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REVIEW

Endothelial oxygen sensors regulate tumor vessel abnormalization by instructing phalanx endothelial cells

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Abstract An ancestral function of vessels is to conduct blood flow and supply oxygen (O_2). In hypoxia, cells secrete angiogenic factors to initiate vessel sprouting. Angiogenic factors are balanced off by inhibitors, ensuring that vessels form optimally and supply sufficient oxygen (O_2). By contrast, in tumors, excessive production of angiogenic factors induces vessels and their endothelial cell (EC) layer to become highly abnormal, thereby impairing tumor perfusion and oxygenation. In such pathological conditions, angiogenic factors act as "abnormalization factors" and promote the vessel "abnormalization switch." Recent genetic data indicate that ECs sense an imbalance in oxygen levels, by using the oxygen-sensing prolyl hydroxylase PHD2. In conditions of O_2 shortage, a decrease in PHD2 activity in ECs initiates a feedback that restores their shape, not their numbers. This induces ECs to align in a streamlined "phalanx" of tightly apposed, regularly ordered cobblestone ECs, which improves perfusion and oxygenation. As a result, EC normalization in PHD2 haplodeficient tumor vessels improves oxygenation and renders tumor cells less invasive and metastatic. This review discusses the role of PHD2 in the regulation of vessel (ab)normalization and the therapeutic potential of PHD2 inhibition for tumor invasiveness and metastasis.

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Introduction

An ancestral function of blood vessels is to supply O_2 and nutrients to cells. In order to do so, vessels and their endothelial lining acquire a streamlined shape so that they can optimally conduct blood flow. During evolution, molecular O_2 sensing mechanisms developed to regulate vessel morphogenesis and readjust endothelial morphology to maximize vessel perfusion. Specialized ECs, each with distinct cellular specifications, contribute to the de novo formation and subsequent perfusion of blood vessels. Rooted on conserved basic principles how tubular airways branch in *Drosophila* [1] and primitive vessels sprout in *Ascidians* [2], a new model of blood vessel branching has recently been proposed. At the forefront of a vessel sprout, highly polarized and motile endothelial "tip cells", which extend filopodia and proliferate minimally, guide the sprouting vessels towards an angiogenic stimulus [3]. Immediately behind the leading tip cells are the follower "stalk cells" which proliferate to elongate the vessel branch and create a lumen. Eventually, after establishing blood flow and stabilizing the vessel wall, ECs return into a dormant "quiescent", nonproliferating, immobile state, a phenotype recently described as "phalanx cells", because these cells are aligned in a tight phalanx of cobblestone cells, similar to how soldiers in the Ancient Greece formed military phalanxes. Phalanx cells sense and regulate perfusion in the persistent sprout [4]. Among other factors, VEGF has a key role in specifying these endothelial phenotypes, by selecting tip cells and stimulating their migration, by inducing proliferation of follower stalk cells, and by transmitting survival signals to phalanx cells.

A hypoxic feedback loop in tumor cells causes tumor vessel abnormalization

In pathological conditions, homeostatic vessel morphogenesis is often deregulated, with vessels and their endothelial layer having an abnormal structure and shape. As a result, their function to supply O_2 is impaired. Once exceeding a volume of 1 mm^3, tumor cell proliferation and metabolic demands outpace the supply of O_2, leading to hypoxia. Tumor cells try to overcome this shortage of oxygen by upregulating additional angiogenic factors to attract even more blood vessels. In addition, oncogenes in malignant cells also upregulate angiogenic factors. Overall, because of their excessive production by hypoxic tumor cells, these angiogenic factors start to act as "abnormalization factors". By hyperactivating ECs, such abnormalization factors cause changes at the level of the vascular network as well as of the EC layer. Indeed, they induce disorganized, complex architectural networks of tortuous and mal-shaped vessels with a highly dysfunctional, leaky EC layer in which hypermotile ECs with irregular shape form pseudo-stratified layers, leave gaps, are loosely connected to each other and obstruct the lumen by extending multiple protrusions [4, 5]. Unlike normal vessels, abnormal tumor blood vessels also have a defective basement membrane and pericyte coverage [6]. Tumor ECs also carry a distinct molecular signature [7], fail to undergo senescence in culture, and are more resistant to genotoxic stress by cytotoxic agents [8]. Moreover, they exhibit aberrant mechanosensing properties because of constitutively high levels of RhoGTPases [9].

