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REVIEW

Targeting endothelial cell metabolism: new therapeutic prospects?

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Abstract **Endothelial cells (ECs) line blood vessels and function as a vital conduit for oxygen and nutrients, but can also form vascular niches for various types of stem cells. While mostly quiescent throughout adult life, ECs can rapidly switch to a highly active state, and start to sprout in order to form new blood vessels. ECs can also become dysfunctional, as occurs in diabetes and atherosclerosis. Recent studies have demonstrated a key role for EC metabolism in the regulation of angiogenesis, and showed that EC metabolism is even capable of overriding genetic signals. In this review, we will review the basic principles of EC metabolism and focus on the metabolic alterations that accompany EC dysfunction in diabetes and vessel overgrowth in cancer. We will also highlight how EC metabolism influences EC behavior by modulating post-translational modification and epigenetic changes, and illustrate how dietary supplementation of metabolites can change EC responses. Finally, we will discuss the potential of targeting EC metabolism as a novel therapeutic strategy.**

Keywords angiogenesis, metabolism, endothelial cell dysfunction, anti-angiogenic therapy

The basics of endothelial cell biology

In healthy adults, ECs can remain quiescent for years. Originally considered only as an inert lining, solely meant to conduct the blood, ECs perform many more functions, among which the supply of oxygen and nutrients, the removal of metabolic waste, the transport of white blood cells for immune surveillance, hemostasis, regulation of the vascular tone (Davignon and Ganz, 2004) and the control of transendothelial transport of macromolecules (Pober et al., 2009). Moreover, ECs rapidly respond to pro-angiogenic stimuli that orchestrate vascularization of tissues deprived of oxygen and nutrients. Vascular endothelial growth factor (VEGF) is a key player in the tightly coordinated process of angiogenesis. Through VEGF/VEGF receptor 2 (VEGFR2) signaling, quiescent ECs adopt a migrating "tip" cell behavior at the forefront of the vessel sprout (Potente et al., 2011). In the elongating sprout, Delta-like 4 (DLL4)-mediated activation of Notch signaling promotes the phenotype of trailing "stalk" cells that proliferate behind the tip cell and extend the vascular sprout (Phng and Gerhardt, 2009). ECs compete for the leading position in a highly dynamic process of repositioning of tip and stalk cells, with non-competitive tip cells being overtaken by fitter stalk cells (Jakobsson et al., 2010). In addition to numerous genetic signals regulating this process (Eelen et al., 2013), a metabolic switch in ECs has been recently postulated to control vessel sprouting as well (De Bock et al., 2013b).

In the adult, new vessels can grow via sprouting (angiogenesis), but bone marrow-derived endothelial progenitors can also contribute to new vessel growth (vasculogenesis), though their relevance remains debated (Moschetta et al., 2014). ECs can also arise from resident stem cells in the vessel wall (Kawabe and Hasebe, 2014), or from endothelial colony-forming cells in the circulation (Pelosi et al., 2014). ECs also establish a vascular niche for stem cells in the brain, hematopoietic system in the bone marrow and cancers (Gómez-Gaviro et al., 2012; Takakura, 2012; Mendelson and Frenette, 2014; Tabe and Konopleva, 2014). Since the role of EC metabolism in vessel sprouting using genetic models has only recently being studied, and

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nearly nothing is known about the metabolism of EC progenitors or vascular niche ECs, we will restrict our review to overviewing the metabolic changes of differentiated ECs during vessel sprouting in health and disease.

Glycolysis fuels vessel sprouting

Despite immediate access to oxygen in the blood, sprouting ECs rely primarily on glycolysis for energy production (De Bock et al., 2013b). VEGF stimulation doubles the glycolytic flux of ECs and increases the expression of the glycolysis regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), an activator of phosphofructokinase 1, itself a rate-limiting enzyme in glycolysis (De Bock et al., 2013b). Genetic or pharmacologic inhibition of PFKFB3 impairs sprouting of ECs *in vitro*, and reduces vessel branching and outgrowth *in vivo* (De Bock et al., 2013b). Importantly, glycolysis can overrule genetic signals that regulate EC specification during vessel sprouting. Indeed, overexpression of PFKFB3 converts genetically-programmed (by Notch) stalk cells into tip cells, while PFKFB3 knockdown diminishes tip cell behavior (De Bock et al., 2013b). Notably, glycolytic enzymes in ECs associate with F-actin and compartmentalize in lamellipodia and filopodia, where rapid and localized ATP production enables cytoskeleton remodeling during cell migration (De Bock et al., 2013b).

In sprouting ECs, glycolysis accounts for the production of 85% of the total cellular ATP content (De Bock et al., 2013b). The role of mitochondria in ECs remains more debated. Certain studies show that mitochondria in ECs are predominantly involved in the production of reactive oxygen species (ROS) for signaling purposes (Quintero et al., 2006; Groschner et al., 2012), while another study documented a role for mitochondrial respiration in the production of ATP in proliferating ECs (Coutelle et al., 2014).

Additional metabolic pathways in ECs

Glycolytic products and intermediates shunted in other metabolic pathways can serve as essential precursors of macromolecules (De Bock et al., 2013a). Glucose-6-phosphate enters the pentose phosphate pathway (PPP), a side pathway of glycolysis that generates NADPH and ribose-5-phosphate, necessary for redox balance and nucleotide synthesis, respectively. The viability and migration of ECs are reduced upon inhibition of both glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the oxidative PPP branch, and transketolase (TKT), a rate-limiting enzyme of the non-oxidative PPP branch (Zhang et al., 2000; Vizán et al., 2009). In addition, the PPP serves as a protector against oxidative stress through NADPH-dependent conversion of oxidized glutathione (GSSG) to its reduced form (GSH), a key cellular antioxidant (Leopold et al., 2003).

The hexosamine biosynthetic pathway (HBP) is another side branch of glycolysis that generates N-acetylglucosamine for protein O- and N-glycosylation. In particular, fructose-6-phospate is shunted in the HBP and converted to glucosamine-6-phospate (GlucN6P), which is in turn metabolized to uridine diphosphate N-acetylglucosamine (UDPGlcNAc), a substrate for glycosylation. As discussed in more detail below, the HBP may serve as a nutrient-sensing pathway that is responsible for glycosylation of proteins that influence angiogenesis (Merchan et al., 2010).

