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Review

Regulation of Angiogenesis by Oxygen and Metabolism

Peter Fraisl,[1,2] Massimiliano Mazzone,[1,2] Thomas Schmidt,[1,2] and Peter Carmeliet[1,2,*]
[1]Vesalius Research Center (VRC), VIB, 3000 Leuven, Belgium
[2]Vesalius Research Center (VRC), K.U.Leuven, 3000 Leuven, Belgium
*Correspondence: peter.carmeliet@med.kuleuven.be
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Blood vessels form an important interface between the environment and the organism by carrying oxygen and nutrients to all cells and thus determining cellular metabolism. It is therefore not surprising that oxygen and metabolism influence the development of the vascular network. Here, we discuss recent insights regarding the emerging crosstalk between angiogenesis and metabolism. We will highlight advances in how oxygen and metabolism regulate angiogenesis as well as how angiogenic factors in turn also regulate metabolism.

Due to the high energy potential of oxygen (O_2), numerous organisms have become dependent on this gaseous substance for their survival. As multicellular organisms attained sizes beyond the diffusion limit of O_2, they developed means to transport O_2 to distant cells, i.e., through an elaborate network of airways in insects and via blood vessels in vertebrates. As the primary function of blood vessels is to transport the O_2 we breathe and the nutrients we eat, it is not surprising that their formation, a process termed angiogenesis, is regulated by variations in O_2 tension and metabolic factors. In this review, we highlight emerging evidence demonstrating how regulation by O_2, and metabolic intermediates and regulators dynamically shapes the microvasculature.

Oxygen Sensing by Endothelial and Smooth Muscle Cells

As angiogenesis is regulated by changes in O_2 tension, and endothelial cells (ECs) and smooth muscle cells (SMCs) constitute the first-line interface with the blood, it is not surprising that these cells are equipped with mechanisms to sense differences in the O_2 supply. ECs have various O_2-sensing mechanisms, including O_2-sensitive NADPH oxidases, endothelial nitric oxide synthase (eNOS), and heme oxygenases, to name a few (Ward, 2008). A substantial body of evidence suggests that mitochondria also participate in sensing O_2 gradients in vascular cells (Archer et al., 2008; Weir et al., 2005). For instance, in response to the increase in O_2 tension upon breathing at birth, the ductus arteriosus constricts (Weir et al., 2005). In these vascular cells, an increase in O_2 tension activates complexes I or III of the mitochondrial electron transport chain, with resultant generation of H_2O_2; this diffusible redox mediator inhibits voltage-gated K^+ channels, causing membrane depolarization, activation of Ca^{2+} channels and, ultimately, vasoconstriction (Archer et al., 2008; Weir et al., 2005).

In addition to these O_2-sensing mechanisms, vascular cells also express a different recently characterized class of O_2 sensors that interface with the hypoxia-inducible transcription factor (HIF) family, which in turn is an important molecular interface for relaying adaptations to changes in O_2 tension. Each of the three isoforms of HIFα (HIF-1–3) can heterodimerize with the HIFβ/ARNT subunit to form an active transcriptional complex that initiates expression of hundreds of genes, including those regulating cell survival, metabolism, and angiogenesis (Semenza, 2003). The activity of these HIFs is regulated by various O_2-sensing enzymes, including prolyl hydroxylase domain proteins (PHD1–3) and factor inhibiting HIFs (FIH) (Kaelin and Ratcliffe, 2008), all of which belong to the 2-oxoglutarate-dependent iron(ii) dioxygenase superfamily. So far, the primary function of these molecules appears to be to act as O_2 sensors. During normoxia, PHDs use O_2 to hydroxylate specific proline residues in their HIF target proteins (Kaelin and Ratcliffe, 2008), marking these targets for recognition by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex, which leads to proteasomal degradation. FIH hydroxylates a conserved asparagyl residue in HIF-1α (Kaelin and Ratcliffe, 2008), which impairs the interaction of HIF-1α with its transcriptional coactivator p300, thereby abrogating the induction of HIF-1α-dependent genes (Kaelin and Ratcliffe, 2008). Thus, when O_2 levels drop, PHDs/FIH become progressively inactive, allowing stabilization of HIFs and triggering downstream activation of the HIF transcriptome. In addition to HIFα, PHDs and FIH also hydroxylate other proteins, some of which are associated with angiogenesis (Kaelin and Ratcliffe, 2008). PHDs exhibit some degree of specificity regarding HIFα subunit recognition, with PHD2 hydroxylating primarily HIF-1α, PHD3 targeting predominantly HIF-2α, and PHD1 showing a slightly higher preference for HIF-2α over HIF-1α (Appelhoff et al., 2004). PHDs and FIH are expressed in vascular cells. As discussed below, O_2 sensing (via the PHD/HIF system) regulates several stages of vessel formation, ranging from EC fate decisions to vasculogenesis and angiogenesis.

Hypoxia Controls Endothelial and Smooth Muscle Cell Fates

In the early embryo, endothelial progenitor cells (EPCs) share a common origin with hematopoietic progenitors; these progenitors differentiate into ECs and become assembled into a primitive vascular plexus (a process termed "vasculogenesis") that later develops via sprouting, branching, and remodeling into a more elaborate, mature vascular network (the process of angiogenesis). In the embryo, low levels of O_2 (~25 mmHg) stimulate the differentiation of various progenitors, including that of early mesoderm into hemangioblasts (Ramirez-Bergeron et al., 2004). Genetic studies show that HIFβ coordinates the early steps of vessel development through paracrine release by hematopoietic cells of angiogenic cytokines, such as VEGF

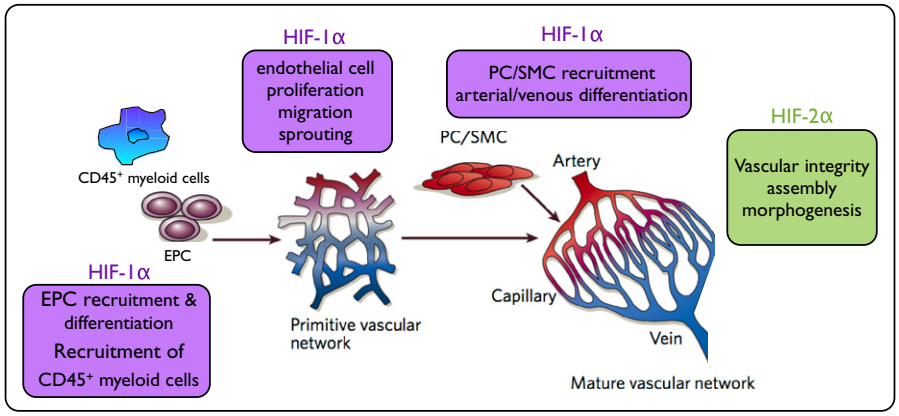

Figure 1. Differential Role of HIF-1α and HIF-2α in the Vascular Development

HIF-1α and HIF-2α have distinct, nonredundant roles in the formation of the vascular tree. While endothelial cell proliferation, migration, and vessel sprouting rely more on HIF-1α, HIF-2α plays a larger role in controlling vascular morphogenesis, integrity, and assembly. HIF-1α also promotes the recruitment of endothelial progenitor cells (EPCs), which physically constitute the vessel wall, and $CD45^+$ myeloid cells, which release angiogenic cues and matrix proteases. HIF-1α favors pericyte (PC) and smooth muscle cell (SMC) recruitment, which cover and stabilize the vessels. HIF-1α also regulates the arterial versus venous fate, promoting the formation of arteries.

