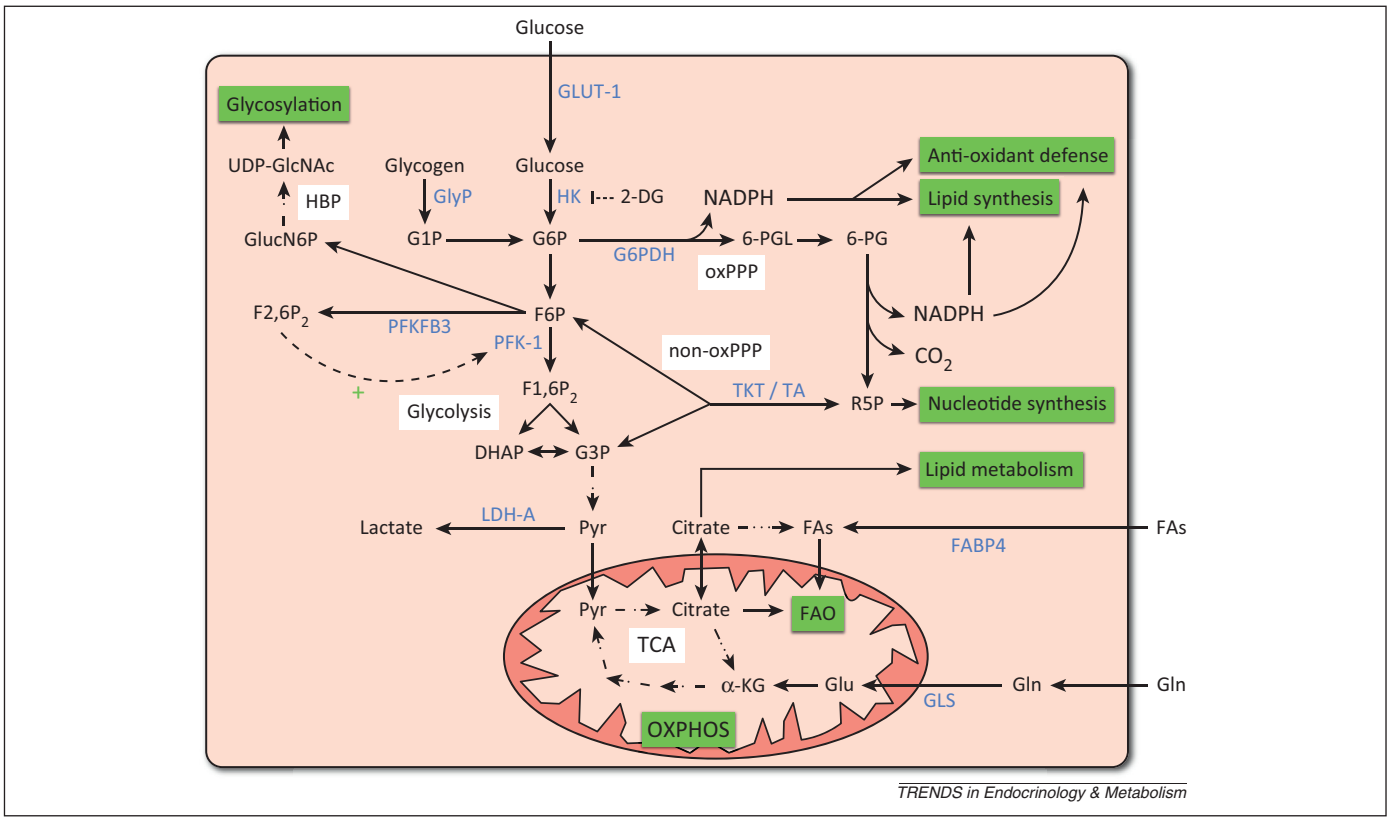

Figure 1. Endothelial cell (EC) metabolism. Schematic overview of the principal metabolic pathways and key enzymes in ECs. Glucose is taken up via the GLUT-1 transporter and subsequently phosphorylated by hexokinase. Quiescent ECs mainly rely on glycolysis to generate the energy needed to perform their functions, whereas sprouting ECs even further upregulate their glycolysis rate. Glycolysis intermediates are shunted into side-branches for anti-oxidant defense and lipid synthesis (oxPPP) as well as nucleotide synthesis (both oxPPP and non-oxPPP) and glycosylation purposes (HBP). Upon glucose scarcity, intracellular reserves can be broken down to sustain the rate of glycolysis. ECs also use FAs and glutamine as energy sources via FAO and glutaminolysis respectively. The latter is required to maintain the functionality of the TCA cycle (anaplerosis) as TCA cycle intermediates (e.g., citrate) exit for anabolic purposes. Abbreviations: 2-DG, 2-deoxy-D-glucose; DHAP, dihydroxyacetone phosphate; $F1,6P_2$, fructose-1,6-bisphosphate; $F2,6P_2$, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; FABP4, fatty acid binding protein 4; FAs, fatty acids; FAO, fatty acid oxidation; G1P, glucose-1-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; G6PDH, G6P dehydrogenase; GLS, glutaminase; Gln, glutamine; Glu, glutamate; GlucN6P, glucosamine-6-phosphate; GLUT-1, glucose transporter 1; GlyP, glycogen phosphorylase; HBP, hexosamine biosynthetic pathway; HK, hexokinase; α-KG, α-ketoglutarate; LDH-A, lactate dehydrogenase A; NADPH, nicotinamide adenine dinucleotide phosphate; (non-)oxPPP, (non-)oxidative branch of the pentose phosphate pathway; OXPHOS, oxidative phosphorylation; 6-PG, 6-phosphogluconate; 6-PGL, 6-phosphogluconolactone; PFK-1, phosphofructokinase-1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; Pyr, pyruvate; R5P, ribulose-5-phosphate; TA, transaldolase; TCA, tricarboxylic acid; TKT, transketolase; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

ECs increase their glycolysis rate by upregulating the expression of, amongst others, glucose transporter 1 (GLUT-1: facilitates the transport of glucose across the plasma membrane), lactate dehydrogenase-A (LDH-A: converts pyruvate and NADH to L-lactate and NAD^+), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) [8,9,12,13]. PFKFB3 synthesizes fructose-2,6-bisphosphate ($F2,6P_2$), an allosteric activator of phosphofructokinase-1 (PFK-1) and the most potent stimulator of glycolysis [14]. Reducing glycolysis by PFKFB3 gene silencing in ECs *in vitro* decreases EC migration and proliferation, and impairs vessel sprouting in EC spheroid assays. Furthermore, PFKFB3 gene inactivation in ECs *in vivo* impedes vessel sprouting, branching, and outgrowth [8].