Amino acid metabolism can be used for energy production, redox homeostasis and biosynthesis of macromolecules, though its role in ECs remains largely enigmatic. L-arginine is used by endothelial nitric oxide synthase (eNOS) to produce citrulline and nitric oxide (NO) (Tousoulis et al., 2012). In addition, glutamine metabolism is critical for ECs, since inhibition of glutaminase-1, which converts glutamine into glutamate as rate-limiting enzyme of glutaminolysis, causes premature senescence of ECs (Unterluggauer et al., 2008). In addition, ECs rely on glutamine for the synthesis of ornithine, a precursor of pro-angiogenic polyamines (Wu et al., 2000). Nevertheless, the precise role of glutamine metabolism in vessel sprouting remains to be determined.

The role of fatty acid β-oxidation (FAO) in ECs has not been clearly defined as well. Knockdown of the fatty acid binding protein 4 (FABP4), which mediates intracellular transport and metabolism of fatty acids, decreases VEGF-dependent proliferation in ECs (Elmasri et al., 2009). FABP4 gene expression is upregulated by Notch (Harjes et al., 2014). In certain conditions, FAO might represent a fuel source for ECs, such as during glucose deprivation, when ECs increase the FAO flux (Dagher et al., 2001). However, in normal conditions, FAO contributes to $< 5\%$ of the total amount of ATP in ECs (De Bock et al., 2013a). Whether FAO regulates redox homeostasis in ECs, as it does in stressed cancer cells (Jeon et al., 2012; Carracedo et al., 2013), remains unknown.

Metabolic features of ECs in disease

In cancer, inflammation and ocular disorders, angiogenesis is excessive and deregulated. Conversely, in diabetes, atherosclerosis and other cardiovascular disorders, the vascular endothelium is dysfunctional, though vessel overgrowth also occurs in a context-dependent manner (Rajendran et al., 2013). In the following sections, we will focus on the metabolic features of ECs in diabetes and cancer as examples of, respectively, EC dysfunction and vessel overgrowth. As a general consideration, metabolism of ECs in disease has been mostly studied *in vitro* by mimicking disease-like conditions, for instance by exposing healthy ECs to high glucose, when studying diabetes. It should be mentioned however that high glucose cannot adequately and comprehensively reflect the more complex, multifactorial situation of diabetes *in vivo* and the different mechanisms in diabetes type I and type II.

Another consideration is that the relative contribution of different metabolic pathways in ECs may vary between species, EC subtypes or the experimental conditions (Oldendorf et al., 1977; Parra-Bonilla et al., 2010). Nonetheless, despite these limitations, exciting insights have been obtained from the available studies.

EC dysfunction in diabetes

Experimental and clinical evidence indicates that hyperglycemia is a major risk factor for EC dysfunction in diabetes (Funk et al., 2012). Hyperglycemia contributes to maladaptive changes of EC metabolism, resulting in macro- and microvascular complications in diabetes. It is widely accepted that the functional perturbation of the endothelium under hyperglycemic conditions is to a large part attributable to the generation of reactive oxygen species (ROS) and oxidative stress, a finding that has been confirmed in diabetic mice and patients, *in vivo* or *ex vivo* after isolation of ECs (Fig. 1) (Wu and Meininger, 1995; Meininger et al., 2000; Meininger et al., 2004; Kizhakekuttu et al., 2012; Tian et al., 2012; Cho et al., 2013; Hernandez-Mijares et al., 2013; Mackenzie et al., 2013; Sudhahar et al., 2013; Venkatesan et al., 2013). So far, various metabolic changes in ECs have been suggested (Brownlee, 2001; Rask-Madsen and King, 2013). First, high glucose levels cause mitochondrial dysfunction, leading to increased production of ROS via protein kinase C-dependent activation of NADPH-dependent oxidases (Brandes et al., 2014).The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is then indirectly inactivated by ROS (Du et al., 2003) and, in parallel, high glucose levels inhibit G6PD, a rate limiting PPP enzyme, thus decreasing the production of NADPH for glutathione reduction (Zhang et al., 2000). The stalling of the PPP and glycolysis in turn shunts more glucose to the polyol pathway, where it is converted to sorbitol by aldolase reductase (ALR2), further depleting NADPH stores and increasing ROS levels (Lorenzi, 2007).

Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO), an important vasodilator that also inhibits platelet aggregation (Forstermann and Sessa, 2012). The activity of eNOS is dependent on the availability of the cofactors tetrahydrobiopterin (BH4) and coenzyme Q (CoQ10) (Gorren et al., 2000; Crabtree et al., 2009; Mugoni et al., 2013). Thus, eNOS becomes "uncoupled" when BH4 is limiting, resulting in the production of excess ROS instead of NO (Stroes et al., 1997; Mugoni et al., 2013). High glucose levels uncouple eNOS, because of reduced BH4 availability and elevated levels of oxidizing peroxynitrite, and favor O-glycosylation of eNOS, further impairing its activity and hence increasing ROS levels (Du et al., 2001; Zou et al., 2002; Cai et al., 2005; Beleznai and Bagi, 2012). eNOS is also uncoupled in atherosclerosis (Takaya et al., 2007). ROS also impair angiogenesis via ligand-independent phosphorylation of VEGFR2's cytosolic domain by Src family kinases, leading to its reduced availability at the cell surface (Warren et al., 2014).