and angiopoietin-1 (Ramirez-Bergeron et al., 2006). Hypoxia also promotes the conversion of pulmonary artery endothelial cells into SMC-like cells in vitro (Zhu et al., 2006), while hyperoxia inhibits angioblast differentiation into ECs (Uno et al., 2007; Figure 1).

In the adult bone marrow (BM), progenitors are exposed to O_2 tensions as low as ~20 mmHg. Through activation of HIFs, hypoxia promotes the transition of vascular progenitors to more differentiated cells expressing CD31, VEGFR2, endothelial NO synthase (eNOS), and other mature endothelial markers (Du et al., 2008; Tillmanns et al., 2008). Differences in O_2 tension also alter the specification of EPCs toward an arterial versus venous fate. EPCs contain high amounts of COUP-TFII, a regulator of vein identity (for more details see review by De Val and Black, 2009, this issue of *Developmental Cell*), and low levels of the arterial regulators Delta-like 4 (Dll4; a ligand of the Notch receptor) and Hey2 (a downstream target of Notch) (Diez et al., 2007). Hypoxic activation of HIF-1α elevates the expression of Dll4; this results in induction of the Notch target genes Hey1 and Hey2, and repression of COUP-TFII in EPCs, thus promoting an arterial fate and the formation of arteries (Diez et al., 2007), perhaps to restore the blood and O_2 supply (Figure 1). Transplantation of HIF-1α-overexpressing EPCs improves revascularization of ischemic hindlimbs because the hypoxia-induced differentiation, proliferation, and migration of the EPCs are enhanced (Jiang et al., 2008). Interestingly, when implanted in ischemic myocardium in vivo, c-Kit^+ cardiac progenitor cells form into vascular cells; this conversion of cell fate relies on the induction of stromal-derived factor 1 (SDF-1) by HIF-1α (Tillmanns et al., 2008).

HIF-1α also promotes the recruitment of various BM progenitors to ischemic sites. Circulating endothelial and pericyte progenitors, as well as angiocompetent $CD45^+$ myeloid cells that express the SDF-1 receptor CXCR4, are recruited to and retained in ischemic tissues and tumors through HIF-1α-mediated upregulation of the chemoattractant cue SDF-1α at these sites (Ceradini et al., 2004; Du et al., 2008; Figure 1). Inhibition of SDF-1 in ischemic tissue or of CXCR4 in circulating cells prevents progenitor cell recruitment to sites of injury (Ceradini et al., 2004). In addition, overexpression of VEGF, a target of HIF-1α, stimulates angiogenesis by upregulating SDF-1 and, thereby, recruits CXCR4-positive proangiogenic myeloid cells (Grunewald et al., 2006). In addition, HIF-1α induces the expression of the intracellular adhesion molecule ICAM-1, which serves as a docking site for mobilized EPCs in ischemic tissues (Lee et al., 2006). Similar mechanisms may be operational in hemangiomas (Kleinman et al., 2007) or hemangioblastomas among patients with loss-of-function mutations in the VHL tumor suppressor gene (Zagzag et al., 2005).

Excess O_2 supply may also affect vascular cell function in adults. Indeed, exposure of cultured ECs to hyperoxia leads to dysfunction and death (Xu et al., 2008), similar to the pathological changes that occur in pulmonary arteries during the development of bronchopulmonary dysplasia (Bhatt et al., 2001). EC death due to hyperoxic conditions has been related to a reduction in endothelial expression of epidermal growth factor-like domain 7 (EGFL7), a molecule that was originally implicated in vascular morphogenesis (Parker et al., 2004b). Moreover, when overexpressed, EGFL7 attenuates EC apoptosis by decreasing proapoptotic Bax and raising antiapoptotic Bcl-xL protein levels (Xu et al., 2008).

Does Oxygen Regulate Endothelial Tip Cell Fate Specification?

Blood vessel sprouts are characterized by leading cells (tip cells) and trailing cells (stalk cells). The tip cell at the forefront of the growing vessel navigates alongside gradients of angiogenic factors, such as VEGF. It has not yet been established whether O_2 levels regulate the differentiation of these endothelial subpopulations during sprouting. Given the striking similarities to airway branching in *Drosophila*, which is regulated by O_2, this is an intriguing possibility. In *Drosophila*, branching of airways into hypoxic sites requires differentiation and sprouting of specialized terminal epithelial cells (equivalent to endothelial tip cells). This process is induced by the fibroblast growth factor (FGF) homolog *Branchless* (*Bnl*), which is expressed by neighboring

cells in response to hypoxia (Jarecki et al., 1999); this ligand binds to its receptor *Breathless* (*Btl*), which is also induced by hypoxia, on tracheal cells (Centanin et al., 2008). Hypoxic induction of Btl and Bnl is mediated by the *Drosophila* HIF ortholog, *Sima*, which is in turn controlled by the oxygen sensor *Fatiga* (fly PHD ortholog) (Centanin et al., 2008). These responses promote outgrowth of terminal branches to O_2-starved cells. During this process, Notch signaling instructs airway cells, trailing behind the leading cell, to adapt a stalk cell phenotype, thereby ensuring tube morphogenesis (Ghabrial and Krasnow, 2006).

It is tempting to speculate that some parallels might exist between the fly airway system and the mouse vascular system. In the latter, hypoxia upregulates VEGF expression in cells adjacent to the endothelial tip cell (Gerhardt et al., 2003). VEGF induces expression of Dll4 in endothelial tip cells, which, via Notch1 signaling, instructs the trailing EC to become a stalk cell (Hellstrom et al., 2007; reviewed in detail by Phng and Gerhardt, 2009, this issue). HIFs induce VEGFR2 expression (Elvert et al., 2003), which allows tip cells to sense VEGF gradients (Gerhardt et al., 2003). Considering that HIF-1α interacts with Notch and is recruited to Notch-responsive promoters upon Notch activation under hypoxic conditions (Gustafsson et al., 2005), but that FIH may also inhibit Notch activity (via hydroxylation of critical asparagine residues in its intracellular domain) (Kaelin and Ratcliffe, 2008), it will be interesting to elucidate whether regulation of Notch by HIFs, PHDs, and FIH co-determines the tip versus stalk EC fate.

