THE METABOLIC ENGINE OF ENDOTHELIAL CELLS

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ABSTRACT

Endothelial cells (ECs) line the quiescent vasculature, but can form new blood vessels (a process termed angiogenesis) in disease. Strategies targeting angiogenic growth factors have been clinically developed for the treatment of malignant and ocular diseases. Studies over the past decade documented that several pathways of central carbon metabolism are necessary for EC homeostasis and growth, and that strategies that stimulate or block EC metabolism can be used to respectively promote or inhibit vessel growth. In this review, we provide an updated oversight of our growing understanding of central carbon metabolic pathways in ECs and the therapeutic opportunities of targeting EC metabolism.

INTRODUCTION

Blood vessels supply oxygen and nutrients, drain waste, regulate vascular patency, produce angiocrine signals for tissue regeneration and serve as gateways for immune cell trafficking [1]. They are lined by endothelial cells (ECs), the name of which is deduced from the Greek word "ενδον (endon)", meaning "inside", referring to their inner location in the blood vessel. While ECs are quiescent in healthy adults (except in a few conditions such as pregnancy, etc.), they become rapidly activated in response to signals, produced by tissues in need of vascularization, which results in the formation of new blood vessels – a process referred to as angiogenesis [1,2]. When activated, ECs undergo a phenotypic switch [3] and differentiate into proliferating stalk cells to elongate the sprout and into migratory tip cells to guide the sprout at the forefront, a position that is adopted by the fittest cell via dynamic competition (Fig. 1) [4].

Dysfunction of ECs has been implicated in the pathophysiology of diabetes [5] and atherosclerosis [6], and contributes to the development of peripheral artery disease [7], coronary heart disease and myocardial infarction [8,9], cerebrovascular disease [10], as well as kidney disease [11]. Excessive angiogenesis, on the other hand, promotes diseases such as cancer, blinding eye disease and pulmonary arterial hypertension (PAH) [12-14].

Traditional anti-angiogenic therapies (AATs) target EC-activating growth factor pathways, such as VEGF signaling. However, while clinically used for cancer and blinding eye disease treatment, these approaches lack sufficient efficacy and treated patients develop resistance – partly due to the compensatory effect of other signaling pathways that activate ECs [1,15]. In search of new therapeutic opportunities that may bypass these limitations, a new concept, proposed in 2009, postulated that ECs rely on metabolic adaptations to execute the instructions of angiogenic signals to form new blood vessels (Fig. 1) [16]. In this review, we provide an update of our current understanding of how ECs utilize

metabolism, often in a particular manner, and discuss possible therapeutic opportunities of targeting EC metabolism for the development of alternative therapeutic strategies to promote or inhibit vessel growth. Rather than providing a historical, all-encompassing overview, we highlight principles by illustrating key examples.

Endothelial Metabolism

Glycolysis and Side Pathways

Blood vessels deliver oxygen to nearly all tissues in the body, yet ECs are not fully taking profit of the access to oxygen in the blood and generate most of their ATP anaerobically via glycolysis (Fig. 2,3) – in contrast to many other cell types [17]. This is striking, as the conversion of glucose to pyruvate during glycolysis yields only 2 moles of ATP per mole of glucose, making it a less efficient means for ATP production than oxidative phosphorylation (OXPHOS), yielding 36 moles of ATP. However, this – at first sight – apparent contra-intuitive behavior offers advantages. Indeed, reliance on anaerobic metabolism lowers the production of reactive oxygen species (ROS) and enables ECs to vascularize regions deprived of oxygen and nutrients, which would otherwise be impossible if their energy production was primarily dependent on oxidative glucose metabolism. Consistently, in glucose-replete conditions, ECs are relatively resistant to hypoxia, but they become sensitive to oxygen depletion when glucose levels drop [18]. Another advantage is that ECs, by consuming little oxygen, can transfer larger amounts of oxygen to perivascular cells, a primary objective of why ECs developed during evolution.

While glycolytic flux is high in all types of ECs, it is lower in quiescent ECs due to downregulation of glycolytic gene expression by the transcription factors FOXO1 and Notch [19,20]. In contrast, growth factors like VEGF upregulate glycolytic gene expression in angiogenic ECs, and both proliferating stalk cells and migratory tip cells rely on glycolysis [17,21]. In tip cells, glycolytic energy production

not only takes place in the cytosol, but also in local “ATP hot spots” in lamellipodia and filopodia, which facilitate energy-intensive actin cytoskeletal remodeling and promote tip cell competitiveness [17].

Hyperproliferative ECs in cancer and PAH have high rates of glycolysis and employ this pathway not only for energy production but also for biomass synthesis [22,23]. In fact, ECs are hyperglycolytic compared to many other cell types [17]. Hence, the upregulated glycolysis represents an attractive metabolic target for manipulating pathological angiogenesis in these diseases [24]. Subject of intense research is, for instance, the role of the glycolysis stimulator 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (PFKFB3, Fig. 2), a VEGFR target [25] that is upregulated in tumor ECs (TECs) [22]. Inhibiting or silencing PFKFB3 reduces the growth and migration of ECs *in vitro*, rendering them more quiescent [17,25,26]. Of relevance, pharmacological blockade of PFKFB3 in mouse tumor models normalizes abnormal tumor vessels by tightening vascular barrier integrity and improving vessel maturation [27]. Extending these observations, overexpression of PFKFB3 in turn enhances glycolysis, which promotes vessel sprouting [17]. Indeed, mosaic overexpression of PFKFB3 in an EC spheroid sprouting model *in vitro* or in a transgenic zebrafish angiogenesis model *in vivo* boosts tip cell behavior, which provided the first evidence that a change in EC metabolism by itself, even without a change in angiogenic signaling, was capable of altering vessel sprouting [17], showcasing the important role of EC metabolism.

Further illustrating the reliance of angiogenic ECs on glycolysis, inhibition of other glycolytic proteins, like hexokinase 2 (Fig. 2) [21], which catalyzes an initial step in glycolysis, and adenosine receptor A2a [28], an upstream activator of glycolysis, similarly impairs angiogenesis. Surprisingly, the anti-angiogenic effect observed by inhibiting another glycolytic enzyme, PKM2, is mechanistically based on altered NFkB signaling instead of glycolytic attenuation or impairment [29]. In glucose-limiting conditions, such as during vessel sprouting into hypoglycemic tissues, angiogenic ECs rely on utilization of

glucose stored in internal glycogen reservoirs, as impairment of glycogen breakdown by inhibiting glycogen phosphorylase (GP) reduces EC viability and impairs EC migration [30].

Intermediates of the glycolytic pathway are utilized as substrates for side pathways, like the pentose phosphate pathway (PPP) (Fig. 2). The PPP is involved in nucleotide biosynthesis and provides NADPH for fatty acid synthesis, redox homeostasis and the synthesis of nitric oxide (NO), a key regulator of vascular tone, by endothelial nitric oxide (NO) synthase (eNOS) [31]. Indeed, increasing the PPP flux reduces ROS accumulation in ECs exposed to high glucose levels [32]. Hyperproliferative ECs in PAH feature an elevated PPP flux, presumably in order to meet the cells' demand for nucleotides during DNA replication (Fig. 3) [33]. In line, inhibition of the PPP by silencing rate-limiting enzymes in oxidative or non-oxidative side arms (respectively, glucose-6-phosphate dehydrogenase (G6PDH) [32] or transketolase (TKT) [30], Fig. 2) reduces proliferation of angiogenic ECs. Considering that inhibiting the glycolytic flux in angiogenic ECs not only impedes energy production but also PPP-dependent biomass synthesis [22], glycolysis manipulation may represent a therapeutic approach for targeting hyper-activated ECs (Fig. 3A).

Another glycolytic side pathway implicated in angiogenesis is the hexosamine biosynthesis pathway (HBP, Fig. 2). The HBP produces UDP-GlcNAc (uridine diphosphate N-acetylglucosamine) inducing protein glycosylation, which regulates the activity of target proteins, like for example VEGFR2 [34]. In ECs, HBP-mediated protein glycosylation might have an inhibitory effect on angiogenesis, since blocking glycosylation by silencing the HBP key enzyme glutamine:fructose-6-phosphate amidotransferase 1 (GFAT1) [35] induces vascular sprouting, whereas boosting glycosylation has opposite effects [36]. The synthesis of serine, another pathway utilizing glycolytic intermediates, will be discussed below.