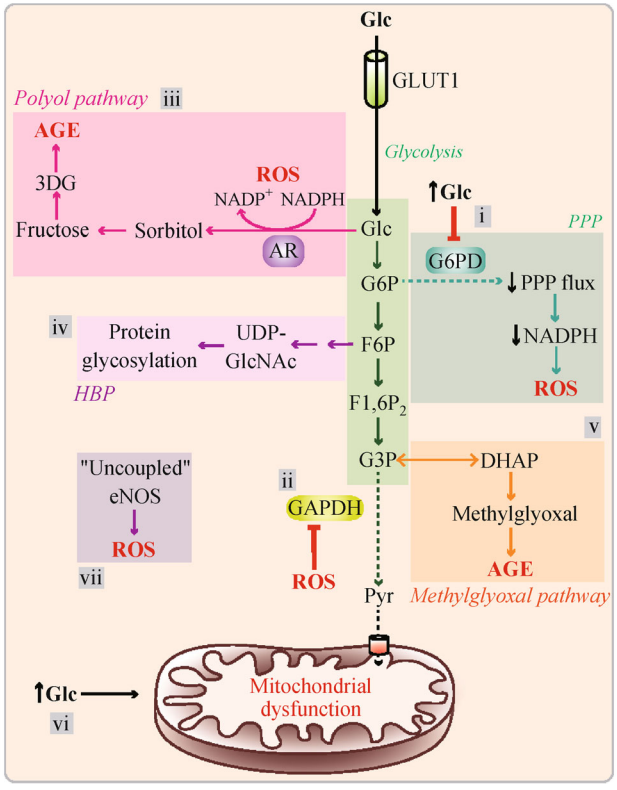

Figure 1 Metabolic perturbations in diabetes. Scheme highlighting the metabolic changes in ECs in diabetes. For clarity, not all metabolites and enzymes of the depicted pathways are shown. Dashed lines indicate downregulation of the metabolic pathway. [i] High glucose levels impair the PPP (greenish-blue), thereby lowering NADPH levels and secondarily elevating ROS levels. [ii] ROS, generated via several mechanisms, likely reduces glycolysis by inactivating GADPH. [iii] Increase in polyol pathway flux (pink) leads to the generation of ROS and AGE. [iv] The flux through the HBP (violet) is also increased, thereby leading to O-glycosylation of eNOS, its uncoupling and further increased levels of ROS. [v] G3P and DHAP are converted to methylglyoxal, leading to subsequent production of AGE (orange). The inhibition of GAPDH further leads to the accumulation of G3P and DHAP phosphate. [vi] High levels of glucose result in mitochondrial dysfunction. [vii] Uncoupling of eNOS is another source of ROS production.
Abbreviations: 3DG: 3-deoxyglucosone; AGE: Advanced glycation end products; DHAP: Dihydroxyacetone phosphate; F6P: Fructose 6-phosphate; $F1,6P_2$: Fructose 1,6-bisphosphate; G3P: Glyceraldehyde 3-phosphate; G6P: Glucose 6-phosphate; G6PD: Glucose 6-phosphate dehydrogenase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Glc: Glucose; GLUT1: Glucose transporter 1; HBP: Hexosamine biosynthesis pathway; NADPH: Nicotinamide adenine dinucleotide phosphate; PPP: Pentose phosphate pathway; Pyr: Pyruvate: ROS: Reactive oxygen species; UDP-GlcNAc: Uridine diphosphate N-acetylglucosamine.

The highly reactive 3-deoxyglucosone derived from the sorbitol pathway promotes the formation of advanced

glycation end products (AGE) (Wautier and Schmidt, 2004). By binding to receptors of AGEs (RAGE), these modified proteins in turn further enhance vascular inflammation, leakage and ROS production (Manigrasso et al., 2014). AGE levels are increased when the AGE precursor methylglyoxal (which is elevated by high glucose levels (Liu et al., 2012)) is formed from the glycolytic metabolites dihydroxyacetone phosphate (DHAP) and glyceraldeyde-3-phosphate (G3P). In diabetic patients, elevated plasma levels of methylglyoxal have been associated with hyperglycemia, and aggravate vascular complications by increasing oxidative stress (Matafome et al., 2013; van Eupen et al., 2013). Methylglyoxal elevates oxidative stress by uncoupling eNOS (Su et al., 2013) and by inhibiting NADPH-generating enzymes (Morgan et al., 2014).

In addition to the aforementioned maladaptive changes of glycolysis and its side pathways, risk factors such as hyperglycemia and hyperlipidemia cause mitochondria damage and subsequent EC dysfunction, which leads to inflammation, oxidative stress and EC death (apoptosis) that in turn result in impaired vasodilation, vascular remodeling, vascular leakage and subsequent vascular diseases (Sena et al., 2013; Tang et al., 2014). High glucose causes mitochondrial dysfunction and fragmentation, defective mitochondrial biogenesis, disturbed mitochondrial autophagy (which results in the accumulation of damaged mitochondria, such as irreversibly depolarized or leaky mitochondria), and mitochondrial ROS production (Makino et al., 2010; Pangare and Makino, 2012; Mishiro et al., 2014; Santos et al., 2014). Atherosclerosis inducers, including oxidized low-density lipoprotein (oxLDL), triglycerides, fatty acids and hyperglycemia, also upregulate mitochondrial production of ROS and inhibit the endothelial ROS buffering system, which causes damage of mitochondrial DNA and other mitochondrial components, important for normal EC function (Tang et al., 2014). Normalization of mitochondrial ROS levels restored the activity of GAPDH (Du et al., 2000), prevented the high glucose-induced activation of the polyol and hexosamine pathway and the formation of AGEs (Nishikawa et al., 2000; Brownlee, 2005).

For a long time, it remained unknown why vascular damage still occurs in diabetes patients even when glycemia levels are under control. Hyperglycemic memory may explain why glucose control has failed to improve cardiovascular outcomes in subjects with diabetes. Indeed, hyperglycemia promotes EC dysfunction even after normalization of glucose levels. Emerging evidence support the notion that ROS-driven hyperglycemic stress is remembered in ECs. The mitochondrial adaptor protein p66Shc is involved in the hyperglycemic memory of ECs, since continued p66Shc activation and mitochondrial translocation are associated with persistent ROS production, decreased NO levels, and EC apoptosis (Tang et al., 2014). In the future, it will be relevant to assess the relative importance of each of these maladapted metabolic pathways described above.

While ECs become dysfunctional in many organs in diabetic patients, in the retina, elevated ROS levels cause apoptosis of pericytes (mural cells covering microvessels), leading to vessel disintegration, bleeding and aneurysms (Rask-Madsen and King, 2013). This impairs perfusion, causing retinal tissue ischemia, which in turn is a strong stimulus for neovascularization, overall leading to overgrowth of leaky vessels (diabetic retinopathy) (Antonetti et al., 2012).