These EC and vascular changes impair perfusion and the supply of oxygen, which aggravates hypoxia in tumor cells that, in turn, upregulate more abnormalization factors. This vicious self-perpetuating cycle further aggravates tumor hypoxia, often approximating anoxia (<0.1 mmHg; 0.01 kPa) [10]. A possible consequence of severe hypoxia is, however, that tumor cells switch on invasive and metastatic programs to escape the hostile hypoxic microenvironment, thereby accelerating tumor cell invasiveness, malignancy, and metastasis [11–13]. A number of recent studies highlighted the importance of normalizing the tumor vessel architecture in improving drug delivery and response to chemo/radiotherapy [14–16], but little information existed about the role and importance of the EC layer itself in tumor vessels. We will discuss the latter in more detail in the next sections.

PHDs are oxygen sensors that control HIF activity

Since O_2 is vital for cells, it is not surprising that ECs and tumor cells are able to sense differences in O_2 supply. During evolution, various mechanisms of O_2 sensing have evolved [17]. It is beyond the scope of this review to discuss them all; instead, we will focus on a recently characterized class of O_2 sensing enzymes, the prolyl hydroxylases domain proteins (PHD1–3) and factor inhibiting hypoxia-inducible factor (FIH) [18], which belong to the 2-oxoglutarate-dependent iron(II) dioxygenase superfamily. For more information on their regulation, the reader is referred to more extensive reviews [19, 20]. Moreover, an overview on the role of PHDs in angiogenesis is provided elsewhere in this review series [21]. Briefly, under well-oxygenated conditions, PHDs hydroxylate two conserved prolyl residues in hypoxia inducible factor α (HIF-α), thereby generating a binding site for the von Hippel–Lindau tumor suppressor protein, which is a member of a ubiquitin ligase complex. As a result, HIF-α becomes polyubiquitylated and targeted for proteosomal degradation. FIH, by contrast, hydroxylates a C-terminal asparagyl residue in HIF-1α, which impairs interaction with the coactivator p300 that is essential for the transcriptional activity of HIF-1α [22]. When oxygen levels drop, the hydroxylation activity of the PHDs and FIH is reduced which results in the accumulation and activation of HIF-α. HIF-α subsequently heterodimerizes with its partner HIF-β; they translocate to the nucleus of the cells and transcriptionally activate hundreds of genes, including those regulating survival, metabolism, and angiogenesis [23]. Although HIF-1α and HIF-2α share a lot of the hypoxia-inducible target genes, differences exist in their molecular targets: whereas HIF-1α activates the expression of genes encoding glycolytic enzymes [24, 25], HIF-2α regulates genes involved in adult erythropoiesis [26] and Oct-4 expression [27]. In addition, they even have opposing roles in the regulation of c-Myc activity [28]. In addition to HIF-α, PHDs and FIH also hydroxylate other proteins, some of which are associated with angiogenesis [18].

Divergent role of HIFs in vessel (ab)normalization

Hypoxia is well known to regulate EC migration, proliferation, and tube formation [29]. Emerging evidence suggests that HIF-1α and HIF-2α have distinct, mostly non-overlapping roles in EC biology in general and in morphogenesis in particular. Loss of HIF-1α in ECs attenuates the expression of hypoxia-inducible angiogenic genes and reduces EC proliferation in hypoxic conditions [30, 31]. EC-specific HIF-1α-deficient mice do not exhibit an overt phenotype in baseline conditions but show reduced tumor growth and vascularization because of attenuated endothelial expression of VEGF and VEGFR2 [30]. HIF-2α levels are not compensatorily regulated by the absence of HIF-1α, suggesting that HIF-2α differentially regulates

EC physiology in vivo. This is in line with previous reports that embryos lacking HIF-1α display severe vascular defects, which are not rescued by HIF-2α [32, 33]. Conversely, HIF-1α stimulates revascularization and improves functional recovery of ischemic tissues in the adult [34]. Thus, HIF-1α seems to have a role in vessel sprouting predominantly.