Distinct Roles of HIF-1α, HIF-2α, and PHDs in Vessel Morphogenesis

HIF-1α and HIF-2α have distinct, nonredundant roles in angiogenesis. Initially cloned from ECs (and assigned as endothelial *PAS* domain protein-*1*, EPAS-1; Tian et al., 1997), HIF-2α is found in a subset of tissues (Wiesener et al., 2003), while HIF-1α is ubiquitously expressed (Semenza, 2003). Emerging evidence suggests that HIF-1α and HIF-2α have distinct, nonoverlapping biological functions, regulate different target genes, and often do not substitute for one another. For example, hypoxic induction of HIF-1α target genes is attenuated in *Hif-1α*-deficient ECs (Tang et al., 2004), indicating that HIF-2α or other hypoxia-induced factors cannot compensate for the loss of *Hif-1α*. The endothelial proliferation and migration response to hypoxia relies on HIF-1α (Sowter et al., 2003; Tang et al., 2004), while the precise role of HIF-2α in EC biology remains more enigmatic.

Genetic and gene transfer studies have shed light on the distinct roles of HIFs in vascular development (Figure 1). Embryonic expression of a dominant-negative HIF that inhibits both HIF-1α and HIF-2α leads to defective vascular remodeling and failed vascular sprouting in the yolk sac and embryo (Licht et al., 2006). *Hif-1β*-deficient embryos also die in utero due to impaired angiogenesis in the yolk sac (Maltepe et al., 1997). Moreover, embryos lacking *Hif-1α* display severe vascular defects (Carmeliet et al., 1998; Iyer et al., 1998; Ryan et al., 1998); these defects are not rescued by HIF-2α, suggesting that HIF-1α and HIF-2α control different target genes. Conversely, *Hif-1α* gene transfer stimulates revascularization and improves functional recovery of ischemic tissues in the adult (Vincent et al., 2000). *Hif-2α*-deficient phenotypes are variable, depending on the genetic background. In one mouse strain, loss of *Hif-2α* causes improper remodeling of nascent vessels into larger conduits; this defect is rescued by EC-specific expression of HIF-2α (Duan et al., 2005). In other murine backgrounds, loss of HIF-2α causes abnormal organ development or homeostasis, without, however, any apparent vascular defects (Compernolle et al., 2002; Scortegagna et al., 2003; Tian et al., 1998). Furthermore, silencing of HIF-2α in ECs leads to the formation of an aberrant vascular network in tumors via reduced expression of ephrin A1 (Yamashita et al., 2008). Because of the emerging role of NO in vessel normalization (Kashiwagi et al., 2008), it is worth mentioning that HIF-2α, but not HIF-1α, is able to induce expression of eNOS (Coulet et al., 2003). Some of these results suggest that HIF-2α participates in the remodeling and maturation of the microvasculature. Consistent with this hypothesis, HIF-2α elevates the expression of the junctional molecule VE-cadherin, which is not regulated by HIF-1α (Le Bras et al., 2007).

Through stabilization of HIF-1α, inhibition of PHDs also stimulates angiogenesis both in vivo and in vitro (Knowles et al., 2004; Milkiewicz et al., 2004). Peptide-mediated inhibition of PHDs leads to the generation of more mature and less leaky vessels than those generated by individual growth factors (Willam et al., 2002). Heterozygous deficiency of *Phd2* in endothelial cells does not affect tumor vessel density or lumen size, but partially "normalizes" their endothelial lining, barrier, and stability (Mazzone et al., 2009). These changes improve tumor perfusion and oxygenation, and, hence, inhibit tumor cell invasion, intravasation, and metastasis. Partial loss of *Phd2* redirects the specification of tip cell-like endothelial cells to a more quiescent fate of tightly aligned filopodia-deprived cells, which resemble "phalanx cells", because of their cobblestone formation (Mazzone et al., 2009). These genetic insights suggest that endothelial cells, by inactivating an oxygen sensor in hypoxic conditions, have feedback mechanisms to readjust their shape and fate (not numbers) to restore oxygen supply. In contrast, conditional widespread loss of *Phd2* during postnatal growth results in hyperactive angiogenesis and angiectasia, potentially due to elevated circulating VEGF levels, raising the question whether PHD2 has gene dosage-dependent activities in regulating vessel morphogenesis versus branching (Takeda et al., 2007). Transient silencing of PHD2 in fibroblasts upregulates the expression of angiogenic factors (VEGF, FGF-2, and angiopoietin-1); when transplanted in vivo, these cells stimulate angiogenesis and pericyte coverage of newly formed vessels (Wu et al., 2008). Several of these findings are consistent with observations of enhanced neovascularization and vessel maturation in transgenic animals overexpressing HIF-1α (Elson et al., 2001) or HIF-2α (Kim et al., 2006). Loss of *Phd1* or *Phd3* does not cause abnormal angiogenesis, at least not under baseline conditions (Aragones et al., 2008; Takeda et al., 2007).

Aerobic Glycolysis Regulates Angiogenesis

In healthy tissues, ECs are quiescent and rarely migrate or divide. However, they exhibit a remarkable phenotypic plasticity in terms of being able to divide rapidly, migrate, and form new vessels during hypoxia or other stress conditions. ECs therefore need to be equipped with metabolic mechanisms to survive in highly hypoxic conditions while sprouting in avascular tissues, and to generate sufficient energy for the biosynthesis of