Vessel overgrowth in cancer

The vasculature of solid tumors is morphologically and functionally aberrant, and characterized by highly permeable and fragile vessels, intense vessel sprouting and loss of hierarchical architecture of the vascular network (Potente et al., 2011; Jain, 2013). To date, the metabolic features of tumor ECs have been poorly characterized. Nevertheless, the switch from a quiescent to an angiogenic phenotype (as in cancer) is metabolically taxing, and tumor ECs are highly activated (Fig. 2). Indeed, VEGF signaling increases glycolytic flux by inducing glucose uptake through elevated expression of the glucose transporter GLUT1 (Yeh et al., 2008). VEGF also upregulates the expression of PFKFB3, which promotes EC specification to sprout-forming tip cells (De Bock et al., 2013b). Elevated intratumoral lactate concentrations are a good indicator of the glycolytic adaptation of tumors and correlate with poor clinical outcome in a variety of human cancers (Hirschhaeuser et al., 2011). Notably, tumor cell-derived lactate can act as *bona fide* signaling molecule for ECs. Once taken up by tumor-associated ECs through the monocarboxylate transporter 1 (MCT1), lactate induces activation of hypoxia-inducible factor-1α (HIF-1α) (Sonveaux et al., 2012). Indeed, lactate inhibits the oxygen-sensing prolyl hydroxylase-2 (PHD2), a negative regulator of HIF-1α, thereby stabilizing HIF-1α levels (Végran et al., 2011). In turn, HIF-1α activation increases the expression of pro-angiogenic factors, such as VEGFR2, basic fibroblast growth factor (bFGF) and nuclear factor kappa B (NFκB), thereby increasing angiogenesis (Végran et al., 2011; Sonveaux et al., 2012).

Besides functioning as a signaling molecule in tumor ECs, lactate can also be converted to pyruvate to fuel the tricarboxylic acid (TCA) cycle. The interconversion of lactate and pyruvate is mediated by lactate dehydrogenase (LDH), a tetrameric enzyme composed of different subunits, which give rise to different isoenzymes depending on the tetramer composition. LDH with a high LDH-A subunit content converts pyruvate into lactate, and is abundant in glycolytic, proliferative pulmonary microvascular ECs (Parra-Bonilla et al., 2010), while expression of LDH-B, the subunit converting lactate to pyruvate, is upregulated in tumor endothelium (van Beijnum et al., 2006), possibly to promote the use of lactate into oxidative metabolism.

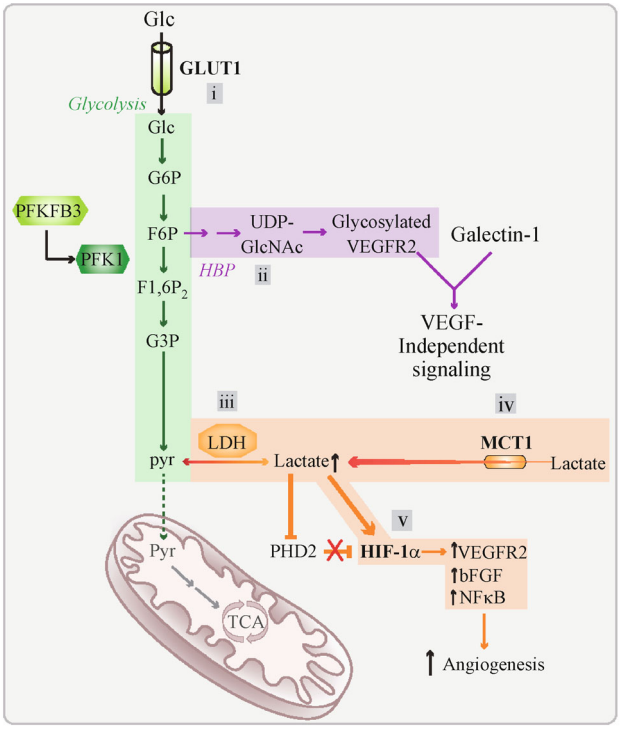

Figure 2 Pathological angiogenesis in cancer. Scheme highlighting the metabolic changes in tumor ECs. For clarity, not all metabolites and enzymes of the depicted pathways are shown. [i] Angiogenic cells are highly glycolytic (green); the glucose transporter GLUT1 is upregulated, thus allowing the uptake of glucose and the increased glycolytic flux. The upregulation of PFKFB3, a regulator of glycolysis, might further increase the conversion of glucose to lactate through the action of LDH-A. [ii] The HBP-dependent generation of UDP-GlcNAc stimulates glycosylation of VEGFR2 (violet), thus favoring its interaction with galectin-1 and subsequent VEGFR2 activation even in the absence of ligand. [iii] LDH drives the interconversion of pyruvate and lactate (orange). In particular, the LDH-B subunit, which converts lactate to pyruvate, is upregulated in tumor ECs. [iv] Upregulation of MCT1 increases lactate influx, further heightening the intracellular lactate levels. [v] Lactate inhibits PHD2 and activates HIF-1α, thus increasing the expression of pro-angiogenic factors and inducing angiogenesis.
Abbreviations: other abbreviations as in Fig. 1. bFGF: Basic fibroblast growth factor; HIF-1α: Hypoxia inducible factor 1a; LDH: Lactate dehydrogenase; MCT1: Monocarboxylate transporter 1; NFκβ: Nuclear factor κβ; PHD2: Prolyl hydroxylase 2; PFK1: Phosphofructokinase-1; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; VEGF: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2.