TCA Cycle and Mitochondrial Oxidative Phosphorylation

A fraction of glycolysis-derived carbons enters the tricarboxylic acid (TCA) cycle in the form of acetyl-CoA or oxaloacetate (Fig. 2) [17,37]. ECs are particular in how they sustain the TCA cycle, as a substantial amount of acetyl-CoA entering the TCA cycle is derived from fatty acids through fatty acid oxidation (FAO, see below) in conjunction with other anaplerotic substrates likely derived from glucose and/or amino acids [37]. The TCA cycle is vital for biomass production in ECs, as its intermediates serve as substrates for various anabolic pathways, such as biosynthesis of nucleotides or amino acids like aspartate [37-39]. Also, the TCA cycle regenerates the reducing agent NADH for OXPHOS. Mitochondrial volume in ECs typically (with an exception of brain ECs) represents only 2–5% of the cytoplasmic volume, in contrast, for instance, to the 32% volume density of mitochondria in oxidative cardiac muscle cells [40,41]. Thus, it is not surprising that mitochondria and OXPHOS were traditionally considered to have only marginal roles in ECs. However, recent reports documenting that OXPHOS is crucial for EC function urge to reconsider the role of mitochondria in ECs [42-46].

Mitochondrial respiration serves various purposes, including ATP production by utilizing reducing agents (Fig. 3). The respiratory machinery consists of multi-protein complexes that transfer electrons derived from NADH and $FADH_2$ while pumping protons across the inner mitochondrial membrane (complexes I-IV). The consequent proton gradient is used by the ATP synthase to generate ATP. Blockade of OXPHOS complexes I and III (by using piericidin A and antimycin A, respectively, or by inactivating ubiquinol-cytochrome C reductase complex III subunit VII (UQCRQ)) compromises EC proliferation and impairs developmental and pathological angiogenesis [47]. In line with these findings, indirect interference with OXPHOS – due to heme synthesis defects, which impedes the activity of heme-containing complexes III and IV (see Serine Metabolism) [48], or by oxaloacetate accumulation as a result of depletion of oxaloacetate decarboxylase FAHD1, which inhibits complex II activity [49] – also causes proliferation defects in ECs.

Mechanistically, inhibition of respiratory complexes compromises recycling of NAD^+, produced

by complex I, which diminishes the TCA cycle flux, lowers aspartate levels and impairs oxidative processes requiring NAD^+ (Fig. 3) [47,50]. Regenerating NAD^+ levels by ectopic expression of electron transport chain-independent NADH oxidase rescues aspartate levels and proliferation in ECs. Therefore, a key function of OXPHOS in ECs is to maintain the NAD^+/NADH ratio in order to sustain biosynthetic processes during proliferation, such as aspartate production [47]. This is in accordance with the fact that, as mentioned above, ECs cover the major part (up to 85%) of their energy demand by glycolysis instead of utilizing OXPHOS (Fig. 3), indicating a negligible role of mitochondrial respiration in ATP production in glucose-rich conditions [17]. Mitochondrial respiration in quiescent ECs (displaying lower proliferation and anabolic needs) is downregulated by FOXO1 [20].

Mitochondrial metabolism offers ECs a flexibility to utilize substrates for energy production, which may become essential for ECs in disease conditions. For instance, hyperproliferative TECs utilize OXPHOS for both biomass synthesis and energy production in glucose-deprived conditions. Indeed, in tumors, glucose levels can be as low as 0.2 mM (in contrast to 5.5 mM in the blood) [51]. Accordingly, proliferating TECs are more sensitive to inhibition of OXPHOS than quiescent ECs [52-54]. Moreover, aberrant mitochondrial metabolism is a hallmark of hyperproliferative PAH-ECs, which rely more on glycolysis for energy production, forfeiting their metabolic flexibility to some extent. PAH-ECs feature reduced mitochondrial ATP production and volume, but increased ROS formation [55,56].

FATTY ACID METABOLISM

Lipids are used by ECs as (i) structural membrane components in the form of phospholipids [57], (ii) as signaling molecules mediating cellular responses such as growth, stress and apoptosis [58,59], and (iii) to sustain the TCA cycle (in conjunction with other anaplerotic substrates) [37]. ECs can take up fatty acids (FAs) directly from the circulation, either passively or by designated transporters (CD36; FATP family), and thereby regulate the lipid content in the blood, an important risk factor of atherosclerosis [60]. De-

spite having access to circulating lipids, ECs can also synthesize FAs endogenously by converting acetyl-CoA to malonyl-CoA, which is then elongated to FAs (Fig. 2) [61].

During angiogenesis, the availability of FAs for ECs is essential [62], and growth factor VEGF-B has been shown to induce FA uptake by upregulation of FATP3 and FATP4 expression [63]. In addition, the synthesis of FAs is upregulated in proliferating ECs [19] and PAH-ECs [64], making manipulation of this pathway a potential target to treat pathological angiogenesis. Indeed, blocking FA synthesis by inhibition of acetyl-CoA carboxylase ACC (by soraphen A or genetic inactivation), the enzyme catalyzing the reaction from acetyl-CoA to malonyl-CoA, decreases EC migration [65]. Mechanistically, the reduced malonyl-CoA levels impede FA- and subsequently phospholipid synthesis [66], leading to a shift in the membrane phospholipid composition that affects membrane fluidity and filopodia formation (Fig. 2,3). Inhibiting fatty acid synthase (FASN), which is crucial for FA elongation from malonyl-CoA, decreases EC migration and/or proliferation [61,67]. Unexpectedly, since the angiogenesis defect cannot be rescued by palmitate supplementation [61], this defect is not a consequence of FA depletion, as observed in cancer cells [68]. Instead, blockade of fatty acid synthesis leads to changes in post-translational protein modifications, i.e. reduced eNOS palmitoylation or increased mTOR malonylation, which reduces their activity and impairs vascular sprouting (Fig. 2,3) [61,67].

FAs intended for different purposes (storage, elongation or oxidation) are shuttled between organelles by fatty acid transporters. Interestingly, the fatty acid transporter FABP4 is upregulated in angiogenesis, while its inhibition in ECs perturbs FA metabolism (by lipolysis of stored FAs, which increases the intracellular FA content and FAO) and induces oxidative stress, leading to reduced EC migration and proliferation [69,70].

For catabolic use, FAs are imported into mitochondria by carnitine palmitoyl transferase 1a (CPT1a), where they are oxidized and enter the TCA cycle in the form of acetyl-CoA (Fig. 2). Whereas

198 many other cell types use FAO for energy production [71-73], FAO in proliferating blood vascular and
199 lymphatic ECs is essential for sustaining the TCA cycle (in conjunction with other anaplerotic sub-
200 strates), which results in the synthesis of aspartate, a precursor of nucleotides needed for DNA repli-
201 cation during EC proliferation (Fig. 3) – a dependency rarely observed in other cell types [37,74]. Im-
202 portantly, the TCA cycle in ECs is not replenished by a net contribution of FA-derived carbons in the
203 traditional meaning of anaplerosis [75]. Although ^{13}C-labelled acetyl-CoA derived from the FA palmitate
204 enters the TCA cycle and labels oxaloacetate, there is no net formation of oxaloacetate from acetyl-
205 CoA, derived from FAs. Rather, in conditions of sufficient availability of anaplerotic substrates (gluta-
206 mine, glucose), FAO provides acetyl-CoA, which in conjunction with an anaplerotic substrate helps to
207 sustain the TCA cycle, necessary for dNTP synthesis for proliferation. Blocking FAO by inhibiting CPT1a
208 (by etomoxir) in angiogenic ECs reduces EC proliferation but not migration [37]. Supplementation with
209 acetate, which can be converted to acetyl-CoA, or with nucleosides rescues the FAO defect [37]. In con-
210 trast to angiogenic ECs, quiescent ECs utilize FAO for NADPH regeneration via the TCA cycle (see Re-
211 dox metabolism, Fig. 3) [19].