In view of the exposure of ECs to oxygen in the blood, the importance of glycolysis in ECs might seem paradoxical given that oxidative phosphorylation (OXPHOS) is more efficient in generating ATP (approximately 20-fold more ATP per glucose molecule [15]). However, this preference can be explained by several reasons. First, endothelial oxygen consumption would decrease the availability for *trans*-endothelial transport to perivascular cells. Second, the low oxygen-dependence of ECs might prepare them for sprouting in hypoxic tissues. As such, ECs are able to quickly sprout without the need to adapt to fluctuating oxygen levels. This is further strengthened by the fact that oxygen is more limiting than glucose in the tumor interstitium (compared to glucose, oxygen has a higher diffusion coefficient, but lower solubility and therefore also lower available tissue concentration, resulting in a shorter diffusion distance and steeper concentration gradient from the vessel to the tissue [16–18]), and because ECs are only susceptible to hypoxia when glucose is limiting [19]. Third, lower oxidative metabolism results in less oxidative stress. Fourth, when glucose availability is unlimited, the glucose uptake and glycolysis rates can be elevated to equal or even exceed the ATP production by OXPHOS [10,15]. Finally, to sustain cell division and migration, glycolysis intermediates can be shunted to side-branches such as the pentose phosphate pathway (PPP) and the hexosamine biosynthetic pathway (HBP).

The PPP is essential for nucleotide, lipid, and aromatic amino acid synthesis as well as for anti-oxidant protection, whereas the HBP is considered a nutrient sensor important in protein glycosylation (Figure 1). The first and rate-limiting step of the oxidative part of the PPP (oxPPP) is

catalyzed by glucose-6-phosphate (G6P) dehydrogenase (G6PDH), the plasma membrane localization and activity of which is regulated by Src-mediated tyrosine phosphorylation, and which plays a role in VEGF-mediated phosphorylation of Akt, VEGFR2, and eNOS, and EC responses such as migration, proliferation, and tube formation [20,21]. This is exemplified by a decrease in EC viability and migration upon G6PDH inhibition by either a pharmacological compound [22] or by high glucose levels [23]. Inhibition of transketolase, an enzyme involved in the non-oxidative part of the PPP (non-oxPPP), also reduces EC viability and migration [22]. Interestingly, inhibition of the non-oxPPP has a more pronounced effect on EC viability and migration than inhibition of the oxPPP, suggesting that the reversible non-oxPPP compensates for the reduced nucleotide synthesis via the oxPPP [22]. However, it is worth mentioning that the relative importance of the oxPPP *in vitro* might have been underestimated because of the high glucose concentrations used in many studies, which can suppress this branch [23]. Hence, both arms may be important, but in different metabolic contexts.

Cells store energy in glycogen reservoirs that can be used when extracellular glucose levels are insufficient to meet the energy demand (Figure 1). VEGF signaling and glucose deprivation induce glycogenolysis, which is the breakdown of glycogen to G6P in ECs [22]. Further, pharmacological inhibition of glycogen phosphorylase (and thus of glycogenolysis) reduces EC viability and migration [22]. Although glycogenolysis-derived G6P only has a limited contribution to glycolysis and energy production [24], it may have more importance in glycolysis side-branches such as the oxPPP. This has recently been shown to be the case for tumor cells [25], but similar evidence in ECs is still lacking. Overall, it is not yet clear which role glycogen has in vascular sprouting.

Other metabolic pathways

In addition to glucose, ECs also use glutamine and fatty acids as fuels (Figure 1). ECs are well-equipped to take up and metabolize glutamine [26,27], a conditionally essential amino acid [28]. Glutaminase catalyzes the conversion of glutamine to glutamate, the first step of glutaminolysis that yields α-ketoglutarate. The latter is used in the tricarboxylic acid (TCA) cycle to replace TCA cycle intermediates that have exited for anabolic purposes, thus sustaining the functionality of the TCA cycle (anaplerosis) [28]. In addition, glutamine is a key nitrogen source [28] and contributes to improved viability of ECs under oxidative stress [29]. The glutaminase activity of ECs has been reported to be high [10] and its inhibition induces EC senescence [30].

ECs can also obtain energy via the oxidation of fatty acids from intra- and extracellular sources [31] (Figure 1). Endothelial fatty acid oxidation (FAO) is increased *in vitro* upon glucose deprivation through activation of AMP-activated protein kinase (AMPK), and upon stimulation with carnitine [31] which is required for mitochondrial import of fatty acids. VEGF-B is a poor angiogenic factor with relatively restricted activity on coronary vessels, but VEGF-B signaling via VEGF receptor 1 (VEGFR1)/neuropilin 1 increases the uptake and *trans*-endothelial transport of fatty acids in ECs [32], but without influencing FAO in ECs [33]. VEGF induces the expression levels of fatty acid binding protein 4 (FABP4). This protein is involved in uptake and trafficking of intracellular fatty acids, and knockdown of FABP4 reduces EC proliferation [34], indicating the importance of lipid metabolism.

Sprouting in a metabolically taxing environment: tip versus stalk cells

Genetic signals

In response to hypoxia, the key angiogenic signal VEGF is secreted in an attempt to restore tissue oxygenation by promoting vessel sprouting. When VEGF reaches the vascular front it binds to VEGF receptor 2 (VEGFR2) on ECs. The EC exposed to the highest level of VEGF is selected to become a tip cell. The current model of vascular branching is based on the coordinated actions of migrating tip cells, taking the lead at the vascular front, and proliferating stalk cells elongating the newly developing branch [1], with the fate of both cell types being dynamically interchangeable rather than being permanently fixed [35] (Figure 2A).

The specification of tip versus stalk cells is dictated by VEGF–Notch signaling. VEGFR2-activated tip cells express the Notch ligand Delta-like 4 (Dll4), which binds to Notch receptors (single-pass transmembrane receptor proteins) in neighboring ECs. This results in the release of the receptor intracellular domain from the membrane, Notch intracellular domain (NICD), which translocates to the nucleus and regulates transcription of target genes [36]. As a result, VEGFR2 expression is lowered whereas levels of the VEGF trap VEGFR1 increase, which renders ECs less responsive to VEGF, thus out-competing their ability to become a tip cell and promoting a stalk cell fate (Figure 2A). Dynamic tip cell–stalk cell fate interchange or 'cell shuffling' occurs at the vascular front to allow the ECs with the highest VEGFR2 and lowest VEGFR1 expression levels to occupy the tip position to ensure maximal fitness of leading the sprout [35,37]. There are many more molecules regulating tip versus stalk cell behavior, such as for instance VEGF-C/VEGFR3, the angiopoietins, the TGF-β/BMP9 receptor, and others [1], but these signals are not known to affect EC metabolism in physiological conditions.