Control of post-translational modifications by metabolism in ECs

Post-translational modifications (PTMs) regulate the biological activity of multiple proteins, also of angiogenic proteins. Protein acetylation, a common PTM, requires acetyl-CoA as substrate for lysine acetyltransferases, thus linking metabolism to the regulation of multiple cellular functions (Friedmann and Marmorstein, 2013; Choudhary et al., 2014). Not surprisingly, acetylation of several angio-modulatory proteins in ECs influences angiogenesis. This has been demonstrated for Notch (Guarani et al., 2011), HIF-1α (Lim et al., 2010), cortactin (Kaluza et al., 2011), VEGFR2 (Zecchin et al., 2014) and the forkhead transcription factor 1 (Foxo1) (Potente et al., 2007). Interestingly, the NAD^+-dependent deacetylase SIRT1, which acts as a sensor of energy and redox state, reverses the acetylation status of many of these proteins (Fig. 3A). Inactivation of SIRT1 favors acetylation of the signaling-active Notch intracellular domain (NICD), thereby preventing ubiquitination and proteasomal degradation of NICD; the resultant increase in Notch signaling promotes differentiation to an endothelial stalk cell phenotype, which impairs vessel sprouting (Guarani et al., 2011). SIRT1 also deacetylates and inactivates Foxo1, a negative regulator of angiogenesis (Potente et al., 2007). It also deacetylates HIF-1α, thereby suppressing its transcriptional activity and impairing tumor growth and angiogenesis (Lim et al., 2010). Furthermore, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a target of SIRT1, and deacetylation of PGC-1α modulates its activity (Rodgers et al., 2005). In normal conditions, PGC-1α stimulates the formation of blood vessels (Arany et al., 2008; Saint-Geniez et al., 2013), while in hyperglycemic conditions, PGC-1α induces EC dysfunction and blunts angiogenesis (Sawada et al., 2014). Whether SIRT1-dependent deacetylation of PGC-1α has a role in diabetes-induced EC dysfunction is unknown.

The link between metabolism and epigenetics is also becoming increasingly evident, as recent reports indicate that ATP citrate lyase (ACLY) and the pyruvate dehydrogenase complex (PDC), two enzymes that generate acetyl-CoA for metabolic purposes, have been shown to exert also non-metabolic functions by influencing the rate of histone acetylation in the nucleus in response to nutrients and growth factors, thereby modulating the expression of target genes (Wellen et al., 2009; Lee et al., 2014; Sutendra et al., 2014). It will be interesting to explore whether such mechanisms are also operational in ECs and regulate angiogenesis.

As mentioned above, the HBP is involved in glycosylation of angiogenic molecules (Takahashi and Shibuya, 1997; Du et al., 2000; Benedito et al., 2009). For instance, glycosylation of VEGFR2's extracellular domain facilitates an interaction with galectin-1, which activates VEGFR2 signaling, even in the absence of VEGF (Fig. 3B). This finding might help to explain part of the tumor refractoriness to anti-VEGF treatment and clarify why blood vessels can still grow in the presence of anti-VEGF therapies (Croci et al., 2014). Glycosylation of other angio-modulatory proteins (eNOS, Akt, Notch1) influences their pro-angiogenic activity in a context-dependent manner (Takahashi and Shibuya, 1997; Du et al., 2001; Federici et al., 2002; Luo et al., 2008; Benedito et al., 2009).