212 **FATTY ACID AND KETONE BODY METABOLISM IN LYMPHATIC ECS**

213 Besides its importance in blood vascular ECs, CPT1a regulates lymphatic EC (LEC) development, not
214 only by sustaining the TCA cycle and promoting nucleotide synthesis via the above described non-
215 anaplerotic mechanism, but also by regulating venous-to-lymphatic EC differentiation via an epigenet-
216 ic mechanism (Fig. 4) [74,76]. During embryonic development, LECs differentiate from venous ECs in a
217 process driven by the transcription factor PROX1, which upregulates the expression of a lymphangio-
218 genesis program, e.g. VEGF receptor 3 (*VEGFR3*) and others [77]. PROX1 binds to the CPT1a promotor
219 and enhances its expression, leading to increased acetyl-CoA levels. Acetyl-CoA is then used for his-
220 tone acetylation of PROX1-binding sites via direct interaction of PROX1 with the histone acetyltrans-

ferase p300. As a result, PROX1 makes its target genes more accessible for itself, thereby potentiating its own transcriptional activity [74].

The fact that the lymphangiogenesis defect caused by FAO inhibition can be rescued by acetate supplementation highlights the importance of acetyl-CoA for LECs. Primed by these results, a recent study showed that acetyl-CoA derived from ketone body oxidation is crucial for lymphangiogenesis [6]. Ketone bodies are energy-rich metabolites secreted by the liver, which are oxidized in mitochondria of extra-hepatic tissues into two molecules of acetyl-CoA, which can then enter the TCA cycle (Fig. 2) [78]. LEC-specific loss of 3-oxoacid-CoA-transferase-1 (OXCT1), a key enzyme of ketone body oxidation[78], reduces LEC proliferation, migration and vessel sprouting *in vitro* and impairs lymphangiogenesis in development and disease in Prox1$^{\Delta OXCT1}$ mice, lacking Oxct1 in LECs [76]. Mechanistically, OXCT1 silencing lowers acetyl-CoA levels, TCA cycle metabolite pools, and aspartate and dNTP levels, required for LEC proliferation (Fig. 4). Ketone body supplementation to LECs induces opposite effects. Notably, elevation of lymph ketone body levels in wild type (but no longer in Prox1$^{\Delta OXCT1}$) mice by a high-fat, low-carbohydrate ketogenic diet or by administration of the ketone body β-hydroxybutyrate (β-OHB) increases lymphangiogenesis after corneal injury and myocardial infarction [78]. Intriguingly, in a mouse model of microsurgical ablation of lymphatic vessels in the tail, which recapitulates features of acquired lymphedema in humans, the ketogenic diet improved lymphatic vessel function and growth, reduced infiltration of anti-lymphangiogenic immune cells, and decreased edema [78], suggesting a novel metabolite dietary therapeutic opportunity (see also Concluding Remarks) [76].

GLUTAMINE AND ASPARAGINE METABOLISM

Of the set of amino acids analyzed, cultured ECs consume glutamine (the most abundant non-essential amino acid in the circulation) to the largest extent and use it, as many other cell types, not only for protein and nucleotide synthesis but also as a carbon source to replenish the TCA cycle via

glutaminase 1 (GLS1) (Fig. 2, 5) [39,79]. As such, GLS1 supports nucleotide synthesis and – to a certain extent – citrate production and lipid biosynthesis via reductive carboxylation, though the latter contextual finding requires confirmation [38,39]. Glutaminase 2, on the other hand, provides glutamine carbons and nitrogen for glutathione synthesis used for redox homeostasis in non-EC types, but its role has not been studied in ECs yet (see Redox Metabolism). Accordingly, in *in vitro* and *in vivo* models, glutamine catabolism blockade (by metabolic, genetic or pharmacological means) in ECs impairs protein and nucleotide synthesis, and renders ECs susceptible to oxidative stress, which leads to proliferation arrest and/or affects EC motility [38,39].

Glutamine is essential for the synthesis of other amino acids and consequently proteins, and is a nitrogen donor for the *de novo* synthesis of asparagine in ECs. Notably, supplementation of asparagine plus α-ketoglutarate rescued the phenotypes induced by glutamine starvation [38,39]. In contrast, single agent supplementation with anti-oxidants or TCA cycle replenishment sufficed to rescue the glutamine depletion-induced phenotype in cancer cells but not in ECs, suggesting a strong reliance of ECs on glutamine [39]. This can be even more apparent in disease conditions, as for example ECs infected by Kaposi 's sarcoma-associated herpes virus depend on glutamine breakdown for survival [80]. Asparagine is believed to serve as a signaling metabolite (rheostat) that senses the availability of TCA intermediates for non-essential amino acid synthesis and coordinates a homeostatic response (Fig. 5) [81]. Even though asparagine can be taken up by ECs, synthesis of this particular amino acid seems to be critical, as inhibition of asparagine synthetase (ASNS, the enzyme producing asparagine from glutamine and aspartate) impairs EC proliferation, even in conditions where asparagine is not limiting [39].

Intriguingly, despite the high levels of glutamine in the circulation, ECs express glutamine synthetase (GS), allowing them to synthesize glutamine from glutamate. However, even in glutamine-deprived conditions (which upregulates GS expression), ECs still only synthesize negligible amounts of glutamine [82]. Nonetheless, GS is of particular importance during vessel growth as EC loss of GS impairs

EC migration, but not proliferation (Fig. 5) [82]. The underlying mechanism relates, at least in part, to perturbed actin remodeling, caused by decreased localization in the plasma membrane of RhoJ, a Rho GTPase enriched in ECs and an assembly regulator of cytoskeleton proteins [83]. RhoJ requires palmitoylation for plasma membrane localization and activity, which is lost upon GS blockade. Molecular characterization and structural modeling identified GS as a previously unrecognized palmitoyl transferase [82]. While RhoJ was one identified target of the palmitoyl transferase activity of GS, it is likely that other targets are also affected.

SERINE METABOLISM

ECs can take up serine from the extracellular milieu or synthesize it *de novo* from the glycolytic intermediate 3-phosphoglycerate (3PG) (Fig. 2, 5) and utilize it for the synthesis of heme, glutathione and nucleotides (dATP, dGTP, dTTP) [84]. The *de novo* synthesis pathway starts with a rate-controlling step controlled by phosphoglycerate dehydrogenase (PHGDH) [85]. Loss of endothelial PHGDH impairs EC proliferation and survival, causing severe vascular defects and neonatal lethality upon Phgdh gene inactivation in ECs [48]. The underlying mechanism of the PHGDH inhibition defect is a depletion of the cellular heme pool, which impedes the activity of heme-containing OXPHOS complexes III and IV, and thus results in electron leakage and defective mitochondrial respiration (Fig. 5) [48]. Escaped electrons react with oxygen, which – together with decreased glutathione levels – causes ROS accumulation and oxidative stress, and induces EC death [48]. Moreover, PHGDH defective ECs display not only the expected decrease in dATP, dGTP and dTTP levels, but also in dCTP levels (not documented in any other cell type to date). This is due to an impaired activity of dihydroorotate dehydrogenase (DHODH) – a mitochondrial enzyme crucial for dCTP synthesis, the activity of which is coupled to and dependent on functional electron transport in OXPHOS. Treatment of ECs with a heme synthesis blocker phenocopied the PHGDH loss, while supplementation with hemin rescued the proliferation defect in

PHGDH defective ECs, thus illustrating the importance of the role of PHGDH-dependent serine synthesis in heme production [48]. In contrast to other cell types [48,86,87], ECs rely on *de novo* serine biosynthesis in a peculiar manner, as PHGDH knockdown in ECs reduces cellular serine levels and induces apoptosis, which cannot be rescued by supplementation with the amino acids glycine and serine, even though they can take up serine from the extracellular medium [48]. In tumors, the flux of glycolytic intermediates to serine biosynthesis is enhanced to sustain nucleotide synthesis for the hyperproliferative TEC phenotype [22,48]. Consistently, TECs express elevated levels of PHGDH [48], representing a therapeutic vulnerability to be explored for new anti-angiogenic approaches.

REDOX METABOLISM

A balanced redox state is crucial for ECs (for reviews, see [88,89]). At physiological concentrations, ROS serve as key mediators in cellular signaling regulating among others EC growth, survival and angiogenesis [90]. Highly elevated levels of ROS, however, give rise to oxidative stress, reduce bioavailability of the key vascular tone regulator nitric oxide (NO) and lead to EC activation and dysfunction, as has for example been observed in ECs in atherosclerosis [91].