Metabolic signals

Recent findings indicate that VEGF increases glycolysis in ECs by upregulating PFKFB3 mRNA and protein levels before ECs start to proliferate, whereas Dll4-induced pro-stalk Notch signaling induces the opposite effect [8], implying that tip cells have higher glycolysis rates (Figure 2B). Nonetheless, glycolysis is also essential for stalk cell functions. Stalk cells actively proliferate and therefore must generate high amounts of ATP and macromolecules that are derived from metabolic pathways, such as the PPP, that branch off from the main glycolytic pathway [7] (Figure 2C). However, Notch inhibits EC proliferation *in vitro*. Hence, the growth-inhibitory function of Notch signaling must be overridden *in vivo*. Indeed, Notch in stalk cells induces the expression of the Notch-regulated ankyrin repeat protein (Nrarp), which in turn enhances Wnt signaling to promote stalk cell proliferation and vessel stability [38].

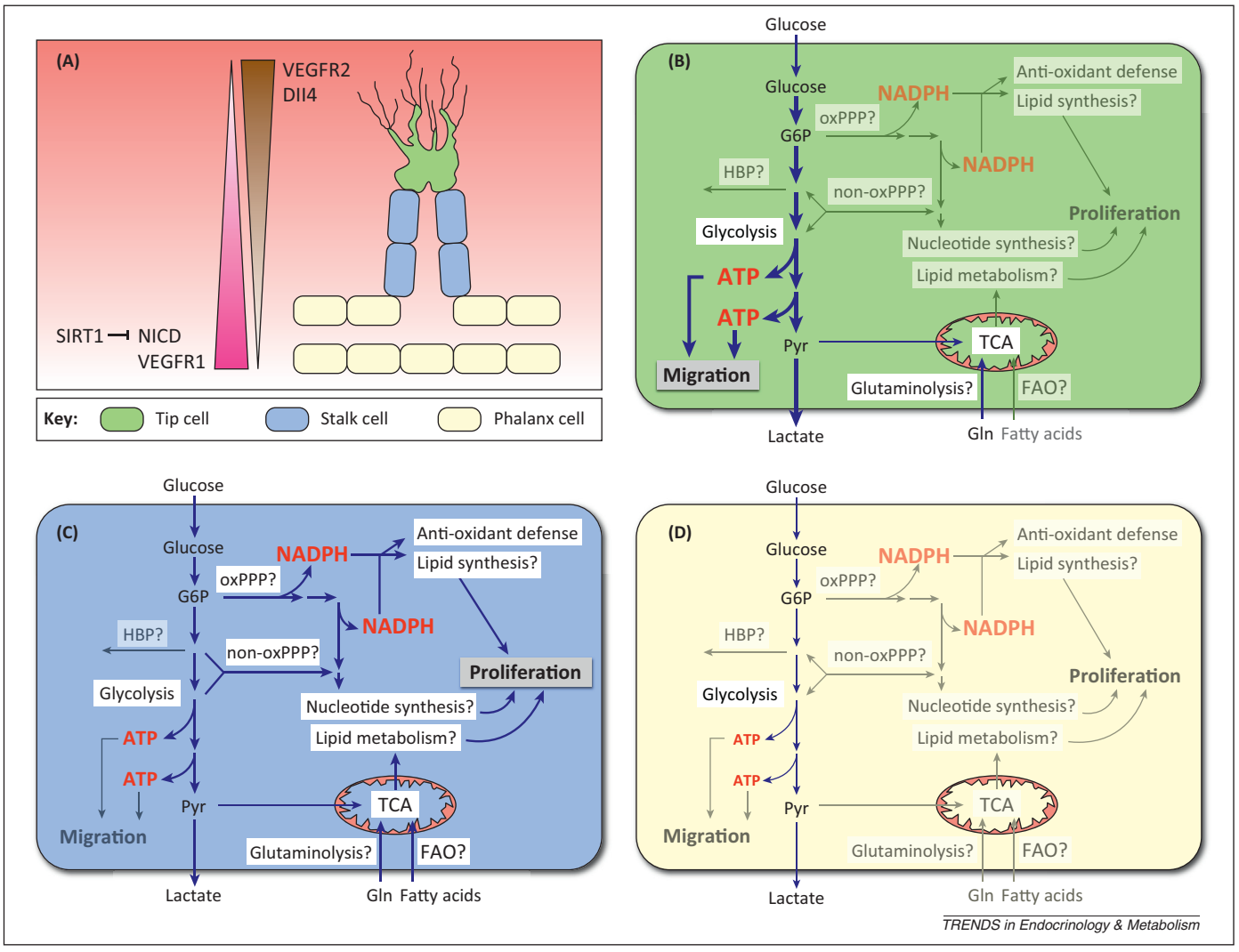

Figure 2. Genetic and metabolic determinants of tip, stalk and phalanx cell behavior. **(A)** Schematic representation of a vascular sprout with tip cell (green), stalk cell (blue) and phalanx cell (yellow) and key genetic fate-determining factors. Upon sensing a VEGF gradient (red background) the tip cell induces Dll4 expression to activate Notch signaling in the adjacent stalk cells (NICD release) and subsequently to decrease VEGFR2 and increase VEGFR1 levels to reduce VEGF sensitivity. SIRT1 translates energy stress into NICD degradation resulting in tip cell behavior. (B–D) Different types of metabolism characterize the different EC subtypes; unknown metabolic changes are indicated by question marks. For their migratory behavior, tip cells require large amounts of ATP. These are generated primarily by the high glycolytic flux **(B)**. Stalk cells divide to elongate the newly formed sprout and consequently require building blocks for rapid macromolecular synthesis. To obtain these precursors, NADPH is generated in the oxPPP, nucleotides are synthesized via the non-oxPPP and/or oxPPP, and fatty acids and ATP are obtained by combining glycolysis and likely also anapleurotic glutaminolysis **(C)**. Phalanx cell metabolism ensures cellular homeostasis and provides energy for vital functions **(D)**. Abbreviations: Dll4, Delta-like 4; FAO, fatty acid oxidation; G6P, glucose-6-phosphate; Gln, glutamine; HBP, hexosamine biosynthetic pathway; NADPH, nicotinamide adenine dinucleotide phosphate; NICD, Notch intracellular domain; (non-)oxPPP, (non-)oxidative branch of the pentose phosphate pathway; Pyr, pyruvate; SIRT1, Sirtuin1; TCA, tricarboxylic acid; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Remarkably, overexpression of PFKFB3 overcomes the pro-stalk activity of Notch signaling and promotes stalk cells to adopt a tip cell behavior, both in EC spheroid sprouting assays *in vitro* and in zebrafish vascular branching *in vivo*, whereas silencing of PFKFB3 causes opposite effects in baseline conditions and upon inhibition of Notch signaling [8]. These findings are intriguing because no other genetic signal is so far known to be able to overrule the pro-stalk activity of Notch. Notably, over- or underexpression of PFKFB3 in baseline conditions or in conditions favoring tip cell behavior (upon inhibition of Notch signaling), or stalk cell behavior (upon activation of Notch signaling), did not alter the molecular signature of genes enriched in either tip or stalk cells, indicating that PFKFB3 did not affect tip and stalk cells through regulation of tip/stalk cell signals [8]. Instead, these studies show for the first time that metabolism (in this case, PFKFB3-driven glycolysis) controls vessel branching in parallel to genetic signals (Figure 3).