Depending on the genetic background, HIF-2α-deficient phenotypes are variable. In one mouse strain, loss of HIF-2α causes improper remodeling of nascent vessels into larger conduits [35]. In other backgrounds, loss of HIF-2α causes abnormal organ development or homeostasis, without apparent vascular deficits [36–38]. In addition, silencing of HIF-2α in ECs leads to the formation of an aberrant vascular network in tumors through reduced expression of Ephrin A1 [39]. Loss of HIF-2α in ECs does not reduce the number of sprouting vessels or hypoxic EC proliferation but impairs vascular remodeling and maturation of the microvasculature. Thus, HIF-2α seems to regulate the form, shape, stability, and, hence, functionality of nascent vessels rather than determining the number of vessel sprouts or microvascular densities.

Haplodeficiency of PHD2 induces tumor vessel normalization

By using tumor vessel abnormalization as a model, a recent study showed that *endothelial* PHD2, via regulation of HIF-2α, senses and readapts O_2 supply in case of O_2 deprivation [4]. Haplodeficiency of PHD2 in ECs does not affect tumor vessel density and area, tortuosity, or lumen size but induces "normalization" of the endothelial lining, barrier, and stability. Indeed, tumor vessels in PHD2 heterozygous deficient mice are lined by a single monolayer phalanx of regular, orderly formed, polarized cobblestone ECs that have few fenestrations (Fig. 1). These changes in EC shape, not numbers, do not affect primary tumor growth but improve tumor perfusion and oxygenation, as evidenced by the reduced staining for the hypoxia-marker pimonidazole as well as the lower O_2 pressure assessed via electron paramagnetic resonance oxymetry [4].

Highly malignant tumor cells generate ATP primarily via glycolysis even though available O_2 levels would allow them to produce energy via oxidative phosphorylation, a process coined the Warburg phenomenon. Because of the improved oxygenation, cancer cells in PHD2 heterozygous deficient mice reprogram their metabolism, away from glycolytic production of ATP to more oxidative generation of energy [4]. Indeed, expression of key glycolytic regulators such as Glut-1 and phosphofructokinase was downregulated; lactate content was also lower, while transcript levels of PDK enzymes (which restrict entry of glycolytic intermediates in the Krebs cycle) were downregulated. The reduced $NADH/NAD^+$ ratio, as a readout of the redox status, further confirmed that tumors in PHD2 heterozygous-deficient mice shifted from glycolytic to more oxidative metabolism, as occurs in more benign tumors.

The improved tumor oxygenation in PHD2 heterozygous-deficient mice prevented a metastatic switch and, overall, prolonged survival [4]. Tumors were encapsulated, grew focally and noninvasively, while metastasis was substantially reduced (Fig. 2). Concomitantly, gene profiling revealed a lower upregulation of HIF-1α-dependent metastatic genes. Moreover, the EC layer in PHD2 heterozygous-deficient mice formed a tighter barrier, not only because ECs expressed more junctional molecules (such as ZO-1 and VE-cadherin) but they were also surrounded by more pericytes and stable basement membranes (Fig. 3). Physically, such a phalanx of impermeable ECs constitutes an obstacle, which impairs cancer cell intravasation and metastasis. These results illustrate that a change in vessel morphogenesis and endothelial shape, even without accompanying alterations in vessel numbers, suffices to induce a shift to a more benign tumor behavior and less aggressive metastasis [4]. Consistent with this study, others have shown that the angiopoietin-1-induced normalization of immature vessels is associated with reduced PHD2 expression [40]. Accordingly, loss of the HIF-1α responsive angiopoetin-2 gene normalizes tumor vasculature via increased coverage of pericytes [41], while angiopoetin-2 plasma levels are a possible biomarker for tumor progression, metastasis and patient survival [42].