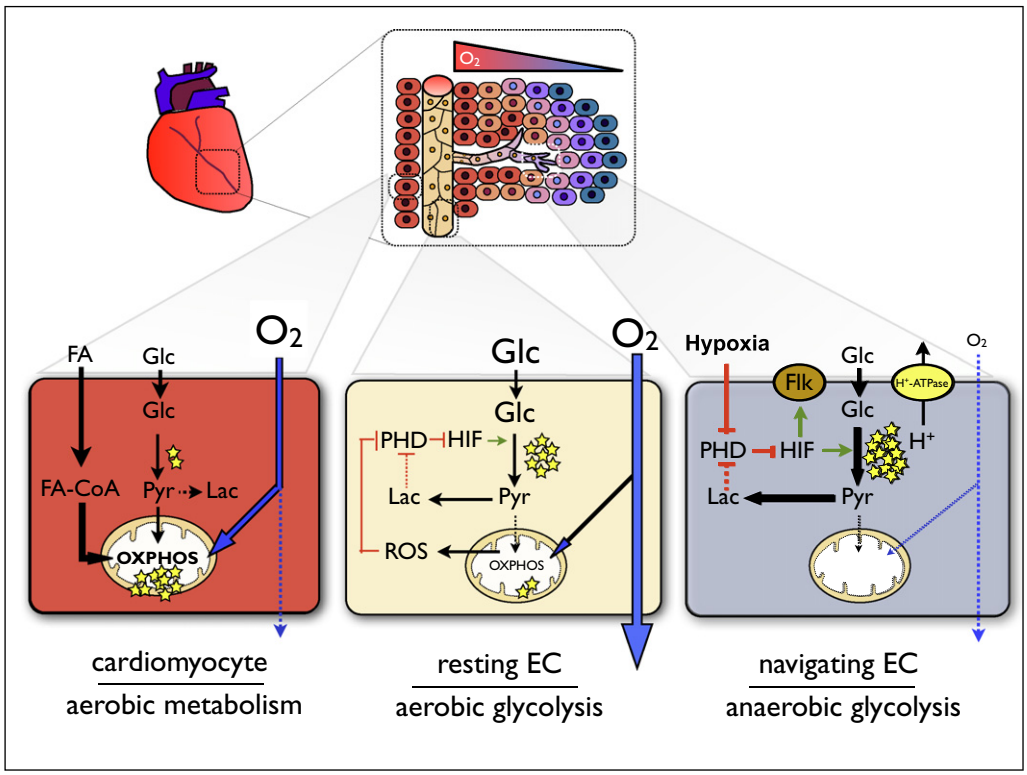

Figure 2. Endothelial Metabolism Correlates with the Abundance of Oxygen

Depending on their localization, endothelial cells (ECs) are exposed to various different oxygen tensions. Resting ECs in mature vessels are in contact with abundant oxygen and ensure oxygen diffusion across the endothelial barrier into the surrounding tissue, where cells subsequently become oxygenized (illustrated as reddish cells). During angiogenesis, ECs start to migrate into less oxygenated/hypoxic areas (blue cells) and establish an oxygen gradient along their migratory path (gradient indicated as turning from well-oxygenated red to poorly oxygenated/hypoxic blue). A resting EC (middle cell) generates its energy largely via aerobic glycolysis. Glucose (Glc) is primarily utilized via glycolysis to pyruvate (Pyr), resulting in ATP generation (yellow stars). Pyruvate is converted to lactate (Lac), which can inhibit PHDs, thereby mounting a HIF response, which—in turn—establishes a positive feedback loop that supports glycolysis. Less ATP is generated via mitochondrial oxidative phosphorylation (OXPHOS) due to attenuated entry of pyruvate into OXPHOS (which is mediated via HIF-dependent induction of pyruvate dehydrogenase kinase, an inhibitor of the pyruvate-converting enzymatic complex: pyruvate dehydrogenase). Moreover, inhibition of respiration results in enhanced generation of reactive oxygen species (ROS), which are also known to inhibit PHDs. Since these cells consume overall low amounts of oxygen, the majority of O_2 will pass into the surrounding tissue (blue arrow), enabling oxidative metabolism. In contrast, a cardiomyocyte (left cell) generates the majority of its ATP via aerobic utilization of fatty acids (FA) and glucose (Glc). Most of the oxygen is consumed during mitochondrial OXPHOS. A navigating endothelial cell (right cell) is exposed to hypoxia, and thus relies on anaerobic energy generation. PHDs are substantially inhibited by hypoxia, leading to strong induction of the HIF program, including acceleration of glycolysis and expression of VEGF receptors such as Flk/VEGFR2. These ECs are also equipped with vacuolar-type H^+-ATPases, which foster export of protons that would normally accumulate as a consequence of enhanced glycolysis, and thereby maintain the cytosolic pH in check.

macromolecules required for rapid cell proliferation. In this respect, ECs resemble cancer cells, which are also adapted to sustain rapid cell growth in the severely hypoxic microenvironments in tumors (DeBerardinis et al., 2008), and there are in fact some remarkable parallelisms between the metabolic machineries of endothelial and tumor cells, as discussed below.

ECs consume O_2 when forming either sprouts in vitro (Helmlinger et al., 2000) or a vascular network in vivo (Hansen-Algenstaedt et al., 2000). Strikingly, however, despite having immediate access to O_2 in the vessel lumen, they derive most of their energy anaerobically through glycolysis (Quintero et al., 2006). Malignant cells also thrive on ''aerobic'' glycolysis, a phenomenon known as the ''Warburg effect'' (Figure 2; Warburg, 1956). In fact, although ECs possess the machinery to metabolize lipids (Dagher et al., 2001), > 90% of their glucose is metabolized anaerobically in vitro (Krutzfeldt et al., 1990). Future studies are required to determine the relative contribution of fatty acid oxidation as a source of energy in endothelial cells. Consistent with the aforementioned observations, several, but not all, in vitro studies report low O_2 consumption rates in ECs (Tsai et al., 2003). Remarkably, when exposed to an increased supply of glucose, ECs further increase their glycolytic metabolism, and thus reduce their O_2 consumption (''Crabtree effect'') (Dobrina and Rossi, 1983). In certain pathological vessel remodeling conditions, ECs also shift to enhanced glycolysis (Xu et al., 2007). Lactate, a by-product of glycolysis, stimulates angiogenesis by inactivating PHDs as a competitive antagonist of 2-oxoglutarate (Lu et al., 2002), thereby triggering HIF-driven VEGF expression (Hunt et al., 2008; Kumar et al., 2007; Figure 2). This metabolite also inhibits poly-ADP ribosylation of VEGF, resulting in its enhanced angiogenic activity (Kumar et al., 2007).

This metabolic adaptation means that ECs are well suited to growing in the harsh environmental conditions of severe hypoxia, but are still able to survive as quiescent cells in normoxic healthy vessels. Indeed, by shifting to glycolysis, ECs use the most

abundant nutrient, glucose, to produce ATP. Even though the yield of glycolytically generated ATP per molecule of glucose is low, energy demands can be satisfied if the glycolytic flux is adequately accelerated (DeBerardinis et al., 2008). Furthermore, aerobic glycolysis allows ECs to survive in conditions of fluctuating O_2 levels that would kill other types of cells. It remains to be determined whether acidosis, resulting from glycolytic generation of lactate and bicarbonic acid, might be less toxic to ECs than to other cells, thus providing them with a growth advantage equivalent to that suggested for tumor cells (DeBerardinis et al., 2008). In fact, microvascular (but not macrovascular) ECs express vacuolar-type H^+-ATPases at the plasma membrane that export protons, thereby maintaining the cytosolic pH balance (Rojas et al., 2006; Figure 2).