Healthy quiescent ECs lining perfused vessels are exposed to a high oxygen environment, which necessitates protective mechanisms against oxidative damage (Fig. 1). For this reason, upon entering a quiescent state, ECs adapt their metabolism by increasing FAO (Fig. 3) [19]. A similar increase in FAO was documented upon overexpression of FOXO1, which induces EC quiescence [20]. Interestingly, the anti-mitogenic signal Notch, which inhibits nucleotide synthesis, seems to be a major regulator of controlling the switch between the use of FAO for promoting nucleotide synthesis in proliferating ECs (low Notch) *versus* the use of FAO for redox homeostasis in quiescent ECs (high Notch) [19]. Since proliferating ECs utilize FAO to support the TCA cycle for nucleotide synthesis (see above), it is surprising that quiescent ECs would have higher, not lower FAO levels, since quiescent ECs synthesize fewer

nucleotides as they proliferate less. Intriguingly, the function of FAO in quiescent ECs is markedly different. Instead of being used for energy production or TCA cycle support for biomass synthesis, FAO is used to sustain redox homeostasis in quiescent ECs [19]. FA-derived acetyl-CoA is used to sustain the TCA cycle, which generates isocitrate and malate, i.e. substrates of NADPH-producing malic enzyme and isocitrate dehydrogenase (IDH2, Fig. 2), the deficiency of which causes oxidative stress in ECs and vascular inflammation [92]. Indeed, NADPH is used to regenerate reduced glutathione (GSH) from its oxidized form (GSSG) by glutathione reductase (see below) [93]. At the same time, when ECs switch from proliferation to quiescence, they upregulate the expression of other NADPH-generating pathways and vasculoprotective proteins that consume NADPH (e.g. G6PDH, glutaredoxin-2 (GLRX2), glutathione peroxidase 3 (GPx3), peroxiredoxin-1 (PRDX1) and eNOS, Fig. 3) [19]. Hence, to meet the increased demands of NADPH by vasculoprotective genes, quiescent ECs reprogram their metabolism to produce more NADPH via several routes, including increased FAO. The increased demands of antioxidant protection thus seem to relate to the fact that quiescent ECs are exposed to high oxygen levels in the blood stream and hence are in need of mechanisms to protect them from oxidative stress.

This may explain why EC loss of CPT1a aggravates inflammatory bowel disease due to elevated ROS levels [19]. Indeed, EC loss of CPT1a or its pharmacological inhibition *in vivo* induces vascular leakage and EC hyperpermeability (by affecting intracellular Ca^{2+} homeostasis) and leads to EC dysfunction [94,95]. Of interest, treatment of mice, lacking CPT1a in ECs, with acetate (a source of acetyl-CoA) protects them against ROS production, vascular inflammation and EC dysfunction [19], raising the question whether such type of metabolite delivery might be useful for the treatment of vascular complications in diabetes, atherosclerosis, etc.

Several other metabolic pathways are involved in the protection against oxidative damage in ECs: (i) by relying mostly on glycolytic metabolism, ECs limit the levels of ROS produced by OXPHOS; (ii) both glutamine and glycine are required for glutathione synthesis and, thus, deprivation of gluta-

mine or serine (which is converted to glycine via serine hydroxymethyltransferase) are associated with increased ROS levels (see Serine Metabolism) [39,48]; (iii) serine, as one-carbon donor for the folate cycle, contributes to the generation of NADPH; (iv) UbiA prenyltransferase domain-containing protein 1 (UBIAD1), which synthesizes the eNOS cofactor Coenzyme Q10, protects ECs from oxidative stress and ROS-mediated lipid peroxidation [96].

CONCLUDING REMARKS & THERAPEUTIC PERSPECTIVES

Studies during the past decade highlighted the importance of EC metabolism in vessel sprouting *in vitro* and *in vivo*, and illustrated that manipulating EC metabolism can overrule instructions by (lymph)angiogenic signals. While not only providing new fundamental insights in EC biology, these findings also raise the question whether they can serve for the development of novel therapeutic strategies to promote or inhibit growth of blood vascular or lymphatic vessels. We will highlight two complementary examples. First, as an example of an approach to inhibit a metabolic target in ECs, we discuss the potential of blockade of the glycolytic activator PFKFB3 in glycolysis-addicted TECs (Fig. 6A). While a justified concern is that not only TECs but also other healthy ECs and non-EC types may require glycolysis, TECs *rely* on glycolysis more than other cell types, which can switch to other metabolic pathways more readily upon glycolysis blockade; precisely therefore, they are more sensitive to even slight reductions in glycolysis levels [22]. The therapeutic goal is not to *eliminate* glycolysis (which would cause toxic side-effects in other cell types) but rather to *normalize* the hyperglycolysis back to glycolysis levels in quiescent ECs, so that TECs do no longer grow [97]. In fact, lowering TEC glycolysis by 15-25% sufficed to normalize tumor vessel abnormalities, thereby reducing metastasis and improving chemotherapy [22], without inducing systemic toxicity (because such a low level of glycolysis inhibition is readily compensated by cell types that are not addicted to glycolysis). In contrast, lowering TEC glycolysis by a maximally tolerable anti-glycolytic activator dose induced opposite effects due to tumor

vessel disintegration [27]. Clinical development of an inhibitor of a glycolytic activator will be required to prove this hypothesis. Silencing or blocking other EC metabolic targets (hexokinase 2 [21]; PKM2 [29]; GP [30]; PHGDH [48]; CPT1a [37]; ACC1 [65]; FAPB4 [69,70]; GS [82]; GLS1 [38,39]; G6PDH [30,32]; FASN [61,67]; OXCT1 [76]; UQCRQ [47]; FADH1 [49]) has been shown to inhibit vessel sprouting *in vitro* and/or *in vivo*. These targets might deserve future attention for possible AAT development, whereby it would be preferable to develop strategies to target such drugs to the ECs in neovessels in diseased tissues, while sparing normal quiescent ECs in healthy tissues. Indeed, studies showing that, for instance, the inhibition of PKM2 impairs the vascular barrier integrity of quiescent ECs and that CPT1a inhibition leads to oxidative stress and dysfunction of quiescent ECs urge for caution when developing EC metabolism-targeted therapeutics [19,98].

Second, a ketogenic diet showed a promising improvement of lymphedema in a preclinical mouse model, a strategy rationally deduced from obtaining fundamental insights on how lipids and ketone bodies affect LEC biology [74,76]. This has resulted in the initiation of a phase II clinical trial to test the clinical effect of dietary ketone bodies (high fat/low carbohydrate ketogenic diet) in lymphedema patients (Fig. 6B).

Despite recent advances, only few metabolic enzymes have been characterized in ECs to date in sufficient detail. Further research is needed to uncover complex metabolic links and to identify other pathways that can be targeted to control angiogenesis or EC dysfunction. Considering the functional heterogeneity of ECs in different states (quiescent, stalk, tip cells), vascular beds (arterial, venous, capillary, lymphatic vessels) and tissues, multi-omics profiling of ECs including at the single cell level could prove to be invaluable for future development of EC-targeted therapies. Advanced bioinformatics tools allowing easy dataset exploration, like EndoDB [99], and *in silico* approaches like computational genome-scale metabolic modelling of ECs can further help facilitate the discovery of novel unexplored targets and promise to yield exciting new perspective in the field.

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651

FIGURE LEGENDS

FIGURE 1: ECS RELY ON METABOLIC REWIRING FOR ANGIOGENESIS

Quiescent ECs adapt their metabolism to endure high oxygen levels, which they are exposed to. When activated, ECs rewire their metabolism to sprout new vessels. Growth factors (schematically represented by green shapes) like fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) bind to receptors on ECs and induce proliferation of "stalk cells" and migration of "tip cells". This phenotypic switch is dependent on metabolic changes in angiogenic ECs, which ensure amongst other energy and biomass production for proliferation and migration.

FIGURE 2: KEY METABOLIC PATHWAYS IN ECS.

The functions of the major metabolic pathways in ECs are described in the main text. Proteins discussed in the main text are depicted in dark blue font, red symbols in the OXPHOS pathway represent heme cofactors.