These genetic insights might have therapeutic implications. Until recently, anti-angiogenic medicine has been largely focused on pruning the established vasculature, inhibiting neovessel growth, or inducing the formation of hypoperfused vessels (termed "nonproductive angiogenesis"; see below). All these strategies bear, however, the risk of inducing severe tumor hypoxia, which, as discussed above, may promote tumor escape and metastasis. Indeed, recent genetic and pharmacological studies show that hypoxia, induced by VEGF-targeted therapy and pruning of tumor vessels, may indeed fuel tumor invasiveness and metastasis [11, 13]. By contrast, inhibition of PHD2 in ECs would provide a conceptually different strategy, whereby tumor vessel numbers are not targeted, but their function is improved by streamlining the EC layer so that the resultant-improved oxygenation renders the tumor less malignant and metastatic (Fig. 2). An outstanding question is whether tumors in PHD2 haplodeficient mice would respond better to chemo/radiation therapy, not only because of improved drug delivery but also because of increased generation of O_2 radicals.

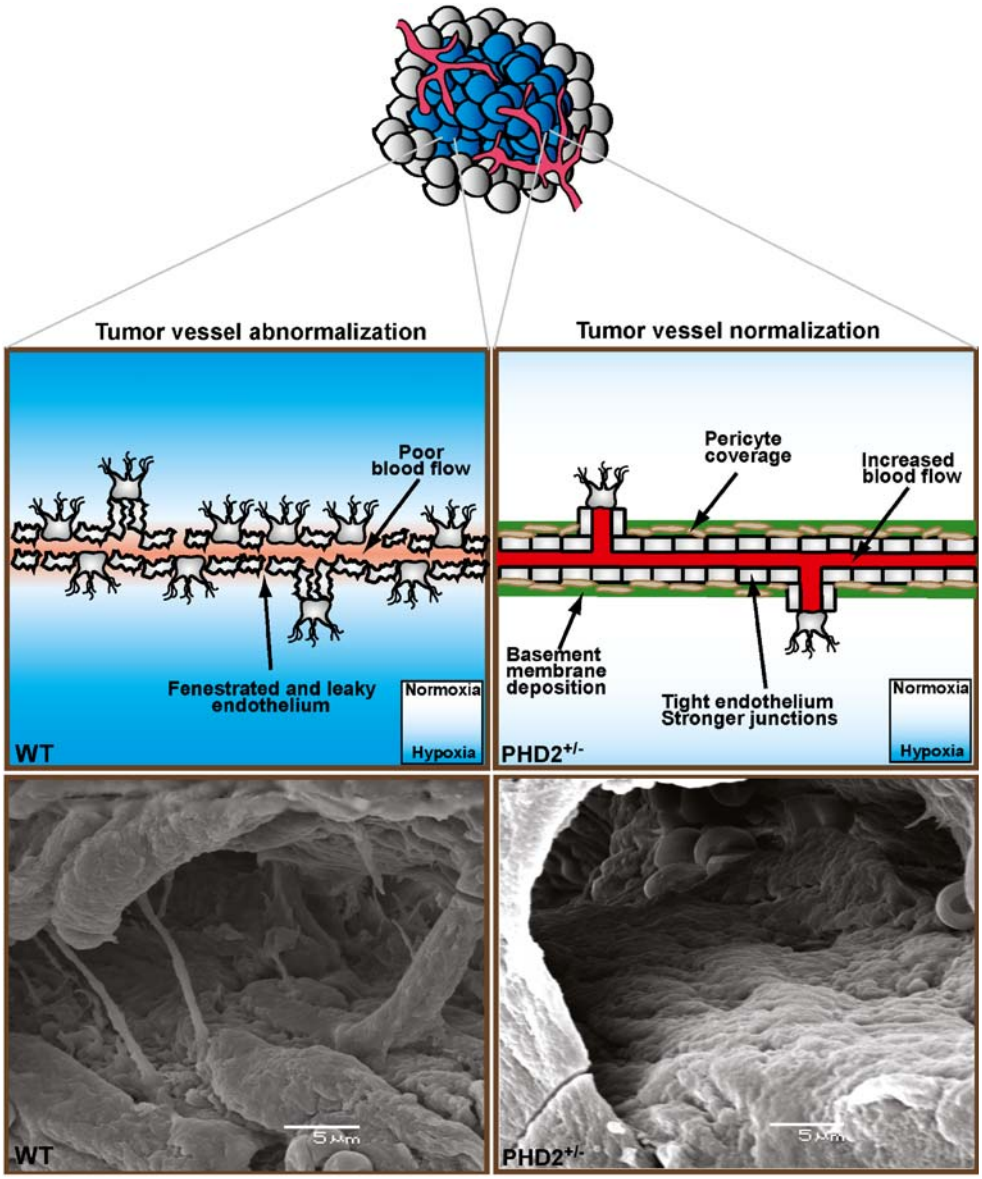

Fig. 1 PHD2 haplodeficiency normalizes tumor vasculature. Haplodeficiency of PHD2 in endothelial cells does not affect tumor vessel density and area, tortuosity, or lumen size but induces "normalization" of the endothelial lining by changing the shape, not the number, of the endothelial cells. These cells have a molecular signature that redirects them from a hypermotile "tip-cell"-like phenotype towards a more quiescent and sessile cell fate. Consequently, tumor vessels are lined by an orderly formed, regular, single EC monolayer with cobblestone-like appearance representing the "phalanx" formation of ancient Greek soldiers. Moreover, ECs are surrounded by more pericytes and well-formed basement membranes