Endothelial Cells Use Oxygen for Signaling Purposes

Even though ECs seem to generate most of their energy anaerobically, they consume oxygen. A model to account for O_2 consumption by ECs is that they use it for other purposes, such as the generation of signaling molecules like reactive oxygen species (ROS) and nitric oxide (NO), which have diverse biological activities in these and other cell types. NO has various effects on blood vessels, ranging from stimulation of angiogenesis and vasodilation to vessel normalization (Fukumura et al., 2006). Molecular studies show that NO produced in response to laminar shear stress or hypoxia reduces EC respiration via inhibition of cytochrome *c* oxidase in the electron transfer chain (Jones et al., 2008). This inhibition would have several implications. First, the cytosolic pool of O_2 would increase as a consequence of the reduced mitochondrial O_2 consumption. This would establish a steeper gradient between ECs and their surrounding cells, allowing O_2 to diffuse further away from the blood vessel lumen into the surrounding tissue (Boveris and Boveris, 2007). Second, inhibition of oxidative phosphorylation by NO could increase the generation of mitochondrial ROS (Zhang and Gutterman, 2007). As ROS can inactivate PHDs by oxidizing their cofactor Fe^{2+}, HIFs would become stabilized and initiate their transcriptome response (Gerald et al., 2004; Guzy and Schumacker, 2006; Figure 2). This response also involves upregulation of eNOS, which, in a positive feedback loop, increases the production of NO and its abovementioned vascular activities which improve overall O_2 delivery. These mechanisms could also explain why NO not only induces vasorelaxation of arteriolar walls, but also improves oxygenation of perivascular tissue (Shibata et al., 2005). Alternative parallel mechanisms that also rely on PHD inactivation may also exist. For instance, NO can inhibit PHD activity under normoxic conditions (Berchner-Pfannschmidt et al., 2007). Excessive amplification of this feedback loop may be tempered by the ability of elevated cytosolic O_2 levels to activate PHDs/FIH, and thereby shutdown HIF signaling in a timely fashion (Hagen et al., 2003). An intricate balance between NO, O_2, and ROS thus seems to determine the overall physiological outcome.

A remarkable finding is that NO production by ECs not only determines the supply of O_2 to tissues through stimulation of angiogenesis and vasodilation (Fukumura et al., 2006), but also influences O_2 consumption in surrounding cells. This regulation occurs in a tissue-specific manner. In the heart, endothelial-derived NO enhances the coupling of O_2 delivery and consumption in cardiomyocytes, and thereby facilitates ATP production at reduced levels of O_2 consumption, improving pump function (Brown and Borutaite, 2007; Williams et al., 2007). In skeletal muscle fibers, *eNOS* deficiency decreases the number of mitochondria and impairs O_2 consumption (Le Gouill et al., 2007). Loss of *eNOS* does not, however, alter hepatic mitochondrial content (Schild et al., 2008). Overall, endothelial-derived NO thus seems to favor not only O_2 delivery to perivascular cells, but also to enhance or improve the efficiency of oxidative metabolism.

ROS, which are generated when O_2 is consumed during mitochondrial respiration, also function as signaling molecules independently from their ability to inactivate PHDs/FIH. For example, ROS stimulate EC proliferation, vessel permeability, and changes in the expression of EC surface adhesion molecules (Ushio-Fukai and Nakamura, 2008). ROS levels also correlate with tumor angiogenesis and growth (Xia et al., 2007), while prevention of ROS formation reduces tumor angiogenesis (Gerald et al., 2004; Ushio-Fukai and Nakamura, 2008).

Modulation of Angiogenesis by Metabolic Regulators

Despite the obvious and widely recognized fact that cells need O_2 for aerobic metabolism and that blood vessels supply O_2, the link between metabolism and angiogenesis has been largely ignored, but it is now being (re)discovered at a rapid pace. An example are the peroxisome proliferator-activated receptors (PPARs), which are transcription factors that regulate nutrient utilization and energy homeostasis. Three isoforms have been described (PPARα, PPARβ/δ, and PPARγ), each with distinct roles (Barish et al., 2006; Evans et al., 2004; Lefebvre et al., 2006); two inhibit angiogenesis and one promotes vessel growth. PPARβ, a regulator of lipid oxidation (Evans et al., 2004), stimulates microvessel maturation (Muller-Brusselbach et al., 2007; Figure 3). Its activators upregulate the expression of VEGF, resulting in enhanced EC proliferation, microvessel sprouting, and tube formation (Piqueras et al., 2007; Wang et al., 2006), while loss of *Pparβ* diminishes tumor perfusion due to abnormal vessel (Muller-Brusselbach et al., 2007). In contrast, PPARα and PPARγ inhibit angiogenesis. PPARα induces a shift from carbohydrate to lipid utilization upon fasting by promoting lipid uptake and oxidation in the heart, liver, and skeletal muscle (Lefebvre et al., 2006). PPARα-selective ligands suppress tumor growth by inhibiting angiogenesis (Panigrahy et al., 2008), in part by suppressing EC proliferation directly or indirectly by lowering the expression of VEGF or its receptor VEGFR2, and by increasing thrombospondin-1 (TSP-1) and other inhibitors (Meissner et al., 2004). PPARγ, a regulator of adipose development and insulin sensitivity (Rosen and MacDougald, 2006), is expressed in tumor ECs; PPARγ-selective ligands inhibit EC proliferation in vitro and exhibit antiangiogenic properties in vivo (Panigrahy et al., 2002). However, the precise mechanisms of action of PPARγ remain unclear, as disparate effects on the expression of VEGF and its receptors have been reported (Chintalgattu et al., 2007; Panigrahy et al., 2002).