ABBREVIATIONS: 3PG, 3-phosphoglycerate; ACC, Acetyl-CoA Carboxylase; ASNS, Asparagine Synthetase; ATP, Adenosine triphosphate; β-OHB, Beta hydroxybutyrate; CD36, CD36 molecule (fatty acid translocase); CoA, Coenzyme A; CPT1A, Carnitine palmitoyltransferase 1A; DHODH, Dihydroorotate dehydrogenase; F2,6BP, Fructose-2,6-biphosphate; F6P, Fructose-6-phosphate; FAHD1, Fumarylacetoacetate hydrolase domain containing 1 (oxaloacetate decarboxylase); FASN, Fatty acid synthase; FATP, Fatty acid transport protein; G3P, Glyceraldehyde-3-phosphate; G6P, Glucose-6-phosphate; G6PDH, Glucose-6-phosphate dehydrogenase; GFAT1, Glutamine:fructose-6-phosphate amidotransferase 1; GLS1, Glutaminase 1; GLS2, Glutaminase 2; GLUT1, Glucose transporter 1; GP, Glycogen phosphorylase; GS, Glutamine synthetase; GSH, Glutathione; HK2, Hexokinase 2; IDH2, Isocitrate dehydrogenase 2; LECs, Lymphatic endothelial cells; ME3, Malic enzyme 3; NAD^+/NADH, Nicotinamide adenine dinu-

cleotide (oxidized/reduced); $NADP^+$/NADPH, Nicotinamide adenine dinucleotide phosphate (oxidized/reduced); NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; OXCT1, 3-oxoacid CoA-transferase 1; OXPHOS, Oxidative phosphorylation (mitochondrial respiration); PEP, Phosphoenolpyruvate; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PHGDH, Phosphoglycerate dehydrogenase; PKM2, Pyruvate kinase isozyme M2; R5P, Ribose-5-phosphate; SDH, Succinate dehydrogenase; TCA, Tricarboxylic acid; TKT, Transketolase; UDP-GlcNAc, Uridine diphosphate N-acetylglucosamine.

FIGURE 3: ADAPTATIONS OF METABOLIC PATHWAYS IN QUIESCENT VERSUS ANGIOGENIC ECS

Quiescent ECs (a) adapt their metabolism to maintain redox homeostasis to cope with their oxidative stress-prone high oxygen environment (e.g. by upregulating the expression of vasculoprotective proteins and regeneration of NADPH, as well as by limiting ROS production). Angiogenic ECs (b) rewire their metabolic pathways for energy and biomass production essential for cell proliferation and migration. The pathways and the corresponding text boxes are highlighted with different colors (yellow, glycolysis; brown, pentose phosphate pathway (PPP); dark green, fatty acid oxidation; light red, redox metabolism; pink, OXPHOS; light green, fatty acid synthesis). The width of the highlighted pathway corresponds to the flux through this pathway. For reasons of clarity, the metabolic pathways are shown only schematically; for names of metabolites and metabolic enzymes, see Figure 2.

ABBREVIATIONS: ACC, Acetyl-CoA Carboxylase; AcCoA, Acetyl-CoA; Asp, Aspartate; ATP, Adenosine triphosphate; CI, Mitochondrial complex I; CIII, Mitochondrial complex III; CPT1A, Carnitine palmitoyltransferase 1A; DNA, Deoxyribonucleic acid; eNOS, Endothelial nitric oxide synthase 3; FA, Fatty acid; FASN, Fatty acid synthase; FOXO1, Forkhead box protein O1; G6PDH, Glucose-6-phosphate dehydrogenase; GLRX2, Glutaredoxin 2; GPX3, glutathione peroxidase 3; HK2, Hexokinase 2; IDH2, Isocitrate dehydrogenase 2; ME3, Malic enzyme 3; NAD+/NADH, Nicotinamide adenine dinucleotide (ox-

idized/reduced); NADP+/NADPH, Nicotinamide adenine dinucleotide phosphate (oxidized/reduced); OXPHOS, Oxidative phosphorylation (mitochondrial respiration); PAH, Pulmonary arterial hypertension; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PRDX1, Peroxiredoxin 1; PTM: post-translational modification (malonylation; palmitoylation); ROS, Reactive oxygen species; TCA, Tricarboxylic acid; TECs, Tumor endothelial cells.

FIGURE 4: ADAPTATIONS OF METABOLIC PATHWAYS IN LYMPHATIC ECS

When stimulated by growth factors, lymphatic ECs (LECs) undergo metabolic adaptations (i.e. upregulation of ketone body oxidation (highlighted in red) and fatty acid oxidation (highlighted in dark green)) to increase the synthesis of acetyl-CoA, which sustains the TCA cycle (in conjunction with anaplerotic substrates), resulting in the production of aspartate, a nucleotide precursor. The lymphatic master regulator PROX1 increases FAO by upregulating Cpt1a gene expression that leads to increased synthesis of acetyl-CoA, which is used for histone acetylation of PROX1 target genes by the histone acetylase p300, thereby facilitating transcription of PROX1 targets and lymphatic differentiation of ECs. For reasons of clarity, the metabolic pathways are shown only schematically; for names of metabolites and metabolic enzymes, see Figure 2.

ABBREVIATIONS: Asp, Aspartate; ATP, Adenosine triphosphate; CPT1A Carnitine palmitoyltransferase 1A; DNA, Deoxyribonucleic acid; OXCT1, 3-oxoacid CoA-transferase 1; p300, Histone acetyltransferase p300; PROX1, Prospero Homeobox 1.

FIGURE 5: AMINO ACID METABOLISM IN ANGIOGENIC ECS

Angiogenic ECs upregulate amino acid synthesizing pathways, which are used for protein and nucleotide synthesis, redox homeostasis or posttranslational protein modification (PTM; palmitoylation). Serine biosynthesis (Ser, highlighted in light blue) promotes nucleotide synthesis via one carbon metabolism and synthesis of the antioxidant glutathione (GSH) and heme, a co-factor of OXPHOS complexes, thereby securing mitochondrial respiration and dCTP synthesis by DHODH. Glutamine (Gln, highlighted dark blue) is taken up from the environment and used to fuel the TCA cycle for biomass production or asparagine synthesis (Asn). In contrast, glutamine synthase (GS) is negligible for glutamine synthesis but regulates EC migration as palmitoyl transferase of RhoJ, a regulator of cytoskeletal organization. For reasons of clarity, the metabolic pathways are shown only schematically; for names of metabolites and metabolic enzymes, see Figure 2.

ABBREVIATIONS: ASNS, Asparagine Synthetase; ATP, Adenosine triphosphate; dATP, Deoxyadenosine triphosphate; dCTP, Deoxycytidine triphosphate; dGTP, Deoxyguanosine triphosphate; DHODH, Dihydroorotate dehydrogenase; dTTP, Deoxythymidine triphosphate; GLS1, Glutaminase 1; Glu, Glutamate; GS, Glutamine synthetase; GS, Glutamine synthetase; PHGDH, Phosphoglycerate dehydrogenase; RhoJ, Ras homolog family member J; TCA, Tricarboxylic acid; TECs, Tumor endothelial cells.

FIGURE 6: THERAPEUTIC STRATEGIES TARGETING EC METABOLISM

A) In response to growth factors released by cancer cells, glycolysis in TECs is hyperactivated, leading to hyperproliferation and impaired vascular barrier integrity, which compromises blood flow and facilitates cancer cell intravasation and metastasis (left panel). Inhibiting glycolysis moderately by approximately 20% (using a low dose of the PFKFB3 inhibitor 3PO) normalizes the glycolytic flux to levels observed in quiescent ECs, which reduces EC proliferation and re-establishes vessel barrier integrity. This reduces metastasis and re-establishes flow in tumor blood vessels, which promotes delivery of anti-

740 tumor drugs (middle panel). In contrast, glycolysis inhibition by 40% or more using a maximally toler-
741 able 3PO dose is toxic for ECs, leading to vessel disintegration and enhanced metastasis (right panel).

742 B) In patients with operable breast cancer, lymph nodes are often surgically removed. This disrupts
743 the lymphatic vessel network, which can lead to fluid accumulation and the development of
744 lymphedema (left). Administration of ketone bodies via a ketogenic diet to the disrupted lymphatic
745 vessels stimulates the formation of new lymphatic vessels (lymphangiogenesis), which reduces
746 lymphedema (right).

747 ABBREVIATIONS: 3PO, Selective inhibitor of PFKFB3; ATP, Adenosine triphosphate; PFKFB3, 6-
748 phosphofructo-2-kinase/fructose-2,6-biphosphatase 3.