The metabolic status of the milieu into which new vessels branch out complements the above mechanisms via a feedback loop involving the NAD^+-dependent deacetylase Sirtuin 1 (SIRT1). Upon sensing energy/fuel stress, SIRT1 activity increases to deacetylate and to pre-destine NICD for proteasomal degradation [39] (Figure 2A). These findings might imply that the subsequent lowering of the Notch-driven pro-stalk activity allows SIRT1 to direct tip cells towards fuel-rich areas. Conversely, ECs with reduced SIRT1 activity fail to sense nutrient stress adequately and are sensitized to pro-stalk Notch signaling, resulting in impaired vessel growth [40].

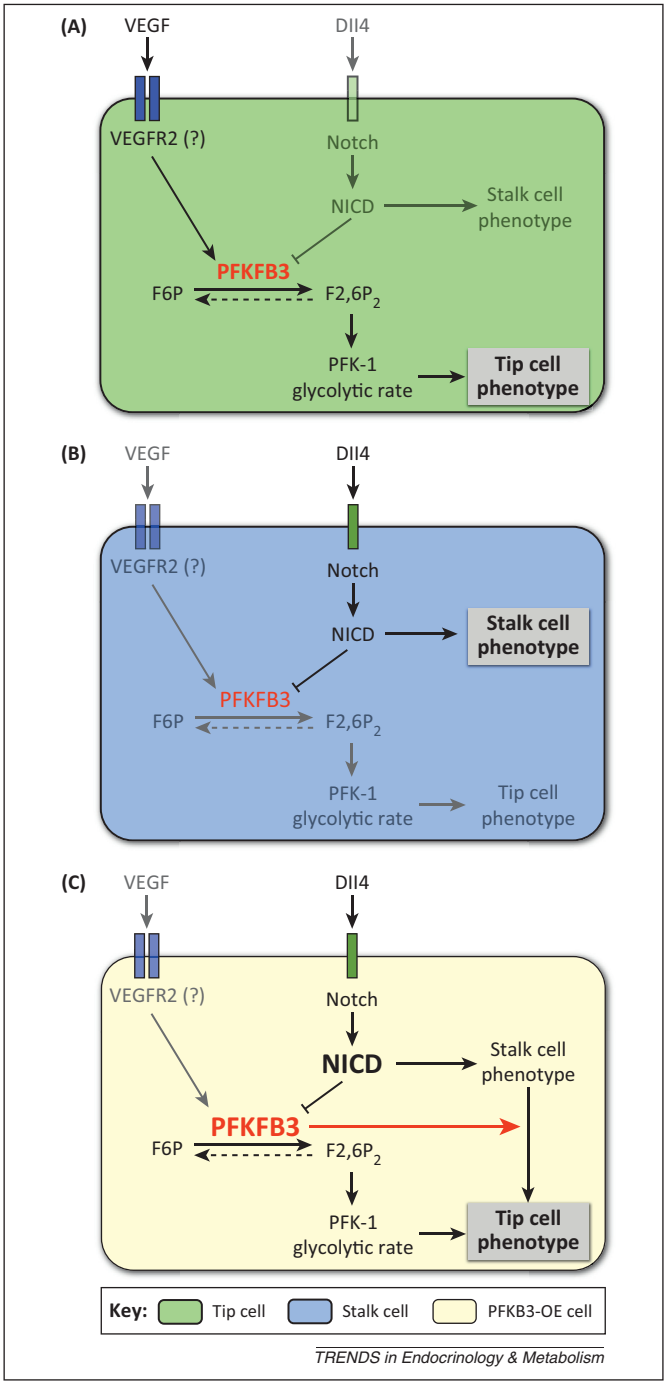

Figure 3. Metabolic switching between tip and stalk cells. **(A)** Metabolic glycolytic signature of tip cells: tip cells are known to have high VEGF/VEGFR2 signaling. Activation of VEGFR2 by VEGF upregulates PFKFB3 levels. PFKFB3 produces $F2,6P_2$, which is the most potent stimulator of PFK-1, a rate-limiting enzyme of the glycolytic pathway. Consequently, the glycolysis rate is elevated which renders ECs more competitive to reach the tip. By contrast, Dll4/Notch signaling, releasing NICD, reduces glycolysis by lowering PFKFB3 expression, but this signaling pathway is less active in tip cells. **(B)** Metabolic glycolytic signature of stalk cells: stalk cells are known to have high Dll4/Notch signaling. Activation of Notch by Dll4 lowers PFKFB3-driven glycolysis, thereby reducing competitiveness for the tip position. **(C)** Overexpression of PFKFB3 induces switching from the stalk to the tip cell phenotype: Overexpression of PFKFB3 in ECs, instructed to adopt a stalk cell phenotype by overexpression of NICD, is capable of overcoming the stalk cell phenotype and increasing the competitiveness of these cells for the tip position. Abbreviations: Dll4, Delta-like 4; $F2,6P_2$, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; NICD, Notch intracellular domain; PFK-1, phosphofructokinase-1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PFKFB3-OE, PFKFB3-overexpressing; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2 (the question mark indicates that VEGFR2 probably mediates the VEGF response, but this needs to be proven in the future).

The HBP pathway is another nutrient sensing pathway. Inhibition of this pathway by 2-DG impedes angiogenesis, which can be rescued by the *N*-linked glycosylation precursor mannose [41]. It is therefore tempting to speculate that the HBP in ECs serves to translate nutrient stress into adjusting blood vessel supply by generating *N*-acetylglucosamine that post-translationally modifies and activates key angiogenic receptors. Indeed, VEGFR2 function requires *N*-glycosylation [42]. Also noteworthy is the evidence that the Notch response to the ligands Dll4 and Jagged1 depends on the level of glycosylation of its extracellular domain [43].

