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STATE-OF-THE-ART REVIEW

Regulation of redox signaling in HIF-1-dependent tumor angiogenesis

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Angiogenesis is the process of blood vessel growth. The angiogenic switch consists of new blood vessel formation that, in carcinogenesis, can lead to the transition from a harmless cluster of dormant cells to a large tumorigenic mass with metastatic potential. Hypoxia, that is, the scarcity of oxygen, is a hallmark of solid tumors to which they adapt by activating hypoxia-inducible factor-1 (HIF-1), a transcription factor triggering *de novo* angiogenesis. HIF-1 and the angiogenic molecules that are expressed upon its activation are modulated by redox status. Modulations of the redox environment can influence the angiogenesis signaling at different levels, thereby impinging on the angiogenic switch. This review provides a molecular overview of the redox-sensitive steps in angiogenic signaling, the main molecular players involved, and their crosstalk with the unfolded protein response. New classes of inhibitors of these modulators which might act as antiangiogenic drugs in cancer are also discussed.

Introduction

Angiogenesis refers to the process of *de novo* formation of blood vessels, which takes place from already existing vessels. It is required for correct delivery of nutrients and oxygen to—as well as removal of waste products of aerobic metabolism from—newly generated cells, such as physiologically, during tissue growth, embryogenesis, wound healing and, pathologically, in age-related macular degeneration, arthritis, and cancer [1].

In tumors, the angiogenic switch, that is, *de novo* vessel formation, is a rate-limiting process triggered at the initial stages of tumorigenesis, when tumor mass reaches about 1–2 mm in diameter. This leads tumor to develop from a dormant cluster of cells to a large defined entity with metastatic potential [2].

Angiogenesis is a complex multistep process regulated by the equilibrium between angiogenic factors,

Abbreviations
ANG-1 and ANG-2, angiopoietin; ATF6, activating transcription factor 6; BIP, binding immunoglobulin protein; CA IX, carbonic anhydrase; CAFs, carcinoma-associated fibroblasts; CHOP, C/EBP homologous protein; $CoCl_2$, cobalt chloride; DPI, diphenyleneiodonium; EGF, epithelial grow factor; ER, endoplasmic reticulum; ERO1, endoplasmic oxidoreduction 1; ETC, electron transport chain; FAD, flavin adenine dinucleotide; Gulo, gulonolactone oxidase; HIF-1, hypoxia-inducible factor-1; LDH-A, lactate dehydrogenase-A; MMP-2 and MMP-9, matrix metalloproteinase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, Nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidases; PDGF, platelet-derived growth factor; PDI, protein disulfide isomerase; PERK, Protein kinase R-like endoplasmic reticulum kinase; PHD, prolyl hydroxylases; PRDX4, peroxiredoxin; ROS, reactive oxygen species; SNO, *S*-nitrosylation; TGF, Transforming growth factor; TME, tumor micro-environment; UPR, unfolded protein response; VEGF, vascular growth factor; VEGFR, VEGF receptor.

whose activity is tightly modulated by their gene dosage, and antiangiogenic factors [1]. Stressful conditions, such as hypoxia or nutrient deprivation, can trigger angiogenic factors that enable the angiogenic switch to take place. Many growth factors [epithelial grow factor (EGF), Transforming growth factor (TGF)α, vascular growth factor (VEGF), nitric oxide synthase (NOS), Platelet-derived growth factor (PDGF)] are also angiogenic and regulate this process.

Oxygen levels range from 3.1% to 8.7% in normal cells; however, it can decrease up to 0.01% in tumor cells, making hypoxia a common hallmark of many solid tumors. Under low-oxygen tensions, hypoxia-inducible factor-1 (HIF-1) is activated and promotes the transcription of angiogenic factors (i.e., VEGF, PDGF, TGF-α) by recognizing a consensus hypoxia response element in their promoter regions, which is a prerequisite for tumor angiogenesis [3].

Given the importance of angiogenesis in tumor growth and spread, Judah Folkman already in 1970 proposed that tumor development could be tackled by antiangiogenic therapy [4]. However, initial enthusiasm clashed against the evidence that selectively targeting a single angiogenic factor—mostly VEGF and its receptor—gave only limited clinical benefits, highlighting the need for a better understanding of the signaling underlying angiogenesis [5] (Fig. 1).

In this framework, it has become evident that changes in the redox state of tumor cells and related micro-environment (e.g., due to the production of reactive oxygen species, *ROS*) regulate HIF-1-mediated transcriptional control of angiogenic factors and also influence downstream angiogenesis signaling where receptor-competent isoforms of angiogenic factors act on tumor angiogenesis. Understanding the full spectrum of redox regulations of tumor angiogenesis