749

750

Figure 1

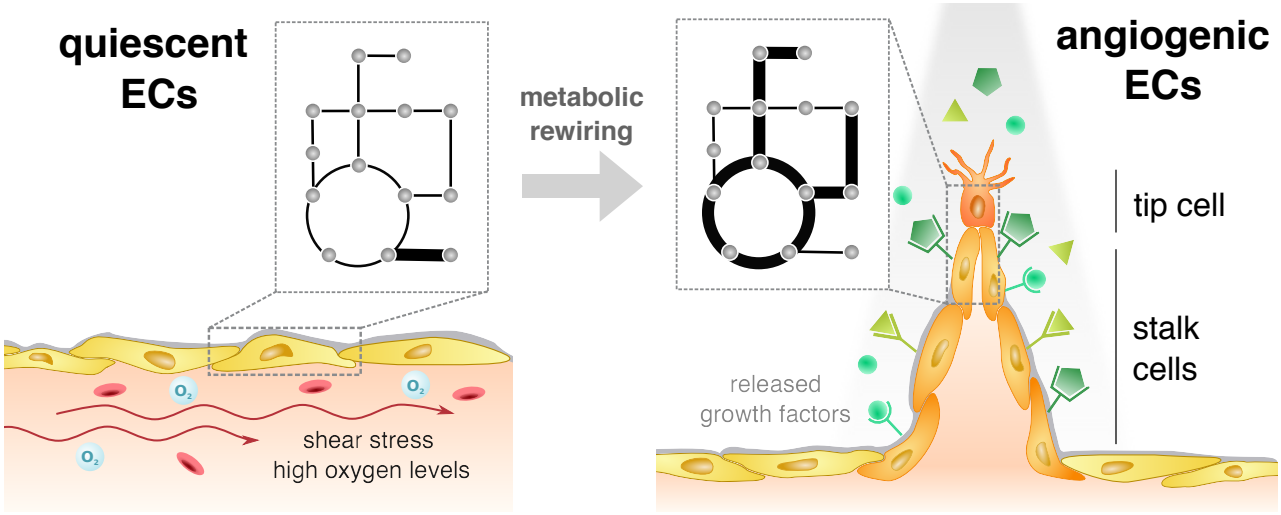

quiescent
ECs
metabolic
rewiring
angiogenic
ECs
tip cell
stalk
cells
released
growth factors
shear stress
high oxygen levels
O2
O2
O2

Figure 2

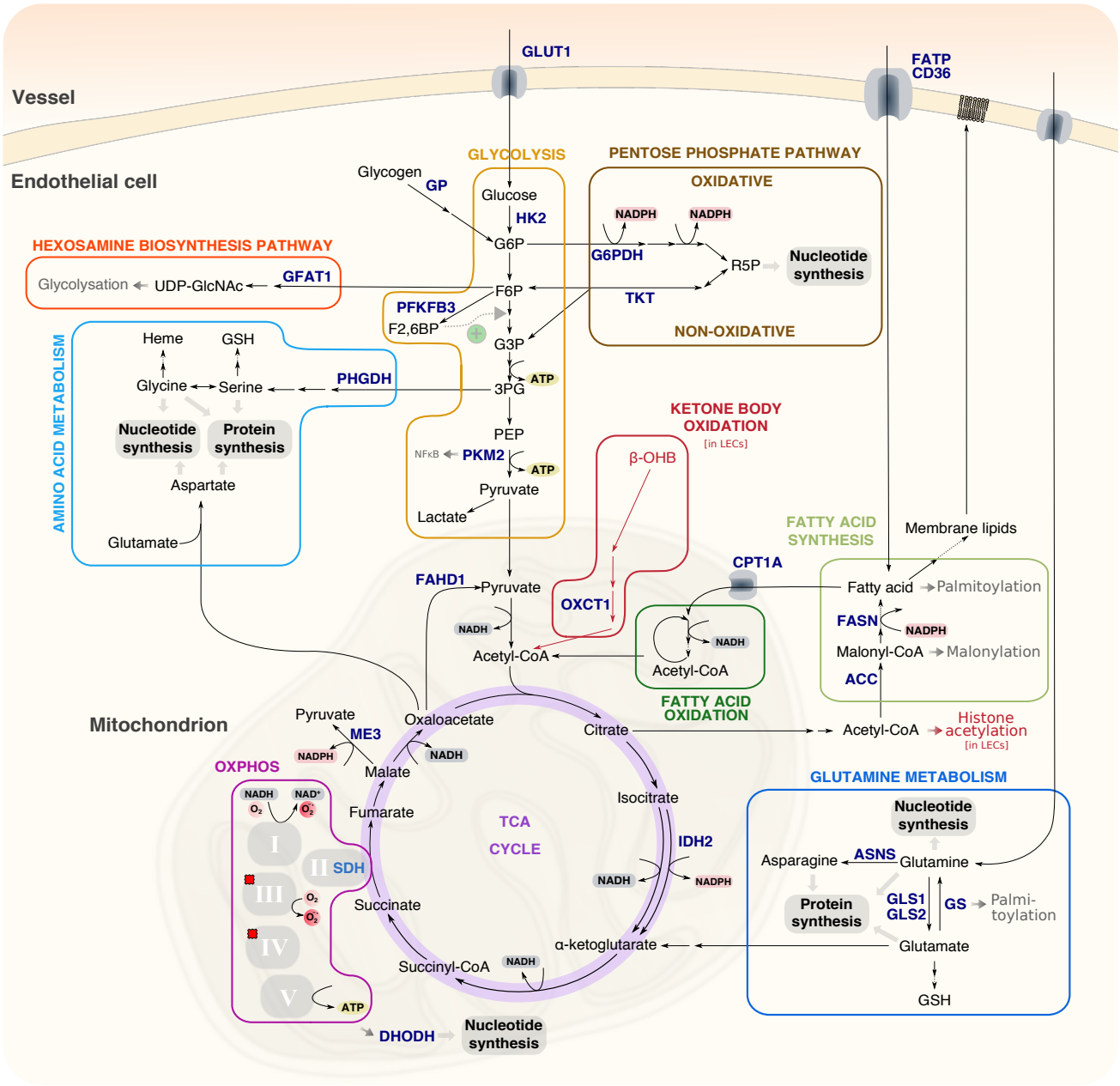

Vessel
Endothelial cell
GLUT1
FATP
CD36
GLYCOLYSIS
PENTOSE PHOSPHATE PATHWAY
OXIDATIVE
NON-OXIDATIVE
Glycogen
GP
Glucose
HK2
G6P
G6PDH
NADPH
R5P
Nucleotide synthesis
TKT
F6P
PFKFB3
F2,6BP
G3P
ATP
3PG
PHGDH
HEXOSAMINE BIOSYNTHESIS PATHWAY
Glycolysation
UDP-GlcNAc
GFAT1
AMINO ACID METABOLISM
Heme
GSH
Glycine
Serine
Nucleotide synthesis
Protein synthesis
Aspartate
Glutamate
PEP
NFκB
PKM2
Pyruvate
Lactate
KETONE BODY OXIDATION
[in LECs]
β-OHB
OXCT1
FATTY ACID SYNTHESIS
Membrane lipids
CPT1A
FAHD1
Pyruvate
NADH
Acetyl-CoA
Fatty acid
Palmitoylation
FASN
Malonyl-CoA
Malonylation
ACC
FATTY ACID OXIDATION
Mitochondrion
Pyruvate
ME3
NADPH
Malate
Oxaloacetate
Citrate
Acetyl-CoA
Histone acetylation
[in LECs]
OXPHOS
Fumarate
TCA CYCLE
Isocitrate
IDH2
Succinate
SDH
α-ketoglutarate
Succinyl-CoA
DHODH
Nucleotide synthesis
GLUTAMINE METABOLISM
Asparagine
ASNS
Glutamine
GLS1
GLS2
GS
Palmi-toylation
Protein synthesis
Glutamate
GSH