Metabolites can also control angiogenesis by acting as *bona fide* signaling molecules. An example is the glycolysis product lactate, which is taken up by ECs via the monocarboxylate transporter 1 (MCT1), and which mainly has a signaling role because it seems to be only marginally metabolized [44], at least at normal extracellular glucose levels [24]. Once taken up by ECs, lactate competitively inhibits the oxygen-sensing prolyl hydroxylase domain protein 2 (PHD2), resulting in activation of hypoxia-inducible factor 1α (HIF-1α) and a secondary increase in VEGFR2 and basic fibroblast growth factor expression [45]. PHD2 inhibition by lactate also triggers IκBα degradation, releasing the transcription factor NF-κB to induce the expression of the pro-angiogenic interleukin-8 [44]. Lactate also engages the PI3K/Akt pathway via upregulation of the ligands and subsequent activation of three receptor tyrosine kinases: Axl, the angiopoietin receptor Tie2, and VEGFR2 [46]. Moreover, lactate signaling induces VEGF expression [47] and reduces post-translational poly-ADP ribosylation of VEGF, thereby making it more potent [48]. Lactate also promotes collagen and proteoglycan matrix deposition [49], and stimulates EC migration and capillary tube formation [44,49], although it remains to be defined if matrix deposition induced by lactate is causally linked to vessel sprouting. Finally, lactate induces pro-angiogenic reactive oxygen species in ECs [44].

Compartmentalization of glycolysis in F-actin-rich lamellipodia

In ECs, both filopodia and lamellipodia have typical actin networks (branched actin for lamellipodia versus aligned filamentous actin for filopodia) that can be assembled within minutes after growth factor stimulation [50]. The highly dynamic nature of these structures relies on active actin cytoskeleton remodeling and requires rapid generation and instantaneous availability of large amounts of ATP; actin polymerization for example is tightly linked to ATP hydrolysis [51–53]. Nevertheless, both filopodia and lamellipodia are devoid of mitochondria [8], likely because mitochondria (ranging in size from 0.5 to over 4 μm) cannot spatially fit into the narrow filopodia and lamellipodia (~250 nm wide and high, respectively) [54] (Figure 4A,C,D).

It is unlikely that sufficient amounts of ATP reach the very tip of the filopodia (up to 10 μm in length) sufficiently fast by diffusion from the distant perinuclear mitochondrial network; but, even so, it is conceivable that the continuous protrusion and retraction of filopodia and lamellipodia locally generates such a high ATP demand that ATP levels

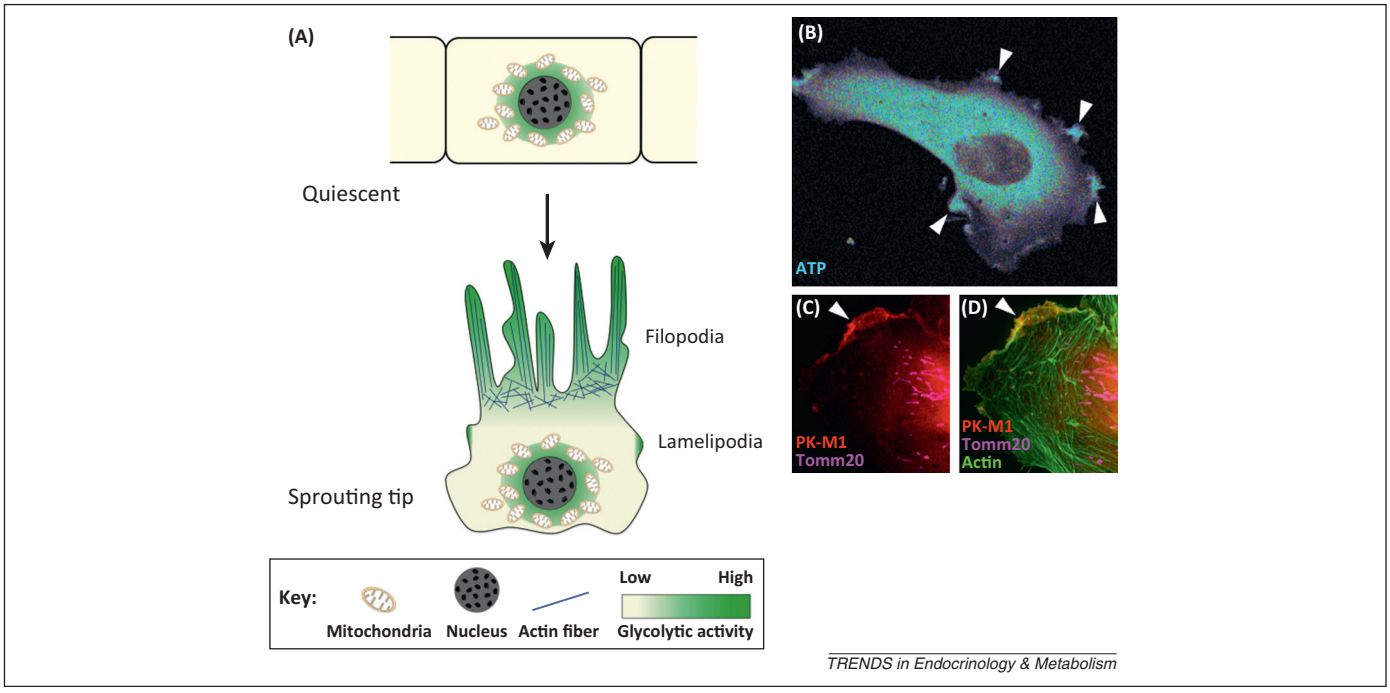

Figure 4. Compartmentalization of glycolysis with F-actin. **(A)** Schematic representation of a quiescent and a tip cell with mitochondria in the perinuclear region but, in the case of the tip cell, not in filopodia or lamellipodia in which glycolytic activity is compartmentalized with F-actin. **(B)** Imaging of intracellular ATP levels in endothelial cells (ECs) with a GO-Ateam ATP fluorescence resonance energy transfer (FRET) probe showing the presence of ATP in lamellipodia (white arrowheads). (C,D) Presence of glycolytic activity in lamellipodia in the absence of mitochondria. EC double-stained for the glycolytic enzyme pyruvate kinase PK-M1 and the mitochondrial marker Tomm20 (lamellipodia indicated by white arrowhead) **(C)** and merged with actin staining **(D)**.