Haplodeficiency of PHD2 induces "phalanx" endothelial cell layers

In vitro studies show that PHD2 heterozygous-deficient ECs are more quiescent, exhibit lower proliferation rates and reduced motility upon VEGF stimulation, and are also more sensitive to its survival activity [4]. Also, they fail to show extensive lamellipodia and filopodia formation. This defect is specific for VEGF as the response to FGF-2 is preserved. Gene profiling of PHD2 heterozygous-deficient ECs, in combination with in situ analysis of ECs in intact tumors, revealed that these cells have a molecular signature that redirects the specification of hypermotile, "tip cell"-like ECs towards a more quiescent and sessile phalanx cell fate. This signature includes the upregulation of VE-cadherin and (s)Flt1 that counteract the abnormalization factors, secreted by tumor cells (Fig. 3). VE-cadherin has also been documented to induce a "normalized, stabilized, quiescent" endothelial phenotype indirectly by modulating VEGF signaling and, hence, inhibiting VEGF-driven proliferation and survival [43, 44]. It also inhibits proliferation of ECs in cooperation with the VEGF trap sFlt to tilt the balance towards quiescence [45]. Consistent herewith, HIF-2α elevates the expression of the junctional molecule VE-cadherin, which is not regulated by HIF-1α [46]. Whether VE-cadherin also inhibits EC migration and lamellipodia formation by modulating VEGF signaling remains to be determined, but these impaired migratory responses in the PHD2 haplodeficient ECs might be attributable, at least in part, to the upregulation of the VEGF-trap (s)Flt1. Likewise, upregulated (s)Flt1 levels could also reduce endothelial proliferation in these cells. The degree of