In addition to *de novo* synthesis of lipids, fatty acid

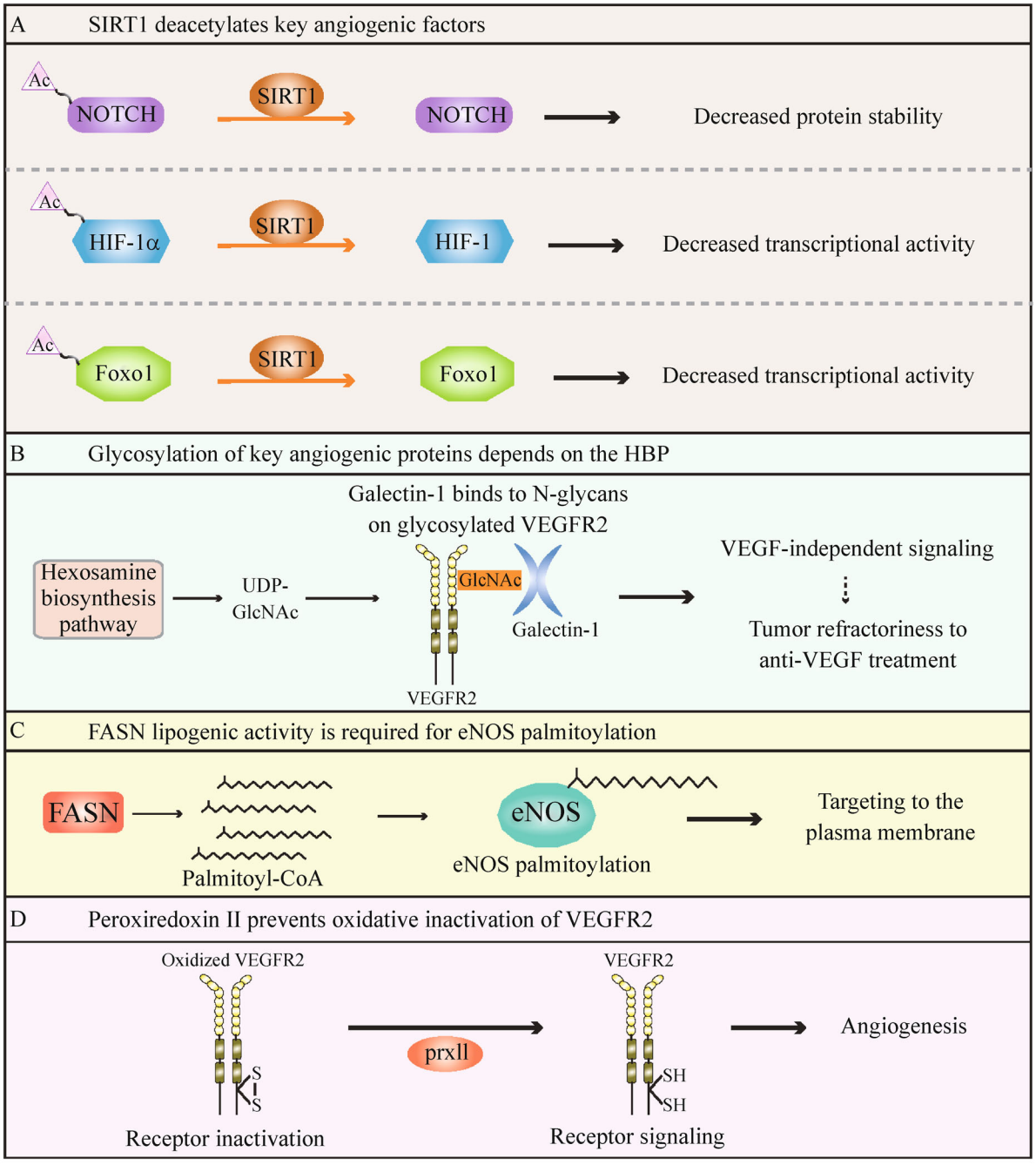

Figure 3 Metabolic pathways control angiogenesis through protein PTM. (A) The NAD^+-dependent deacetylase SIRT1 exerts its activity on key angiogenic factors (Notch, Foxo1, HIF-1α). (B) HBP, a side branch of glycolysis, generates UDP-GlcNAc, a substrate for protein glycosylation. The glycosylation-mediated binding of galectin-1 to VEGFR2 induces ligand-independent receptor activation. (C) FASN produces palmitate, which is utilized for eNOS palmitoylation in ECs to target the enzyme at the plasma membrane. (D) Oxidation of reactive cysteine thiols (cys-SH) in VEGFR2 inactivate its signaling; the antioxidant enzyme peroxiredoxin II (PrxII) protects VEGFR2 against oxidative inactivation, thereby preserving its pro-angiogenic ativity.

Abbreviations: eNOS: endothelial nitric oxide synthase; Foxo1: forkhead transcription factor 1; FASN: Fatty acid synthase; GlcNAc: N-acetylglucosamine; HIF-1α: Hypoxia inducible factor 1α; prxII: Peroxiredoxin II; SIRT1: Sirtuin1; VEGFR2: Vascular endothelial growth factor receptor 2; UDP-GlcNAc: Uridine diphosphate N-acetylglucosamine.

synthase (FASN) generates palmitate, which can be used for palmitoylation of the pro-angiogenic protein eNOS in ECs (Wei et al., 2011) (Fig. 3C). Hence, FASN deficiency impedes angiogenesis by impairing the targeting of the pro-angiogenic VEGFR2 and eNOS to the plasma membrane through reduced palmitoylation (Browne et al., 2006; Wei et al.,

2011). Of note, insulin induces palmitoylation of eNOS, and palmitoylation of eNOS is decreased in insulin-deficient and insulin-resistant mouse models (Wei et al., 2011).

The redox state of the endothelium can also control the activity of angiogenic proteins via oxidation of cysteine residues. For instance, oxidation of C-terminal cysteine residues in VEGFR2 inactivates VEGFR2 signaling, a process that impairs angiogenesis (Kang et al., 2011) (Fig. 3D). The antioxidant enzyme peroxiredoxin II protects VEGFR2 against such oxidative inactivation, explaining why peroxiredoxin II deficiency suppresses tumor angiogenesis (Kang et al., 2011). However, the effects of oxidation are contextual, since in response to VEGF, (presumably other) reactive cysteine thiols (Cys-SH) in the cytosolic domain of VEGFR2 and c-Src become oxidized; this promotes the interaction between VEGFR2 and c-Src, and results in activation of c-Src, a process that stimulates angiogenesis (Lee et al., 2011). Whether the latter phenomenon indeed promotes angiogenesis *in vivo* and how these seemingly opposite findings can be reconciled and whether they occur in different conditions requires further work.

Role of epigenetic DNA methylation in ECs

DNA methylation is an epigenetic modification that regulates various cellular processes and affects progression of several diseases such as cancer and diabetes (Dawson and Kouzarides, 2012; Yang et al., 2012; Dayeh et al., 2014; Nilsson et al., 2014; Stefan et al., 2014). The transfer of the methyl group is mediated by DNA methyltransferases (DNMT), which use as universal methyl donor S-adenosylmethionine (SAM), an intermediate of one-carbon metabolism (Struck et al., 2012; Locasale, 2013). Aberrant epigenetic modifications can also contribute to EC dysfunction – this has been best documented in atherosclerosis (Mitra et al., 2011; Kim et al., 2012; Kumar et al., 2013). DNA methylation patterns in atherosclerotic plaques of human patients and apolipoprotein E-deficient (apoE$^{-/-}$) mice are different from those in healthy tissues (Hiltunen et al., 2002; Castillo-Díaz et al., 2010; Nazarenko et al., 2011; Nazarenko et al., 2013). ApoE$^{-/-}$ mice already display global changes in DNA methylation preceding atherosclerotic lesion formation (Lund et al., 2004). Also, the finding that inhibiting DNA methyltransferase 1 (DNMT1), the enzyme responsible for *de novo* DNA methylation, with 5-aza-2′-deoxycytidine reduces atherosclerotic lesion burden in apoE$^{-/-}$ mice further supports a role for DNA methylation in atherogenesis (Dunn et al., 2014). There is increasing evidence that part of these epigenetic changes in atherosclerosis take place in ECs.

Indeed, exposure of ECs to atherogenic lipoproteins upregulates the expression of p66shc by inducing DNA hypomethylation of the p66shc promoter; since this gene promotes oxidative stress in response to a high fat diet (Napoli et al., 2003), these lipoproteins cause EC dysfunction (Kim et al., 2012). Further, treatment of ECs with low-density lipoproteins (LDL) upregulates the expression of DNMT1. This leads to hypermethylation of the promoter of Kruppel-like factor 2 (KLF2), and thereby a decrease in its expression. Since KLF2 mediates vascular homeostasis and has anti-inflammatory and anti-atherosclerotic activity, LDLs render ECs dysfunctional (Kumar et al., 2013). Disturbed blood flow, another risk factor for atherosclerosis, also induces genome-wide changes in the DNA methylation pattern in ECs, resulting in global changes of gene expression (Dunn et al., 2014). In particular, disturbed blood flow causes hypermethylation of the promoter and thus suppression of the expression of KLF4, another atheroprotective transcription factor in ECs, thus promoting progression of the disease (Jiang et al., 2014).

It is tempting to speculate that aberrant epigenetic DNA modifications may induce maladaptive changes of EC metabolism, which could further contribute to atherosclerosis, but such a link has only recently emerged. Indeed, a recent study demonstrated that KLF2 lowers glycolysis in ECs by downregulating the expression of PFKFB3, an activator of glycolysis (Doddaballapur et al., 2015). However, what precisely the consequences are for glycolysis in ECs in atherosclerotic plaques and how such metabolic changes might modify the cellular activities remains to be explored.