throughout the whole cell body might become compromised during active lamellipodial and filopodial activity. To avoid this, ECs compartmentalize glycolysis with the actin skeleton in cellular protrusions, mediating EC motility [8]. Various glycolytic enzymes contain actin-binding sites, and binding to F-actin enhances their activity [55]. In migrating ECs, both PFKFB3 and other glycolytic enzymes – such as phosphoglycerate kinase and the pyruvate kinase isoenzyme that generates ATP (PK-M1), rather than the isoenzyme involved in biosynthesis of macromolecules (PK-M2) – are enriched in the membrane ruffles at the leading front of lamellipodia, and co-concentrate with F-actin in these structures [8] (Figure 4B–D). Co-immunoprecipitation experiments confirmed the PFKFB3-actin interaction and showed that the fraction of PFKFB3 bound to actin increases during EC motility [8]. Localizing glycolysis-driven ATP production at the site of high ATP demand during motility prevents the cell from succumbing to overall ATP depletion. Of note, compartmentalization of glycolysis in motile structures originated early in evolution: for example, flagella in various microorganisms and tentacles on members of the basal metazoan *Hydra* genus [56,57]. Noteworthy in this respect, astrocytes localize glycogen depots in their lamellipodial and filopodial extensions, and glycolysis and glycogenolysis are essential for astrocytic responses to increasing energy demand [58].

Phalanx cells: quiescent again

Genetic signals

ECs resume a quiescent state once the new vessel branch is formed, and then adopt a cobblestone-like appearance that resembles the ancient Greek military phalanx formation – hence the term 'phalanx cells' [59]. These cells have few filopodia, are non-proliferating, survive for years, migrate poorly, and have an attenuated response to VEGF. Phalanx cells express elevated levels of VEGFR1 to diminish the pro-angiogenic signaling of VEGF [59] (Figure 2A). Elevated signaling through Notch and the Tie2 receptor in response to elevated levels of angiopoietin-1 between ECs in *trans* additionally promotes EC quiescence [60,61].

Phalanx cells form a tight barrier and their monolayer apposition allows conduction of blood flow, which by itself is an important regulator of EC quiescence [1]. Vessel stabilization is further enhanced by the deposition of a basement membrane (BM) around quiescent ECs; this is in part due to the fact that the BM component laminin α4 reduces tip cell specification by inducing Notch signaling [62,63]. The expression of vascular endothelial cadherin (VE-cadherin) and neuronal cadherin (N-cadherin) in quiescent ECs is crucial for cell–cell adhesion and optimal endothelial barrier function, which are required for controlled exchange of fluids and solutes between the bloodstream and the neighboring tissue [64,65]. Lowering of pro-angiogenic signals and increasing pro-quiescence molecules promote ECs quiescence.

Metabolic signals

Less is known about the metabolic changes that precede or accompany the EC's switch to quiescence. Interestingly, cell quiescence is often characterized by a higher level of metabolism than previously anticipated [15]. Quiescent ECs lower their glycolysis rate only by 40% – the proportion of total glycolysis that is required for proliferation and migration [8] (proliferation has been estimated to require a 30% increase in ATP production over quiescence [66]). The remaining 60% of glycolysis in quiescent ECs ensures

cellular homeostasis and provides the necessary energy for vital functions such as safeguarding barrier functions, matrix deposition, and glycocalyx production [1,67] (Figure 2D).

Concluding remarks and future perspectives

Over the past decade the angiogenesis field has gained an enormous insight into the molecular players that control the formation of vessels in physiological and pathological conditions. Metabolism only recently entered the field of angiogenesis as an important player. Although there are some published data on the role of separate metabolic pathways in ECs *in vitro*, a unifying view on how major metabolic pathways converge in *in vivo* vessel branching is almost nonexistent. Recent findings on how PFKFB3-driven glycolysis determines vessel branching have only shown the proverbial tip of the iceberg, and numerous outstanding questions remain to be addressed (Box 1). For example, a 'metabolo-map' of ECs in quiescence versus sprouting state is largely missing, and other pathways besides glycolysis have been only minimally studied. Furthermore, it is currently unknown how other branching signals (aside from VEGF and Notch) control metabolism and vice versa. The importance of metabolic communication between ECs and other cell types, in normal tissues as well as in the tumor microenvironment, remains unclear. In addition, it is crucial to understand how the metabolic adaptations of growing tumor vessels differ from those of rapidly proliferating cancer cells because this will influence our therapeutic targeting strategies. Another outstanding question is whether the definition of an underlying metabolic shift that ECs undergo from quiescence to angiogenesis will allow the development of new 'anti metabolic anti angiogenic' agents, and whether targeting EC metabolism could therefore provide a valuable alternative to overcome resistance to currently approved anti angiogenic therapies, without causing unacceptable toxicity. This is a timely question given that novel anti angiogenic strategies with fundamentally new mechanisms will likely aid in overcoming toxicity and resistance issues of current anti angiogenic therapies [3]. The generation of EC-specific metabolic knockout animals, as well as the continuous improvement of techniques to assess metabolic fluxes of ECs in quiescent and growing vessels *in vivo*, will be required to answer all these intriguing questions.

Box 1. Outstanding questions

- What are the metabolic properties of ECs in quiescent as well as sprouting conditions?
- What other pathways in addition to glycolysis are involved in sprouting?
- How do other branching signals (aside from VEGF and Notch) control metabolism, and vice versa?
- How important is metabolic communication between ECs and other cell types in normal tissues as well as in the tumor microenvironment?
- In which sense do the metabolic adaptations of growing tumor vessels differ from those of rapidly proliferating cancer cells, and will this influence our therapeutic targeting strategies?
- Will targeting EC metabolism provide a valuable alternative for overcoming resistance to currently approved anti angiogenic therapies, without causing unacceptable toxicity?

Disclaimer statement

P.C. declares to be named as inventor on patent applications claiming subject matter related to the results described in this paper.

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Treating Diabetes by Blocking a Vascular Growth Factor

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Ectopic lipid deposition in muscle and liver is associated with the pathogenesis of type II diabetes. Hagberg et al. (2012) report that targeting the vascular endothelial growth factor (VEGF)-B restores insulin sensitivity and glucose tolerance by inhibiting endothelial-to-tissue lipid transport, opening promising avenues for diabetes therapy.

Type II diabetes represents a formidable unmet medical health problem with more than 300 million people estimated to be affected worldwide. Individuals with diabetes have an elevated risk of vascular disease (atherosclerosis, stroke) and other complications. Diabetes-related deaths are expected to rise at an alarming speed over the coming years. Insulin resistance has been linked to accumulation of toxic lipid intermediates (ceramides, diacylglycerol) in skeletal muscle and liver. Hence, strategies preventing lipid deposition may offer therapeutic benefit but are not widely available (Samuel and Shulman, 2012). Hagberg et al. (2012) demonstrate that targeting the vascular endothelial growth factor B (VEGF-B) restores insulin sensitivity and prevents type II diabetes by reducing lipid accumulation in muscle.