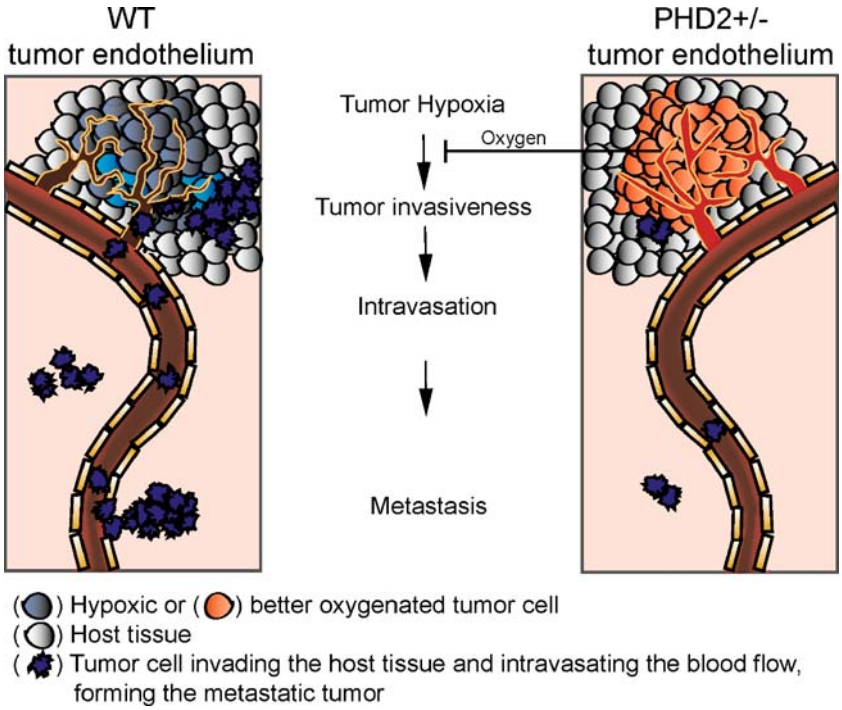

Fig. 2 Vessel normalization improves tumor oxygenation and reduces metastasis. The changes in endothelial cell shape upon PHD2 haplodeficiency do not affect primary tumor size but improve tumor perfusion and oxygenation. Consequently, the primary tumor shows a less malignant phenotype, growing more focally and noninvasively and showing lower induction of pro-metastatic genes. In addition, the phalanx of impermeable ECs constitutes an unusual vascular obstacle, impairing cancer cell intravasation and the formation of metastasis

inhibition of VEGF signaling by the upregulated levels of (s)Flt1 is not sufficient to induce vessel regression and inhibit new vessel sprouting but does suffice to induce vessel and endothelial normalization. The need for such subtle regulation is further suggested by previous findings that a pericellular gradient of (s)Flt1 improves vessel morphogenesis [45], while absence of Flt1 causes aberrant vessel branching. Other factors may contribute to tumor vessel normalization though their relationship with PHD2 remains to be studied. These include matrix metalloproteinases [47], Robo4 [48], Ephrin-A [39], angiopoietin-1 [14], platelet-derived growth factor receptor ß [49], and the regulator of G-protein 5 [50]. In addition, perivascular gradients of nitric oxide (NO) regulate vessel stabilization, as disruption of this gradient (through NO production by tumor cells) causes EC destabilization and impairs vessel maturation [51].

While reduction of PHD2 levels by 50% sufficed to induce EC normalization, complete loss of PHD2 causes other vascular changes, indicating that PHD2 has dose-