Several other regulators of metabolism modulate angiogenesis, including PGC-1α, AMP-dependent kinase (AMPK), and forkhead transcription factors (Foxo). The transcriptional

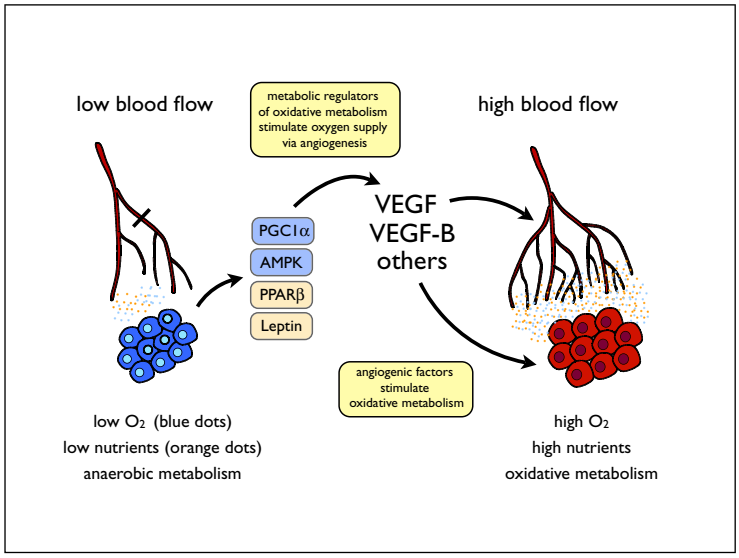

Figure 3. Crosstalk between Metabolism and Angiogenesis

Under physiological or pathological (e.g., vessel occlusion; black bar) conditions of a low oxygen (blue dots) and nutrient (orange dots) supply, metabolic activity is hampered (blue cells). Regulators of metabolism such as PGC1α and AMP-dependent kinase (AMPK), both of which can be induced under conditions of oxygen or nutrient deprivation (indicated in blue), peroxisome proliferator-activated receptor β (PPARβ) and leptin can induce proangiogenic factors (e.g., VEGF). This enhances the availability of oxygen and nutrients, leading to accelerated cellular metabolic activity (red cells). Angiogenic factors such as VEGF and VEGF-B also directly impact cellular metabolism itself, stimulating cellular oxidative metabolism, and thus prime the cells to efficiently utilize the surplus of oxygen and nutrients.

coactivator PGC-1α augments energy production in skeletal muscle and the heart by stimulating mitochondrial biogenesis and cellular respiration (Finck and Kelly, 2006). It also stimulates angiogenesis by inducing VEGF expression through interacting with estrogen-related receptor-α (ERR-α), thereby preparing the tissue for oxidative metabolism (Arany et al., 2008). AMPK is activated upon energy deprivation and promotes nutrient uptake and oxidation, but also attenuates energy-consuming processes (Long and Zierath, 2006). Pharmacological activation of AMPK upregulates VEGF levels, resulting in improved revascularization of ischemic limbs (Ouchi et al., 2005). These findings support the concept that regulators of oxidative metabolism ensure sufficient O_2 and nutrient supply through boosting vessel growth (Figure 3).

Foxo1 and Foxo3 are induced during fasting and upregulate expression of genes involved in lipid oxidation (Barthel et al., 2005). The role of Foxo family members in angiogenesis is complex. Foxo1 destabilizes blood vessels, in part by upregulating the expression of angiopoietin 2 and inducing EC apoptosis (Daly et al., 2004). Conversely, silencing of Foxo1 increases EC migration in vitro and ischemic limb perfusion in vivo (Potente et al., 2005), and *Foxo1*-deficient embryos form dilated and irregularly shaped vessels (Furuyama et al., 2004). Overexpression of Foxo3a induces EC apoptosis and inhibits SMC proliferation (Lee et al., 2007b), while suppression of Foxo3a stimulates angiogenesis in vitro and in vivo, in part via induction of eNOS (Potente et al., 2005). Combined deletion of *Foxo1*, *Foxo3*, and *Foxo4* in various adult tissues results in the formation of hemangiomas, indicating that Foxo members indeed inhibit endothelial growth in vivo (Paik et al., 2007). Less severe lesions were observed in mice lacking only *Foxo3* and *Foxo1*, arguing that Foxo1 is the physiological inhibitor of angiogenesis (Paik et al., 2007). This antiangiogenic activity of Foxo1 has not, however, been reported by others. Indeed, paradoxically, *Foxo1* deficiency impairs angiogenesis during development, while silencing of Foxo1 promotes migration and tube formation of adult ECs; however, the reason for this paradox remains unknown.

Angiogenesis also stimulates the growth of adipose tissue (Cao, 2007), and adipogenic factors have been increasingly recognized as modulators of angiogenesis. For instance, the adipokine leptin, which regulates food intake, stimulates angiogenesis and EC fenestration, in part by inducing VEGF expression (Suganami et al., 2004), and promotes angiogenesis together with FGF2 and VEGF (Cao et al., 2001; Figure 3). Adiponectin also regulates angiogenesis, although conflicting results have been reported. Indeed, it may act as a negative regulator of angiogenesis by inducing EC apoptosis (Brakenhielm et al., 2004), while conversely, it also stimulates vessel shaping and formation by enhancing eNOS activity (Ouchi et al., 2004).

Endothelial-Derived and Angiogenic Signals Regulate Metabolism

Metabolic regulators not only influence angiogenesis, but angiogenic or endothelial factors also influence metabolism. For instance, EC-derived signals regulate pancreatic development (Lammert et al., 2001), while pancreatic islets express VEGF, which stimulates the formation of a fenestrated capillary bed in islets (Lammert et al., 2003). Deletion or reduction of pancreatic or islet-specific VEGF expression reduces islet capillaries and insulin secretion (Brissova et al., 2006; Lammert et al., 2003), while overexpression of VEGF in the pancreas induces the opposite effects (Lammert et al., 2001). Similarly, EC-derived signals regulate hepatic development. In VEGFR2-deficient mice, which exhibit severe EC defects, hepatic specification occurs, but liver morphogenesis is arrested (Matsumoto et al., 2001). Moreover, activation of VEGFR-1 on liver sinusoidal ECs by VEGF stimulates hepatocyte proliferation and regeneration because these ECs produce hepatocyte growth factor (HGF) (LeCouter et al., 2003). VEGF also stimulates mitochondrial biogenesis in ECs via induction of mitochondrial genes (Wright et al., 2008). VEGF-B, a homolog of VEGF, is prominently expressed in highly metabolic tissues and cardiac overexpression results in decreased triglyceride levels, suggesting that VEGF-B is involved in regulating cardiac lipid metabolism (Karpanen et al., 2008). Furthermore, apelin, a ligand of the G protein-coupled receptor AJP, that stimulates glucose utilization and restores glucose tolerance in insulin-resistant mice (Dray et al., 2008), promotes embryonic vascular development or tumor angiogenesis in vivo (Kalin et al., 2007). Consistent with these findings, apelin knockdown inhibits angiogenesis in zebrafish