Figure 3

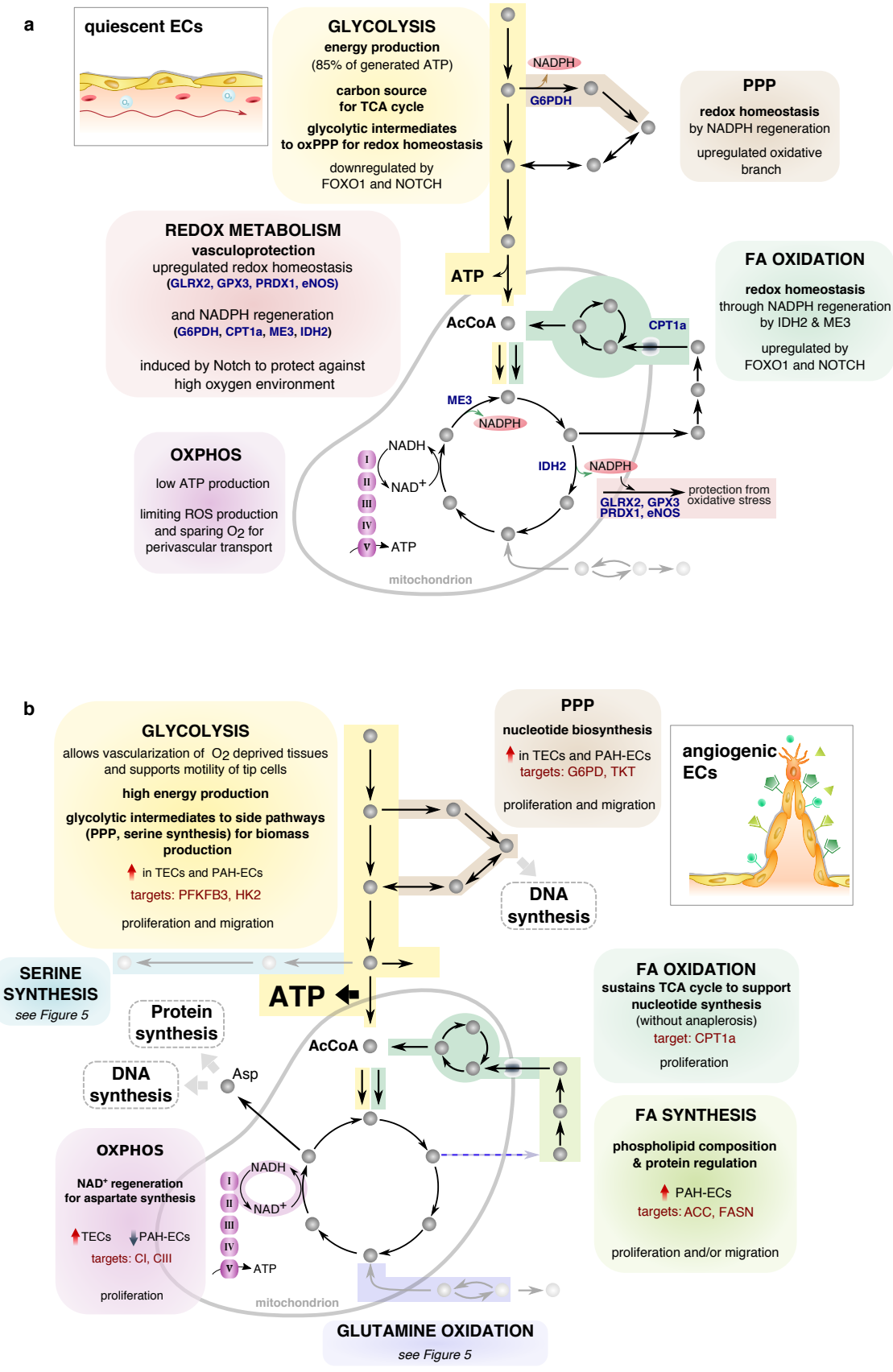

a
quiescent ECs
GLYCOLYSIS
energy production
(85% of generated ATP)
carbon source
for TCA cycle
glycolytic intermediates
to oxPPP for redox homeostasis
downregulated by
FOXO1 and NOTCH
NADPH
G6PDH
PPP
redox homeostasis
by NADPH regeneration
upregulated oxidative
branch
REDOX METABOLISM
vasculoprotection
upregulated redox homeostasis
(GLRX2, GPX3, PRDX1, eNOS)
and NADPH regeneration
(G6PDH, CPT1a, ME3, IDH2)
induced by Notch to protect against
high oxygen environment
ATP
AcCoA
CPT1a
FA OXIDATION
redox homeostasis
through NADPH regeneration
by IDH2 & ME3
upregulated by
FOXO1 and NOTCH
ME3
NADPH
NADH
NAD+
IDH2
NADPH
protection from
oxidative stress
GLRX2, GPX3
PRDX1, eNOS
OXPHOS
low ATP production
limiting ROS production
and sparing O2 for
perivascular transport
I
II
III
IV
V
ATP
mitochondrion
b
GLYCOLYSIS
allows vascularization of O2 deprived tissues
and supports motility of tip cells
high energy production
glycolytic intermediates to side pathways
(PPP, serine synthesis) for biomass
production
in TECs and PAH-ECs
targets: PFKFB3, HK2
proliferation and migration
PPP
nucleotide biosynthesis
in TECs and PAH-ECs
targets: G6PD, TKT
proliferation and migration
angiogenic
ECs
DNA
synthesis
SERINE
SYNTHESIS
see Figure 5
ATP
Protein
synthesis
DNA
synthesis
Asp
AcCoA
FA OXIDATION
sustains TCA cycle to support
nucleotide synthesis
(without anaplerosis)
target: CPT1a
proliferation
FA SYNTHESIS
phospholipid composition
& protein regulation
PAH-ECs
targets: ACC, FASN
proliferation and/or migration
OXPHOS
NAD+ regeneration
for aspartate synthesis
TECs
PAH-ECs
targets: CI, CIII
proliferation
NADH
NAD+
I
II
III
IV
V
ATP
mitochondrion
GLUTAMINE OXIDATION
see Figure 5

Figure 4

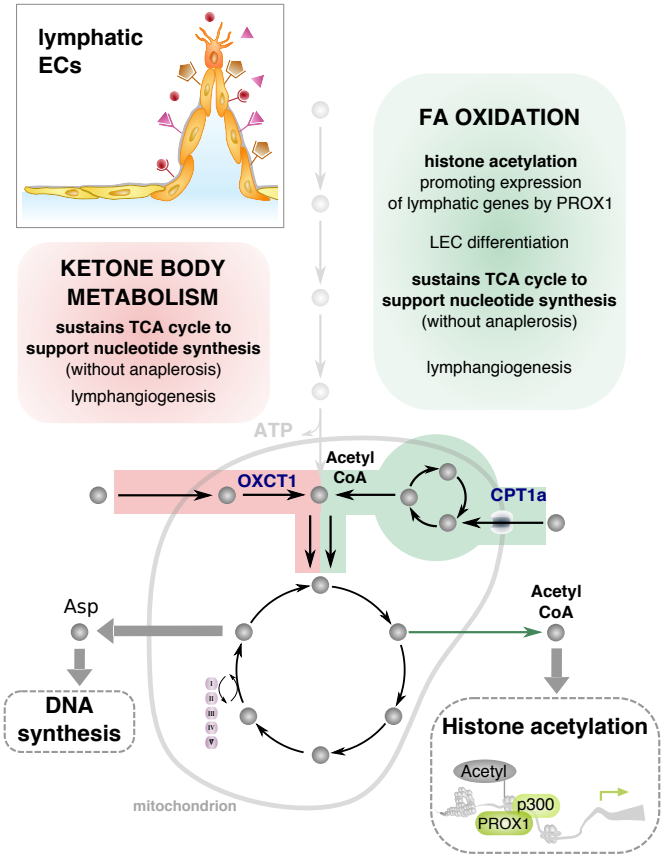

lymphatic ECs
KETONE BODY METABOLISM
sustains TCA cycle to support nucleotide synthesis
(without anaplerosis)
lymphangiogenesis
FA OXIDATION
histone acetylation
promoting expression
of lymphatic genes by PROX1
LEC differentiation
sustains TCA cycle to support nucleotide synthesis
(without anaplerosis)
lymphangiogenesis
ATP
OXCT1
Acetyl CoA
CPT1a
Asp
DNA synthesis
Acetyl CoA
Histone acetylation
Acetyl
p300
PROX1
mitochondrion