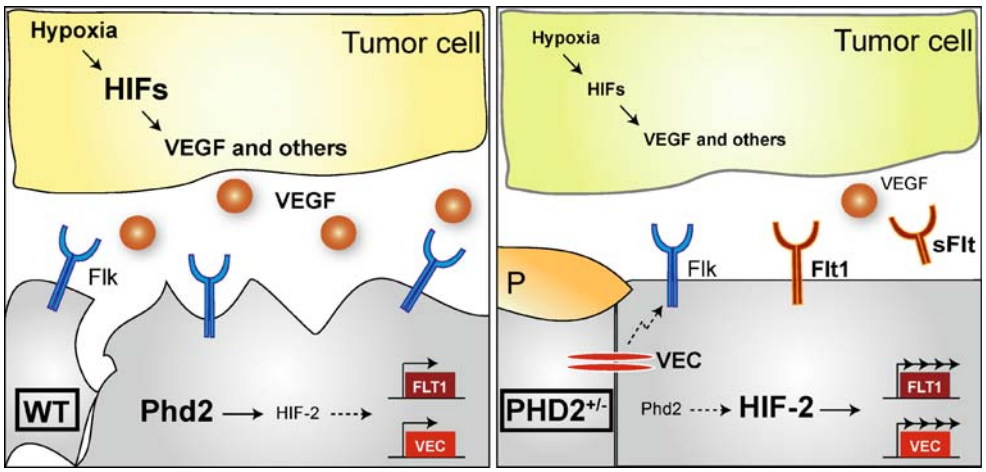

Fig. 3 PHD2$^{+/-}$-mediated tumor endothelial normalization switch. *Left panel* Hypoxic tumor cells (in *yellow*) induce WT endothelial cell (EC; *gray*) abnormalization by the release of excessive amounts of VEGF and other angiogenic factors. *Right panel* PHD2$^{+/-}$ endothelial cells counteract the abnormalization switch, in part via HIF-2α upregulation of (s)Flt1 and VE-Cadherin (VEC), thereby improving vessel lining, pericyte (P) coverage, perfusion, and oxygenation. As a consequence, better oxygenated tumor cells (in *light green*) release less hypoxic-driven angiogenic factors. This pathway is more effective in PHD2$^{\pm}$ endothelial cells, since they reset oxygen sensing and become better (pre)adapted to hypoxia

dependent qualitatively distinct biological effects. Mouse germ-line disruption of PHD2 results in developmental defects and embryonic lethality [52]. Complete inactivation of PHD2 in mice after birth stimulates the growth of supernumerary vessels in healthy organs [53, 54]. It remains to be elucidated whether the vascular changes in these PHD2 conditional knockout mice reflect primary EC-related changes or are secondary to the elevated hematocrit levels and cardiac dysfunction or to elevated plasma VEGF levels [53, 55]. Alternatively, this vessel phenotype may relate to the hydroxylation-independent activity of PHD2, although this effect has been only demonstrated in vitro in immortalized ECs [56]. Another study reports that stabilizing HIF-1α via chemical inhibition of PHDs systematically prevents vessel loss and subsequent aberrant neovascularization in a model of oxygen-induced retinopathy [57].

A "tug of war" between endothelial and tumor cells for oxygen

As discussed above, hypoxic ECs try to counteract the (over) production of abnormalization factors by hypoxic tumor cells by releasing anti-abnormalization factors. In most tumors, ECs loose the "tug of war" against tumor cells, since tumor vessels are highly abnormal [5, 6]. Rephrased tumor cells succeed in turning on the tumor "vessel abnormalization switch". Genetic studies indicate that inactivation of one PHD2 allele is required to restore this imbalance and turn off the abnormalization switch. Thus, by genetically altering oxygen-sensing mechanisms in ECs, such that ECs behave as if they are preconditioned to a more hypoxic state; ECs are capable of overcoming the abnormalization switch, incited by hypoxic tumor cells. These genetic findings also imply that therapeutic inhibition of PHD2 should be restricted to ECs and not occur in tumor cells as the latter would upregulate abnormalization factors and, thereby, nullify the upregulation of normalization factors by ECs. How selective inhibition of PHD2 in ECs can be achieved remains an outstanding, challenging question.

Another implication is that (epi)genetic mutations, which inactivate the oxygen-sensing machinery in tumor cells, may also affect this "tug of war" between ECs and tumor cells and tilt the balance to vessel abnormalization. The expression and activity of PHDs in tumor cells can be modulated by different oncogenes. For instance, v-Src and Ras stabilize HIFs, presumably by blocking prolyl hydroxylation by PHDs, though the precise mechanisms remain to be defined [58]. Oncogenic mutations in the fumarate hydratase and succinate dehydrogenase enzymes have also been associated with cancer because fumarate and succinate inhibit PHDs [59, 60]. Furthermore, through physical interaction, the melanoma antigen 11 suppresses PHD2 activity [61].