How can an angiogenic growth factor like VEGF-B be linked to diabetes? VEGF-B was one of the later members of the VEGF family to be identified, and its activity remains poorly understood (Fischer et al., 2008). It is expressed in the heart, skeletal muscle, and brown fat and binds to VEGF receptor-1 (VEGFR1; flt-1) and its coreceptor neuropilin-1 (NRP1). Genetic studies indicate that VEGF-B's angiogenic capacity is contextual and largely restricted to the heart, while pharmacological blockade inhibits ocular angiogenesis. In cancer, VEGF-B is, however, anti-angiogenic. Overall, its activity is less potent than other traditional angiogenic signals, but it induces strong arterialization of the coronary vasculature in rats (Bry et al., 2010). Importantly, VEGF-B overexpression in mouse but not in rat hearts leads to hypertrophy and signs of mitochondrial lipotoxicity, suggesting a possible role in cardiac metabolism (Bry et al., 2010; Karpanen et al., 2008). However, VEGF-B does not affect mitochondrial function per se (Hagberg et al., 2010; Karpanen et al., 2008). Rather, it promotes lipid transport across the endothelial barrier by upregulating the fatty acid transport proteins (FATP)-3/4 (Hagberg et al., 2010). Indeed, lipid uptake and deposition in muscle were reduced, and lipids were shunted to white adipose tissue in VEGF-B-deficient mice. Consequently, the fat mass and body weight increased, and glucose uptake to the heart was enhanced. These initial observations prompted Hagberg et al. (2012) to investigate the potential of anti-VEGF-B therapy for insulin resistance.

In their follow-up study, Hagberg et al. (2012) demonstrate that genetic deficiency of VEGF-B in mouse models of insulin resistance and type II diabetes, including *db/db* diabetic mice (which carry mutations in the leptin receptor gene) and mice fed a high-fat diet, reduces lipid storage in muscle, heart, and pancreas—but not liver. VEGF-B inhibition reduced plasma triglyceride and non-esterified fatty acid levels and normalized the HDL-c to LDL-c ratio, indicating a reduced risk for cardioavascular pathology. However, the body weight of VEGF-B-deficient mice on a high-fat diet was increased. Nevertheless, VEGF-B deficiency lowered blood glucose levels without increasing insulin secretion and restored insulin sensitivity and glucose uptake in the muscle and heart.

Pharmacological inhibition of VEGF-B via the administration of an anti-VEGF-B antibody to *db/db* mice or rats fed a high-fat diet largely phenocopied the genetic findings and enhanced insulin sensitivity. In a preventive setting (prediabetic *db/db* mice), VEGF-B blockade prevented the development of hyperglycemia, reduced lipid uptake in muscle, improved glucose tolerance, and protected against dyslipidemia. In a therapeutic setting (diabetic *db/db* mice), VEGF-B inhibition halted the progression of hyperglycemia and muscle lipid uptake. In addition, VEGF-B blockade improved pancreatic islet morphology, restored insulin and glucagon expression in the islets, and reduced islet cell death in both models. In another high-fat diet rat model, coincident initiation of the high-fat diet and anti-VEGF-B treatment normalized glucose tolerance and glucose-stimulated insulin secretion, reduced glucose infusion and disposal rates in a hyperinsulinemic/euglycemic clamp study, and promoted glucose uptake in muscle—evidence of improved insulin sensitivity. These results support the idea that VEGF-B regulates lipid uptake by muscles. Given that VEGF-B regulates lipid transport across the endothelium, the findings suggest that the endothelium acts as a prominent barrier controlling muscle lipid uptake. Furthermore, blocking VEGF-B provides a mechanism to ameliorate or even prevent type II diabetes (Figure 1).

As with any breakthrough, this study raises a number of questions. For instance, is the effect of VEGF-B on *trans*-endothelial lipid transport the only mechanism? VEGF-B has a poor angiogenic activity in healthy conditions, but an effect of this growth factor on the vasculature in conditions of insulin resistance remains possible. Another question is whether VEGF-B can control insulin signaling in