Metabolites controling EC function?

By virtue of their localization, ECs are exposed directly to metabolites and nutrients in the blood, such as glucose, lipids and amino acids. Emerging evidence indicates that a change in the availability of blood metabolites can affect the cellular activities of ECs. One example is how lipids affect ECs. In addition to shunting fatty acids from the circulation to surrounding tissues (Hagberg et al., 2010; Hagberg et al., 2012), the endothelium itself also responds to lipids in the circulation. Indeed, cholesterol influences the formation of membrane lipid rafts in ECs, a process that facilitates membrane localization and signaling of the pro-angiogenic VEGFR2 (Fang et al., 2013). Hence, cholesterol efflux from ECs to high-density lipoprotein (HDL) reduces vessel sprouting (Fang et al., 2013). Another example is how circulating apolipoprotein B (apoB) negatively affects angiogenesis by upregulating the expression of VEGFR1 (Avraham-Davidi et al., 2012). While only a few studies to date have documented such a relationship, they open exciting future research avenues on how dietary nutrients or metabolites (or proteins interacting with them) might influence EC dysfunction in diabetes and atherosclerosis, or modulate vessel overgrowth in cancer and ocular disease.

Dietary metabolite supplementation to protect or inhibit ECs?

Given that metabolite scan alter EC functions, the question arises whether dietary supplementation of metabolites might represent a strategy to ameliorate vascular disease. We provide here two examples.

Dietary supplementation of L-arginine to protect ECs in atherosclerosis

In the folate cycle, methyl-tetrahydrofolate (mTHF) donates a methyl group to homocysteine (hCys) to generate methionine (Locasale, 2013). Methionine itself is an important methyl donor for the methylation of lysine and arginine residues in target proteins (Leiper and Nandi, 2011). Due to the turnover of methylated proteins, asymmetric dimethylated arginine (ADMA) is released. This metabolic by-product functions as an endogenous inhibitor of eNOS, leading to uncoupling of this enzyme, a process that results in reduced NO production but increased ROS formation (Dhillon et al., 2003; Leiper and Nandi, 2011). In patients with atherosclerosis who have elevated ADMA plasma levels (Boger, 2009), dietary supplementation of L-arginine increases NO production by directly competing with ADMA for the binding to eNOS (Bode-Böger et al., 2007; Leiper and Nandi, 2011). These elevated NO levels improve endothelium-dependent vasodilation and reverse the hyperadhesive phenotype of monocytes and T-lymphocytes to the endothelium in atherosclerotic patients, processes that counteract disease progression (Chan et al., 2000). Especially atherosclerotic patients with a low ratio of L-arginine/ADMA in the plasma could benefit from L-arginine administration (Bode-Böger et al., 2007).

Dietary supplementation of n-3 PUFAs to reduce pathological angiogenesis

Several studies have reported an anti-angiogenic effect of long-chain n-3, polyunsaturated fatty acids (PUFAs), also known as omega-3 (ω-3) fatty acids, in addition to their anti-thrombotic and anti-inflammatory properties (Calder, 2014; Wang et al., 2014). n-3 PUFAs inhibit EC functions directly, in part by reducing the expression of VEGFR2, matrix metalloproteinase (MMP)-2 and MMP-9, which dampen EC migration and capillary tube formation (Yang et al., 1998; Tsuji et al., 2003; Tsuzuki et al., 2007). Indirect anti-angiogenic effects related to a decrease in VEGF levels have also been ascribed to n-3 PUFAs. All this can explain why an omega-3-enriched diet reduces tumor growth and microvessel density (Rose and Connolly, 1999; Mukutmoni-Norris et al., 2000; Tevar et al., 2002; Calviello et al., 2004; Zhang et al., 2013). n-3 PUFAs also attenuate diabetic retinopathy, a pathological neovascularization in the eye that causes blindness (Connor et al., 2007; Yanai et al., 2014). However, not all studies report an anti-angiogenic effect of n-3 PUFAs (Hu et al., 2014), suggesting that their angio-modulatory activity is context-dependent.

Targeting EC metabolism in vessel overgrowth and EC dysfunction

Vessel overgrowth in cancer

Several agents that block metabolic pathways have been demonstrated to inhibit pathological angiogenesis. Glycolytic blockers, for instance, inhibit vessel overgrowth. The aforementioned findings that glycolysis is essential and necessary for vessel sprouting render this metabolic pathway an attractive therapeutic target. Initial studies showed that the non-metabolizable glucose analog 2-deoxy-D-glucose (2DG), which blocks all cellular glucose metabolism, induces EC cytotoxicity and impairs EC proliferation, migration and vessel formation *in vitro* (Chung et al., 2004; Nef et al., 2008; Merchan et al., 2010). However, adverse systemic effects due to complete and permanent blockade of glucose metabolism by 2DG limit its therapeutic potential (Zhang et al., 2014). More recent studies provided a paradigm shift in the design of anti-glycolytic therapy, utilizing a PFKFB3-blocker to inhibit glycolysis only transiently and partially. Importantly, this strategy reduced only the "hyper-glycolysis," *i.e.* the extra amount of glycolysis that was increased and necessary for ECs to switch from quiescence to sprouting. This transient and partial reduction in glycolytic flux was sufficient to reduce pathological angiogenesis in the eye, inflamed skin and gut (Schoors et al., 2014a, 2014b; Xu et al., 2014), but since it did not eliminate baseline glycolysis entirely, this strategy did not affect quiescent healthy vessels, nor did it induce systemic effects (Schoors et al., 2014b).

EC dysfunction in atherosclerosis and diabetes

By inhibiting HMG-CoA reductase and thereby cholesterol synthesis, statins have been approved for the prevention of cardiovascular events in dyslipidemia patients (Wilkinson et al., 2014). In addition to lipid-lowering, anti-inflammatory and other effects, statins also decrease EC dysfunction by restoring the activity of eNOS (Rolfe et al., 2005; Biasucci et al., 2010; Antoniades et al., 2011). This effect relies on a non-lipid lowering mechanism. Indeed, eNOS mRNA stability is reduced by RhoA signaling; for RhoA to become activated, it must be anchored in the plasma membrane, a process that requires prenylation of RhoA. Prenylation itself requires the substrate geranylgeranyl pyrophosphate (GGPP), an intermediate of the mevalonate pathway, which is inhibited by statins. Hence, by inhibiting RhoA activation in ECs through a decreased availability of geranylgeranyl, statins restore eNOS expression (Rolfe et al., 2005).