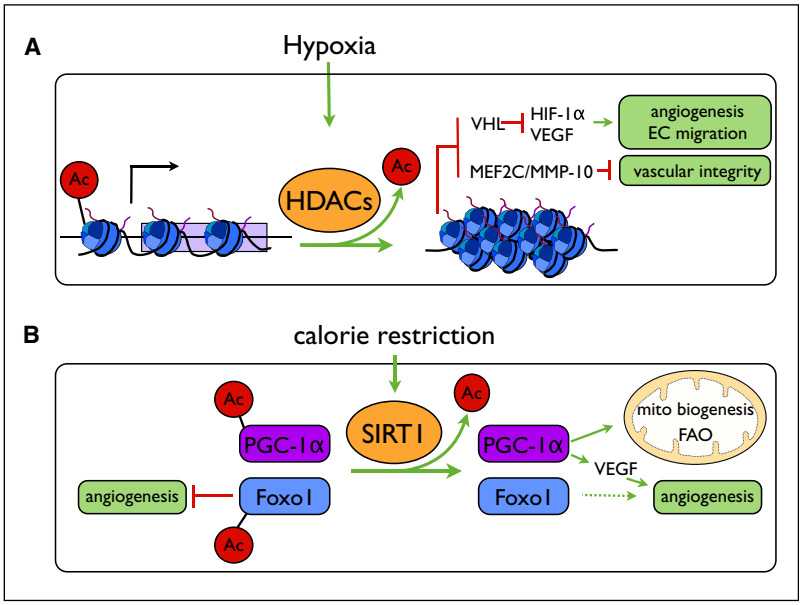

Figure 4. Influence of O_2 and Metabolism on Histone Deacetylases

(A) HDAC activity can be induced by hypoxia, as indicated for HDAC1 and HDAC2. Enhanced HDAC activity consequently leads to epigenetic histone deacetylation and chromatin condensation, reducing the expression of targeted genes (e.g., VHL), and to direct repression of transcription factors (e.g., MEF2C). Epigenetic downregulation of VHL can lead to increased activity of proangiogenic HIF-1α and VEGF, which are usually inhibited by VHL, or the repression of MEF2C leads to reduced transcription of MMP10, and thereby to decreased degradation of extracellular matrix.

(B) The NAD^+-dependent histone deacetylase SIRT1 is induced during calorie restriction and leads to posttranslational histone-independent protein deacetylation of PGC-1α and Foxo1. Protein deacetylation increases the activity of proangiogenic PGC-1α, whereas it decreases the activity of the antiangiogenic transcription factor Foxo1. FAO, fatty acid oxidation.

(Eyries et al., 2008). In conclusion therefore, endothelial-derived or angiogenic factors impact on various aspects of metabolism, as illustrated in Figure 3.

Epigenetic Regulation of Angiogenesis: Influence of O_2 and Nutrients

Environmental factors such as O_2 can regulate the expression of genes epigenetically through modification of DNA and its associated histones. Histone acetylation by histone acetyltransferases (HATs) leads to a relaxation of the chromatin structure, facilitating the recruitment of transcription factors, whereas deacetylation by histone deacetylases (HDACs) results in a more compact chromatin structure, repressing transcriptional activity (Kouzarides, 2007). Emerging evidence indicates that expression of angiogenic genes in response to O_2 fluctuations can be influenced by such epigenetic mechanisms. For instance, in a fraction of individuals with von Hippel-Lindau (VHL) disease, hemangiomas are caused by germline inactivation of one *VHL* allele, and somatic epigenetic silencing of the other, through hypermethylation of a CpG island in the *VHL* promoter region (Herman et al., 1994). Another example is the epigenetic silencing of RUNX3, an inhibitor of angiogenesis (Peng et al., 2006). Hypoxia decreases acetylation of the *RUNX3* promoter in cancer cells via upregulation of HDAC1, thereby stimulating angiogenesis. Hypoxic activation of HDAC1/2 also leads to silencing of the *VHL* gene, resulting in stabilization of HIF-1α and angiogenesis (Pluemsampant et al., 2008; Figure 4A). The promoter region of some hypoxia-inducible genes (VEGF) demonstrates decreased demethylation during hypoxia, which could facilitate HIF binding and gene transcription (Johnson et al., 2008). Interestingly, Jumonji C-domain-containing histone demethylases also belong to the family of 2-oxoglutarate-dependent dioxygenases (Agger et al., 2008); their expression is also upregulated by HIF-1α during hypoxia, perhaps to secure demethylation-dependent regulation of gene expression in these conditions (Pollard et al., 2008). There are many other instances where epigenetic regulation influences angiogenesis, but connections to O_2 or nutrient changes have not yet been established.

Genetic and pharmacological loss-of-function studies have facilitated characterization of the roles of HDACs in angiogenesis. For instance, loss of *Hdac7* causes embryonic cardiovascular defects as a consequence of reduced epigenetic silencing of the endothelial transcription factor MEF2C (Chang et al., 2006; Lin et al., 1998). This results in increased levels of matrix metalloproteinases, removing the matrix support necessary for ECs and preventing them from forming capillary tube-like structures (Chang et al., 2006; Mottet et al., 2007; Figure 4A). HDAC inhibitors also impair angiogenesis (Buysschaert et al., 2008; Deroanne et al., 2002; Michaelis et al., 2004). Their inhibitory capacity relies on silencing angiogenic factors (VEGF, eNOS) and upregulating angiogenic inhibitors (Deroanne et al., 2002; Michaelis et al., 2004). HDAC inhibitors also impair endothelial progenitor cell function, by repressing the homeobox transcription factor HoxA9, which downregulates eNOS, VEGFR-2, and VE-cadherin (Rossig et al., 2005).

Intriguingly, the histone deacetylase machinery may also regulate angiogenesis through alternative mechanisms, independent of their bona fide role in histone deacetylation. For instance, HDACs have been shown to deacetylate HIF-1α, thereby increasing its transcriptional activity (Kim et al., 2001; Qian et al., 2006), although this conclusion is controversial (Arnesen et al., 2005; Jeong et al., 2002). HDACs may also facilitate an interaction between HIF-1α and chaperones such as Hsp90, which protect it from proteasomal degradation (Kong et al., 2006; Qian et al., 2006), or may enhance transcription of HIF-1α target genes during hypoxia by forming a complex with HIF-1α and p300 (Kato et al., 2004). Another histone deacetylase, the NAD^+-dependent SIRT1, promotes vascular development in zebrafish and revascularization of ischemic tissues in mice (Potente et al., 2007). It is expressed in sprouting ECs and activates the angiogenic metabolic regulator PGC-1α and decreases the activity of Foxo1, an inhibitor of angiogenesis (Potente et al., 2007; Figure 4B).