Figure 5

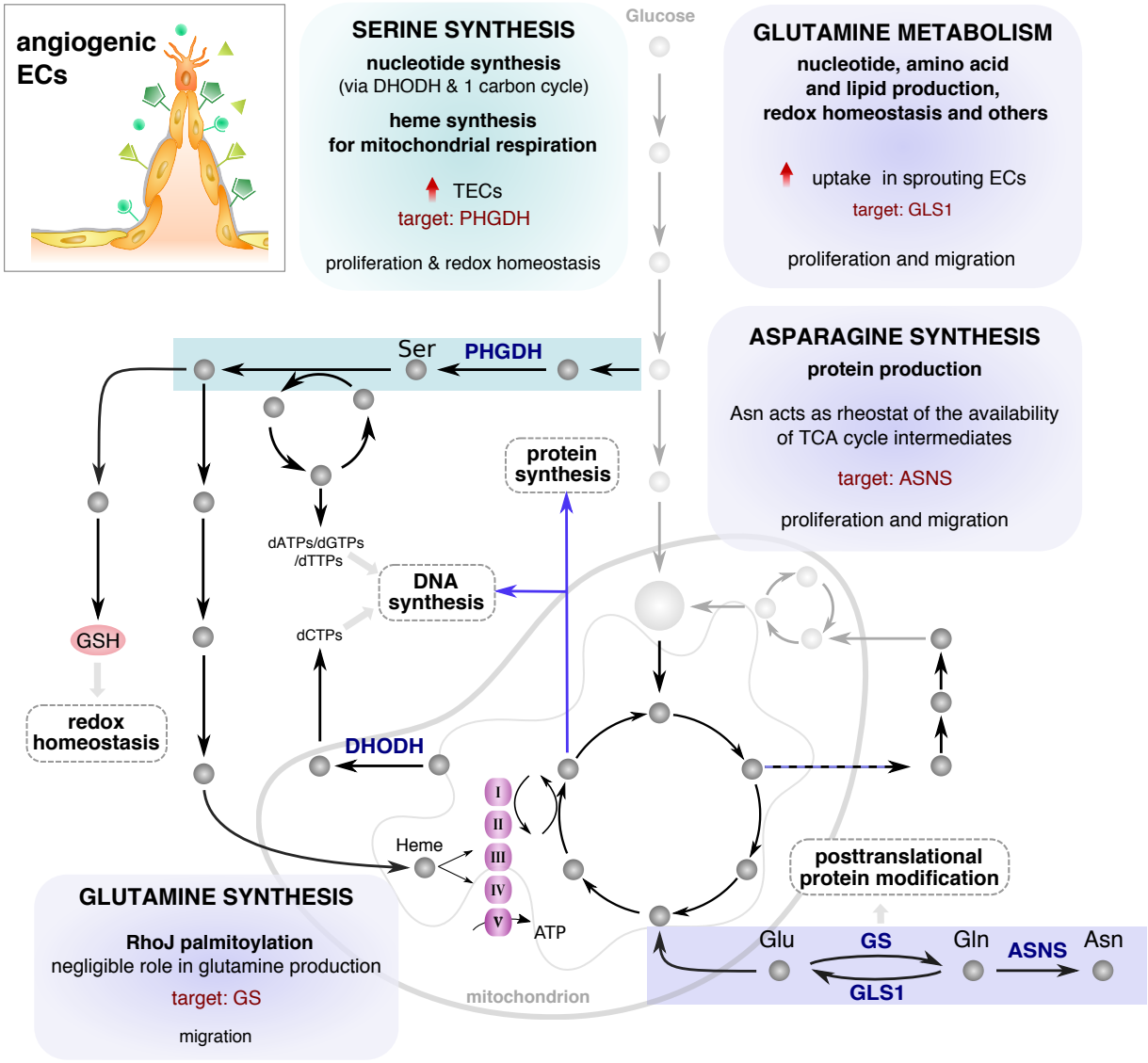
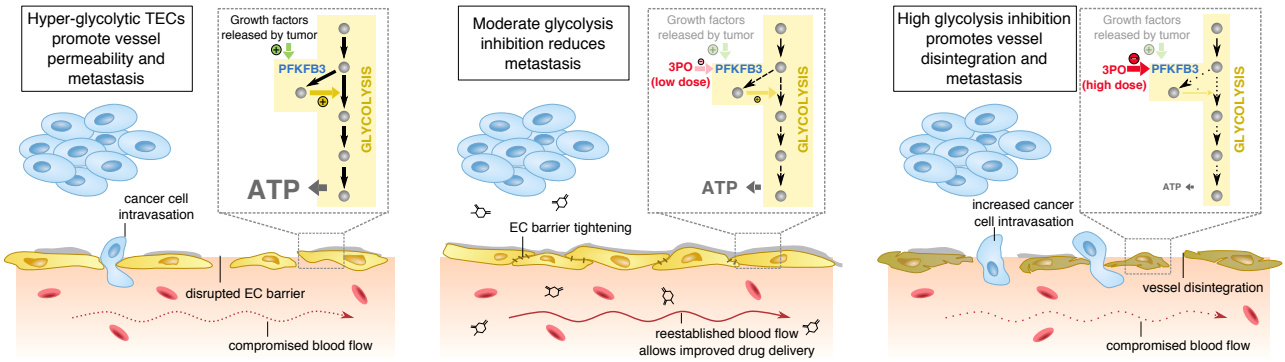
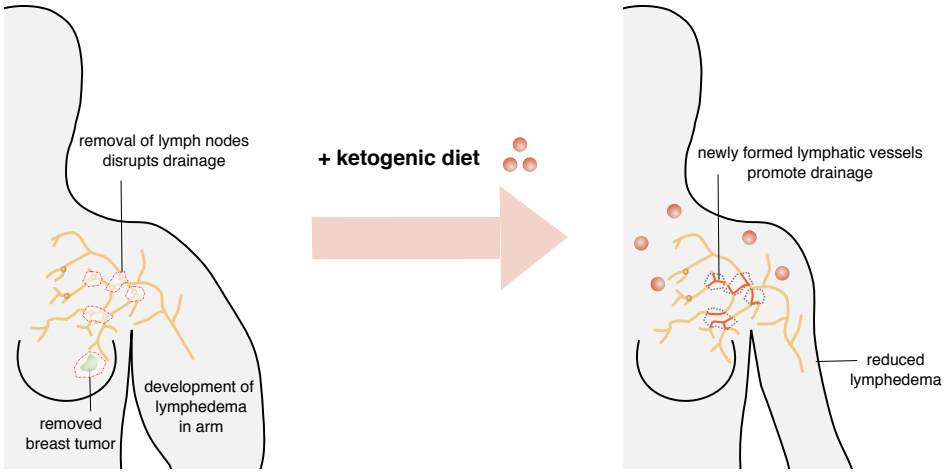

angiogenic ECs
SERINE SYNTHESIS
nucleotide synthesis
(via DHODH & 1 carbon cycle)
heme synthesis
for mitochondrial respiration
TECs
target: PHGDH
proliferation & redox homeostasis
Glucose
GLUTAMINE METABOLISM
nucleotide, amino acid
and lipid production,
redox homeostasis and others
uptake in sprouting ECs
target: GLS1
proliferation and migration
ASPARAGINE SYNTHESIS
protein production
Asn acts as rheostat of the availability
of TCA cycle intermediates
target: ASNS
proliferation and migration
Ser
PHGDH
protein synthesis
dATPs/dGTPs
/dTTPs
DNA synthesis
dCTPs
GSH
redox homeostasis
DHODH
I
II
III
IV
V
Heme
ATP
posttranslational protein modification
Glu
GS
Gln
ASNS
Asn
GLS1
mitochondrion
GLUTAMINE SYNTHESIS
RhoJ palmitoylation
negligible role in glutamine production
target: GS
migration

Figure 6

a


Hyper-glycolytic TECs promote vessel permeability and metastasis
Growth factors released by tumor
PFKFB3
GLYCOLYSIS
ATP
cancer cell intravasation
disrupted EC barrier
compromised blood flow
Moderate glycolysis inhibition reduces metastasis
Growth factors released by tumor
3PO (low dose)
PFKFB3
GLYCOLYSIS
ATP
EC barrier tightening
reestablished blood flow allows improved drug delivery
High glycolysis inhibition promotes vessel disintegration and metastasis
Growth factors released by tumor
3PO (high dose)
PFKFB3
GLYCOLYSIS
ATP
increased cancer cell intravasation
vessel disintegration
compromised blood flow

b


removal of lymph nodes disrupts drainage
removed breast tumor
development of lymphedema in arm
+ ketogenic diet
newly formed lymphatic vessels promote drainage
reduced lymphedema