The anti-diabetic drug metformin is another example of a drug that affects EC functions by acting on metabolism and

critical regulators of cellular metabolism. Metformin inhibits complex I-mediated mitochondrial respiration and, secondary to the resultant drop in energy charge, activates AMP-activated protein kinase (AMPK), a critical energy sensor of cellular energy homeostasis that integrates multiple signaling networks to coordinate a wide array of compensatory, protective and energy-sparing responses (Sena et al., 2011; Foretz et al., 2014; Pernicova and Korbonits, 2014). In the setting of atherosclerosis and diabetes, metformin reduces EC dysfunction and improves vascular homeostasis (Foretz et al., 2014). This effect of metformin is in line with its known effects on stimulating vessel sprouting *in vitro* and angiogenesis/ischemic tissue revascularization *in vivo* (Takahashi et al., 2014; Venna et al., 2014). This protective effect of metformin on ECs relies in part on the reduction of oxidative and endoplasmic reticulum stress (induced by high glucose and lipid levels), inhibition of NAD(P)H oxidases (Batchuluun et al., 2014), activation of eNOS (Davis et al., 2006; Eriksson and Nyström, 2014; Valente et al., 2014), upregulation of VEGF (Cittadini et al., 2012; Martin et al., 2012), decreased expression of RAGEs and on abrogation of the suppression of SIRT1 expression (Arunachalam et al., 2014).

However, in the tumor setting, metformin mostly inhibits tumor growth and in some cases also tumor angiogenesis (Algire et al., 2011; Dowling et al., 2012; Akinyeke et al., 2013; Mohammed et al., 2013; Hadad et al., 2014; Orecchioni et al., 2014; Qu and Yang, 2014). This is at least in part due to direct inhibitory effects on ECs, though indirect effects on cancer cells have been also documented, albeit at high metformin concentrations (Foretz et al., 2014). Indeed, metformin inhibits proliferation and migration of ECs, and induces endothelial progenitor apoptosis *in vitro* (Tan et al., 2009; Esfahanian et al., 2012; Dallaglio et al., 2014; Orecchioni et al., 2014). It remains however to be explored whether metformin treatment of ECs induces the same switch from anabolic to catabolic metabolism as occurs in other cell types (Foretz et al., 2014), and whether these changes in EC metabolism also contribute to its observed contextual effects.

As discussed above, hyperglycemic ECs redirect excess glucose-derived metabolites into the ALR2-dependent polyol pathway, leading to increased production of AGEs and ROS. Ocular neovascularization is a common complication in diabetes. Preclinical findings indicate that inhibition of ALR2 reduces the progression of early diabetic retinopathy (Obrosova and Kador, 2011). In human diabetics, the ALR2 inhibitor epalrestat delays the progression of retinopathy (Hotta et al., 2012). Overall, these examples illustrate the therapeutic potential of targeting EC metabolism for the treatment of vascular disorders.

Conclusions and future perspectives

Current strategies to inhibit pathological angiogenesis rely primarily on the inhibition of growth factor (VEGF) signaling (Singh and Ferrara, 2012). However, refractoriness and drug resistance limit the efficacy of these treatments. There is thus a need for anti-angiogenic therapies that act via complementary mechanisms. It will be interesting to explore whether drugs targeting EC metabolism could enhance the response of cancer patients to current anti-angiogenic agents. Preclinical animal studies have already provided initial proof of concept that glycolytic PFKFB3 blockade enhances the anti-angiogenic effects of VEGF inhibitors (Schoors et al., 2014a).

But metabolic inhibitors may also improve anti-angiogenic therapy in other ways. For instance, patients suffering wet age-related macular degeneration (AMD), the most frequent blinding disease in the elderly population, receive frequent intra-ocular injections of anti-VEGF therapies, causing local adverse effects and personal discomfort from the needle injections in the eye. An alternative to avoid recurrent intra-ocular injections would rely on the use of a drug that can be intravenously administered without causing systemic adverse effects. Recent studies revealed that systemic administration of the glycolytic PFKFB3 blocker 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) caused a partial and transient reduction of glycolysis in ECs, which sufficed to inhibit choroidal neovascularization in a preclinical mouse model of wet AMD, without a need for intra-ocular injections (Schoors et al., 2014b).

It will be also interesting to characterize how current anti-cancer and anti-angiogenic drugs affect EC metabolism, since recent studies documented substantial changes of cancer cell metabolism upon treatment with anti-angiogenic drugs (Keunen et al., 2011; Sounni et al., 2014; Curtarello et al., 2015). In tumor models, anti-VEGF therapy induced a selection of highly glycolytic and tumorigenic cancer cells, which showed reduced responsiveness to VEGF blockade (Curtarello et al., 2015). Also, withdrawal of VEGF receptor tyrosine kinase inhibitors induced a metabolic shift toward lipid metabolism in cancer cells, suggesting that inhibition of lipid metabolism might represent a promising strategy to overcome the tumor aggressiveness induced by the withdrawal of anti-angiogenic agents (Sounni et al., 2014). Understanding the effects of anti-cancer treatments on EC metabolism promises to improve overall anti-cancer treatment options.

Finally, there is nearly nothing known about the metabolism of the different types of endothelial progenitors or vascular niche cells, neither it is known how these cells adapt their metabolism when they proliferate and differentiate to ECs, or maladapt their metabolism in disease. It is also not known how quiescent vascular niche ECs harboring dormant disseminated breast cancer cells adapt their metabolism when these niche ECs start to sprout to promote micrometastatic outgrowth (Ghajar et al., 2013). EC metabolism is a young field with multiple opportunities to address novel questions, and offering unexplored translational opportunities for improving anti-angiogenic or EC-protective therapy.

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Compliance with ethics guidelines

Peter Carmeliet is named as an inventor on patent application regarding subject matter related to the findings reviewed in this publication. Annalisa Zecchin and Aleksandra Brajic declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review boards or ethics committees.

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