Emerging evidence suggests that epigenetic regulation may also be relevant in angiogenic disorders, although much still needs to be learned. For instance, ECs in tumor vessels express

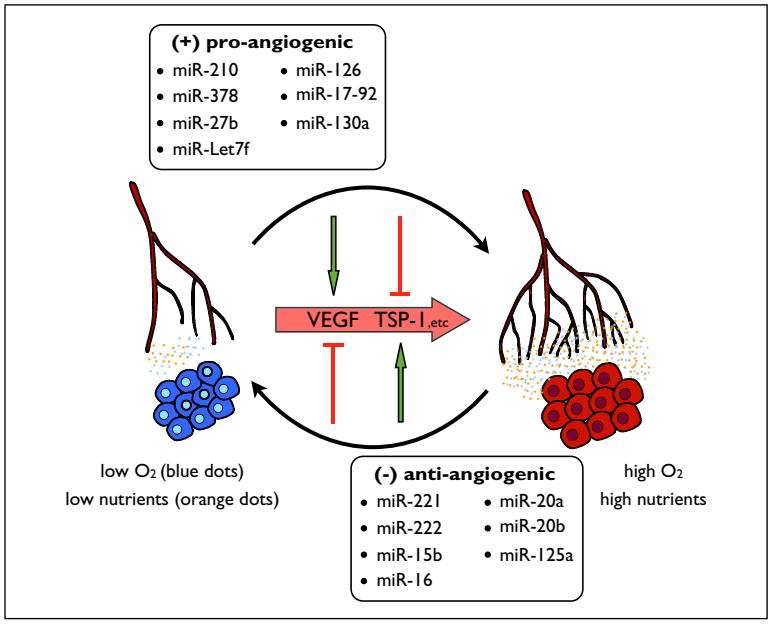

Figure 5. miRNAs Modify the Angiogenic Response
Blood vessel formation is regulated by miRNAs. Several proangiogenic miRNAs stimulate angiogenesis by regulating proangiogenic factors, such as VEGF. This results in accelerated vessel formation, and thus in an enhanced supply of oxygen (blue dots) and nutrients (orange dots), promoting oxidative metabolism (red cells). Conversely, antiangiogenic miRNAs inhibit vessel formation via induction of the antiangiogenic factors thrombospondin-1 (TSP-1) and related proteins such as connective tissue growth factor (CTGF).

a selective subset of genes that are not normally found in quiescent cells of healthy vessels (Madden et al., 2004; Parker et al., 2004a; St Croix et al., 2000). Some of this altered gene expression pattern has been attributed to epigenetic regulation, for instance, by EZH2, a protein that regulates chromatin remodeling and is upregulated in tumor ECs (Lu et al., 2007). Inhibitors of DNA methyltransferase and HDAC indirectly affect angiogenesis by silencing the expression of angiogenic genes and reactivating the expression of antiangiogenic genes (Hellebrekers et al., 2006; Kim et al., 2001). Moreover, hypermethylation of the angiogenic inhibitors thrombospondin 1 and ADAMTS-8 suppresses their expression and is associated with the progression of human cancers (Buysschaert et al., 2008). Interindividual heterogeneity in hypoxic VEGF induction is associated with epigenetic changes to explain differences in vessel formation in patients with coronary artery disease (Schultz et al., 1999); however, final proof for this assumption is missing. At this point, it is also unclear to what extent metabolism intersects with pathology via this type of mechanism.

Regulation of Angiogenesis by microRNAs

MicroRNAs (miRNAs) are noncoding small RNAs of ~22 nucleotides that modulate posttranscriptional gene expression by degrading mRNA transcripts or preventing their translation. In mammalian cells, the ribonuclease Drosha processes miRNAs from longer transcripts in the nucleus. These pre-miRNAs are further processed in the cytosol by the ribonuclease Dicer to form mature miRNAs, which become integrated into a ribonucleoprotein complex (RISC); target mRNAs are then degraded, leading to silencing of the target genes. Emerging evidence indicates that the expression of several of these miRNAs is regulated by hypoxia. For instance, miR-210 is induced by hypoxia in ECs; this leads to increased capillary-like tube formation and EC migration, in part through downregulation of Ephrin-A3 (Fasanaro et al., 2008; Figure 5). Notably, the expression of several other miRNAs is also regulated by hypoxia; these are termed "hypoxia-regulated miRNAs" (HRMs) (Kulshreshtha et al., 2007).

Other miRNAs also regulate angiogenesis, although it remains unclear whether they are regulated by hypoxia. Let-7f and mir-27b miRNAs are proangiogenic as a consequence of their ability to silence the expression of the endogenous angiogenesis inhibitor TSP-1; these miRNAs are downregulated upon loss of *Dicer*, alone or together with *Drosha*, which partially explains why angiogenesis is inhibited in these conditions (Kuehbacher et al., 2007; Shilo et al., 2008; Suarez et al., 2007). Likewise, overexpression of miR-378 in tumor cells enhances EC survival and tumor angiogenesis by promoting VEGF expression. This results from the fact that miR-378 competes with miR-125a (which silences VEGF expression) for the same RNA-binding sequence in the 3′UTR region of the VEGF gene (Hua et al., 2006; Lee et al., 2007a). Other miRNAs also stimulate angiogenesis by suppressing the expression of the antiangiogenic homeobox genes GAX and HOXA5 (miR-130a) (Chen and Gorski, 2008) or Spred-1, an intracellular inhibitor of MAPK signaling (miR-126), by increasing the EC response to VEGF and FGF-2 (miR-126) (Fish et al., 2008; Wang et al., 2008), or by downregulating the antiangiogenic factors TSP-1 and related proteins, such as connective tissue growth factor (CTGF) (miR-17-92 cluster) (Dews et al., 2006; Figure 5). Antiangiogenic miRNAs (miR-221, miR-222) inhibit endothelial sprouting in vitro (Poliseno et al., 2006). Overexpression of several other miRNAs (miR-15b, miR-16, miR-20a, and miR-20b) also represses VEGF expression (Figure 5). Notably, the hypoxia mimetic and PHD-inhibitor desferrioxamine downregulates these miRNAs in vitro, suggesting that hypoxia stimulates angiogenesis in part by suppression of these antiangiogenic regulators (Hua et al., 2006).

Conclusions and Perspectives

Based on the abovementioned findings, it is evident that more crosstalk occurs between angiogenesis and metabolism than previously anticipated. However, the mechanistic aspects of this crosstalk have only started to become apparent, with numerous questions remaining to be addressed. For instance, how are differences in O_2 tension connected to variations in cellular responses? What are the executive pathways of the different O_2 sensing systems and how are they connected, if at all? How are nutrients sensed in growing vessels, and are these signals linked to the availability of O_2? The answers to these questions might provide useful information for translation into

therapeutic opportunities for the treatment of angiogenic or metabolic diseases. As a consequence, improved understanding of the players and deciphering of the cues involved will hopefully assist in the medical battle against the increasing number of angiogenesis-related disorders.

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