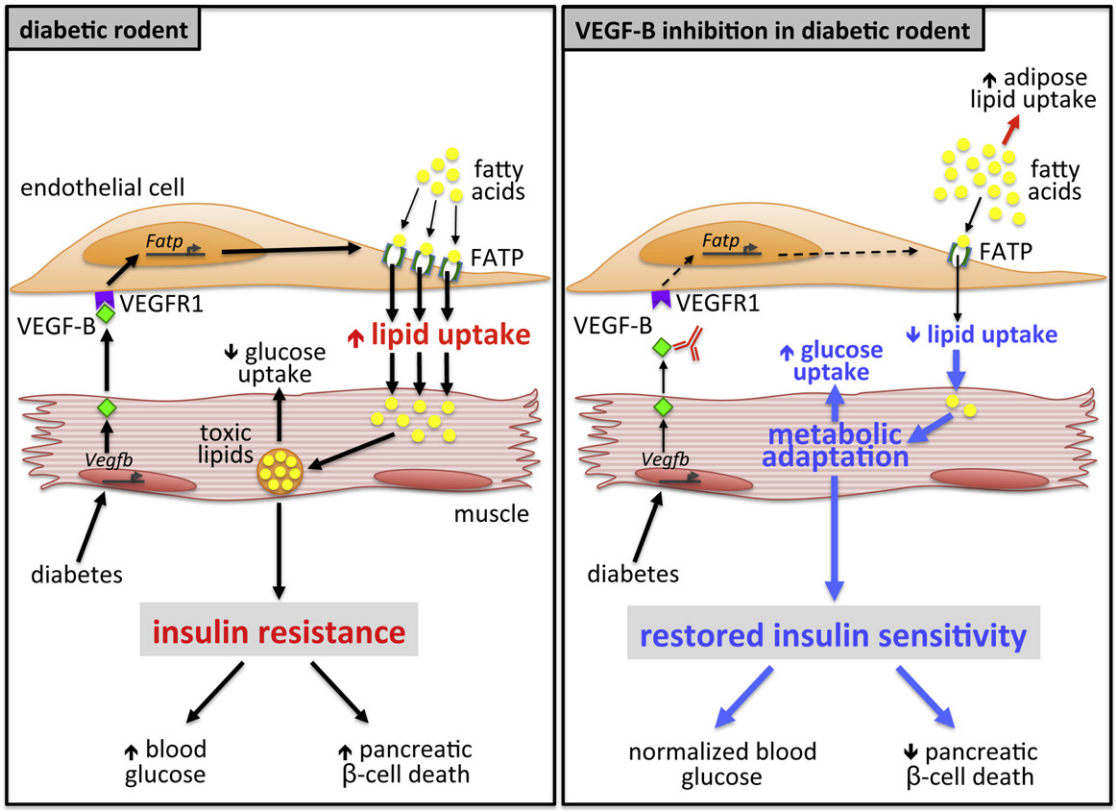

Figure 1. VEGF Promotes Diabetes by Facilitating Lipid Uptake by Muscle

In diabetic rodents (left panel), vascular endothelial growth factor (VEGF)-B, expressed in muscle, binds to VEGF receptor-1 (VEGFR1; flt-1) on endothelial cells to increase the expression of fatty acid transport proteins (FATP). FATP increase lipid transport across the endothelium to the muscle, where it accumulates as lipid droplets, resulting in insulin resistance and leading to increased blood glucose and pancreatic β cell death. VEGF-B blockade (right panel), using a neutralizing antibody, prevents VEGF-B-mediated upregulation of FATP in the endothelium, thus reducing lipid transport to the muscle. As a result, lipid uptake in the muscle is decreased, with a compensatory increase in glucose uptake, resulting in restored insulin sensitivity and leading to normalized blood glucose and preservation of pancreatic islet area and function. Of note, peripheral adipose lipid uptake is consequently increased, leading to increased body weight. Similar data were obtained after genetic deletion of VEGF-B.

endothelial cells, a process known to increase insulin delivery to muscle (Kubota et al., 2011). Since insulin also promotes lipid uptake in muscle, VEGF-B's activity might also depend on effects on insulin. It would also be interesting to test whether endothelial deletion of NRP1 or VEGFR1, known to reduce the levels of the FATP4 transport protein in vitro (Hagberg et al., 2010), phenocopies the protective effects of VEGF-B blockade in vivo. Given that different VEGF-B-deficient mouse strains exhibit distinct phenotypes in baseline conditions (Aase et al., 2001; Bellomo et al., 2000), a confirmation of the antidiabetic phenotype in another VEGF-B-deficient strain would strengthen the current findings. It would also be interesting to explore if VEGF-B has additional prodiabetic effects through other changes in endothelial cells, perhaps modifying endothelial cell metabolism or endothelial-to-adipocyte differentiation, or altering vascular inflammation, a process known to modify insulin sensitivity.

For anti-VEGF-B treatment to be clinically relevant, the data of the Hagberg et al. (2012) paper should be relevant for humans. While the authors paid great effort to test various rodent models, genetic association studies have not yet overwhelmingly identified VEGF-B as a possible risk factor for diabetes in humans. Another clinically relevant question is whether therapeutic blockade of VEGF-B therapy is safe. Deficiency of VEGF-B is well tolerated. Nonetheless, VEGF-B has been implicated in neurogenesis and neuroprotection (Poesen et al., 2008) and ischemic cardiac revascularization, raising the question whether VEGF-B blockade might worsen diabetic neuropathy or ischemic heart disease. The role of VEGF-B in inhibiting tumor angiogenesis will also deserve consideration. Overall, this work places the endothelium and its regulation by the VEGF-B ligand at the center stage of tissue lipid homeostasis, with promising therapeutic potential in the context of diabetes, obesity, and dyslipidemia.

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Mining Genes in Type 2 Diabetic Islets and Finding Gold

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Pancreatic β cell failure is central in the pathogenesis of type 2 diabetes (T2D), but the mechanisms involved remain unclear. Mahdi and colleagues (2012) couple global evaluation of gene expression with coexpression network analysis of human islets from T2D patients to identify SFRP4 as an early mediator of β cell dysfunction in T2D.

Genome-wide association studies for T2D have so far identified 65 susceptibility loci for the disease (Morris et al., 2012), but together these loci account for less than 10% of the variance in disease susceptibility. This contrasts with the situation in type 1 diabetes (T1D), where such studies have identified 50 loci across the human genome associated with T1D that explain nearly 80% of the heritability (Pociot et al., 2010). Other approaches to identify basic mechanisms of disease are therefore needed (Taneera et al., 2012). Furthermore, direct studies of the diseased human tissue—in the case of T2D, human islets are the gold standard given the central role of β cell dysfunction in its pathogenesis—are essential to further our understanding of human diabetes (Cnop et al., 2005; Kahn, 2003). In this issue of *Cell Metabolism*, Mahdi and colleagues tackle these challenges by performing microarray analyses of human islets isolated from T2D and normoglycemic individuals (Mahdi et al., 2012) (Figure 1). They identify a group of T2D-associated genes related to interleukin-1 (IL-1), a proinflammatory cytokine, and show that secreted frizzled-related protein 4 (SFRP4) is highly associated with T2D.

Previous work already suggested that IL-1β plays a role in β cell dysfunction and death in T1D and T2D. However, whereas a role for inflammation in β cell loss is well established in the context of T1D (Eizirik et al., 2009), it has remained controversial for T2D (Cnop et al., 2005; Donath et al., 2008). To explain local IL-1β production in the islets of T2D individuals, one model proposed that IL-1β production was induced by glucose, leading to upregulation of the apoptotic Fas receptor and ligand and β cell "suicide" (Donath et al., 2008), but this was not confirmed by other groups (reviewed in Cnop et al., 2005). In recent years a more nuanced view of inflammation in T2D islets emerged, consisting of mild upregulation of cytokines and chemokines in islets from T2D patients, possibly mediated by increased circulating concentrations of the free fatty acid palmitate that induces islet IL-1β and TNF-α expression (Igoillo-Esteve et al., 2010). Experimental findings show that this metabolic "T2D-like" stress induces a mild inflammatory response, representing around 5%–10% of the proinflammatory response of human islets exposed to "T1D-like" conditions (Cnop et al., 2005; Igoillo-Esteve et al., 2010). The role for this "low-intensity" innate immunity-mediated inflammation in β cell dysfunction and death in T2D remains unclear. For instance, an IL-1 receptor antagonist blocked palmitate-induced chemokine expression, but failed to prevent apoptosis (Igoillo-Esteve et al., 2010).

Using gene expression topology with weighted gene coexpression network analysis (in which coexpressed genes are clustered into gene modules based on their connectivity), Mahdi et al. now identify a T2D-related gene module enriched for IL-1-related genes. Among the most connected hub genes, the authors identify SFRP4 as highly associated with T2D, HbA1c (a measure of average glucose levels over the past 2 months), and insulin secretion (Figure 1). Subsequent functional studies then show that the SFRP4 protein is induced by the cytokine IL-1β. Furthermore, the authors show