Another consideration is that the activity of PHDs in cancer cells might be more complex than anticipated. For instance, a recent paper reports that PHD2 levels are inversely correlated with tumor-forming potential, HIF-1α activity, glycolytic rates, VEGF expression, and ability to grow under hypoxic stress [62]. However, when PHD2 is completely inhibited, tumorigenesis is reduced, presumably because of a HIF-dependent pro-apoptotic response [62]. These findings about a dual role of PHD2 in tumor biology highlight that PHD2 may regulate, in a dose-dependent mechanism, different oncogenic versus tumor-suppressing mechanisms. Another example is the candidate tumor suppressor ING4, which suppresses the expression of HIF target genes. However, interaction of ING4 with PHD2 relieves this suppressor activity [63]. Silencing PHD2 might, therefore, increase the ING4 availability and repress, not induce, HIF activity.

Abnormalization of tumor vessels by inhibiting notch: another process?

Other types of tumor vessel abnormalization have been reported. For instance, upon genetic inactivation or pharmacological inhibition of Dll4/Notch signaling in ECs, the tumor vascular network contains supernumerary, hypoperfused vessel branches [64, 65]. Unlike the tortuous, large, leaky vessels with an abnormal EC layer that obstructs blood flow in normal tumor vessels, perfusion in these Dll4/Notch-inhibited tumor vessels is reduced because their size is too narrow. Moreover, newly forming vessels in Dll4 haplodeficient mice have a reduced pericyte recruitment [66]. Consequently, the tumor is more hypoxic than in control tumors, leading to reduced tumor growth [64, 65]. These observations led to the hypothesis that inhibition of Notch or Dll4 constitute an alternative anti-angiogenic strategy [67], though the effects on metastasis need to be further analyzed.

Since inhibition of Notch signaling induces a different type of tumor vessel abnormalization, we will briefly describe its role in vessel morphogenesis and how Notch signaling might interface with hypoxia signaling during this process. Following VEGF stimulation, Dll4 expression is induced in tip cells [68]; it subsequently binds to the Notch receptor on neighboring cells, which thereby acquire the stalk cell phenotype; the stalk cell, in turn, downregulates VEGFR2 signaling and, thus, prevents itself from becoming a tip cell [69, 70]. Hence, upon inhibition of this intercellular communication, a supernumerary amount of tip cells is formed, explaining excessive vessel branching. Since tip cells are formed at the expense

of stalk cells, these branches are thin, small, and slender and, hence, hypoperfused.

There is emerging evidence that the Notch-signaling pathway is modulated by the cellular hypoxia-sensing machinery and vice versa. Interestingly, some components of this machinery have been shown to modulate the Notch-signaling pathway. First, HIF-1α and HIF-2α can directly activate the promoters of Notch-signaling downstream targets hey1, hey2, and Dll4 [71, 72]. Second, HIF-1α directly interacts with Notch, stabilizing the latter and consequently increasing its activity [73]. Finally, FIH-1 also hydroxylates the Notch intracellular domain, and independently from its hydroxylation status, their interaction negatively regulates Notch-signaling [74, 75]. However, so far, the hypoxia-sensing machinery is not known (yet) to affect tumor angiogenesis via regulation of Notch.

Conclusions

It has been traditionally believed that pruning tumor vessels as maximally as possible would starve the tumor to death by depriving it from O_2 and nutrients. However, emerging evidence indicates that such anti-angiogenic strategies induce severe hypoxia, resulting in increased tumor invasiveness and metastasis [11, 13]. By contrast, antitumor vessel abnormalization strategies, for instance, by reducing PHD2 in the endothelial cell layer would not target tumor vessel density but improve their perfusion, which renders tumors less invasive, metastatic, and malignant. From a clinical perspective, a more encapsulated, less invasive primary tumor might be easier to surgically resect. It is also tempting to speculate that this strategy could increase delivery of cytotoxic agents to the cancer cells. Moreover, enhanced tumor oxygenation may improve the efficacy of radiation therapy by increasing the amounts of lethal reactive oxygen species [5]. Nevertheless, before vessel normalization via reducing PHD2 will become a feasible treatment strategy, a critical threshold for the reduction of PHD2 should be defined in order to confer its dose-dependent actions in normalizing the endothelial cell layer. It also remains an outstanding challenge how it will be possible to selectively and precisely reduce PHD2 levels in endothelial but not in tumor cells.

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