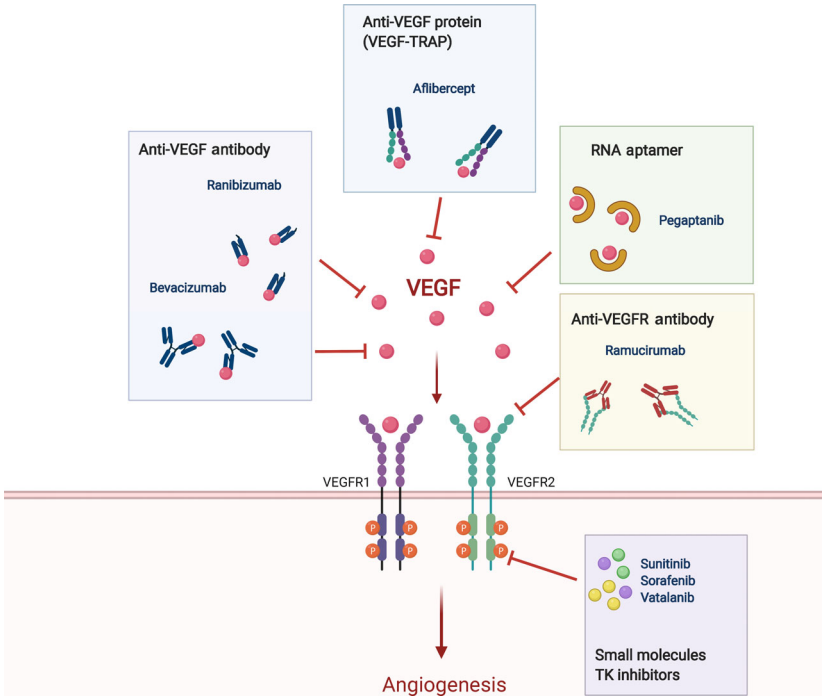

Fig. 1. VEGF targeting antiangiogenic therapy. Schematic representation of the VEGF inhibitors. From left to right: Bevacizumab is a recombinant humanized monoclonal antibody which directly binds soluble VEGFA, preventing receptor binding. Bevacizumab has been approved by Food and Drug administration (FDA) and European Medicines Agency (EMA) for therapeutic use in metastatic tumors. Ranibizumab is a monoclonal antibody fragment (Fab) against all isoforms of soluble VEGF. Ranibizumab has been approved by FDA and EMA for the treatment of macular degeneration and macular edema. Aflibercept is a recombinant fusion protein consisting of the Fc portion of IgG linked to the extracellular domains of human VEGFR1 and 2. Aflibercept's therapeutic use includes wet macular degeneration and metastatic colorectal cancer. Pegaptanib, a pegylated aptamer that binds VEGF, is approved for the treatment of age-related macular degeneration. Ramucirumab is a monoclonal anti-VEGFR2 antibody which prevents VEGF/VEGFR2 signaling activation and has been approved by FDA and EMA for the treatment of solid tumors. Sunitinib, Sorafenib, and Vatalanib are small molecules inhibiting the protein kinase domains including those of VEGFR1 and 2, employed in the treatment of solid tumors. Of note, Vatalanib is still under investigation in clinical trials and has not been officially approved by EMA or FDA.

signaling and their mediators is, therefore, crucial for an optimal design of angiogenesis-targeted therapies.

This review aims at providing a general picture of basic principles and emerging concepts underlying redox regulation of angiogenesis in cancer, pointing to the effects that redox modifications can induce to molecular modulators and pharmacological inhibitors used in the clinics.

ROS and tumor micro-environment crosstalk

The central role of transformed cells (parenchyma) in the biology of cancer has been changing during the last decades, and nowadays, it is commonly accepted that the stroma, usually referred to as the tumor micro-environment (TME) contributes significantly to the development of a wide variety of tumors. TME constitutes the surrounding support of the tumor mass, which contains extracellular matrix along with blood vessels, several cell types, and subtypes enabling tumor growth and progression. Consistently, it has been reported that the expression profile of genes involved in angiogenic and hypoxic response reveals a strong prognostic capacity in predicting breast cancer outcome [6].

Fibroblasts are a common type of stromal cells extracted from invasive carcinomas and referred to as carcinoma-associated fibroblasts (CAFs). At variance with normal fibroblasts derived from nontumor areas, active CAFs can promote the growth of mammary carcinoma cells and enhance tumor angiogenesis [7]. In this context, ROS play a primary role as they functionally interact with CAFs in a complex crosstalk that sustains tumor growth. If, indeed, from one side, fibroblasts are induced by ROS, which are generated by the activity of a plethora of enzymes (see below), to turn into active CAFs via HIF-1α signaling (with the consequent angiogenesis), from another side CAFs promote ROS production and aggressiveness of cancer [8,9].

In this regard, it is emblematic the role of the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2), which is a master regulator of the cellular response to oxidative stress, whose ROS-dependent activation in cancer cells promotes cancer progression whereas in TME has onco-suppressor effects [10,11]. ROS in TME affect also the oxidative status of T_{reg} cells enhancing their immunosuppressive function and promoting resistance to PD-L1 blockade therapy [12].

Since increased ROS production is associated with cancer development and resistance to therapy, pharmacological research aims to modulate the oxidative landscape of tumor and TME by acting at different levels on HIF-1 signaling, ROS-generating enzymes and the crosstalk between ROS and HIF-1 signaling.

Redox regulation of HIF-1 signaling

HIF-1 is a heterodimer composed of an α and β subunit, the former being the O_2-regulated and the latter the constitutively active. In the presence of oxygen, HIF-1α is hydroxylated on conserved proline (Pro^{402}, Pro^{564}) and asparagine (Asn^{803}) residues by *Fe*(II)- and 2-oxoglutarate-dependent dioxygenases (also called prolyl hydroxylases, PHDs and asparaginyl hydroxylase, respectively) [13]. HIF-2α shows 48% amino acid sequence homology with HIF-1α, and it has a similar domain arrangement [14].

Once hydroxylated, HIF-1α is ubiquitinated and directed to proteolysis by binding to the von Hippel-Lindau protein (pVHL)-E3 ubiquitin ligase complex, which in turn targets HIF-1α to degradation via the ubiquitin-proteasome pathway. In hypoxic conditions (i.e., when oxygen is scarce and not available to PHDs), the rate of prolyl hydroxylation is reduced, thus preventing HIF-1α degradation [15]. Thus, stabilized HIF-1α accumulates in the nucleus and binds to HIF-1β to induce the expression of a large number of proangiogenic factors including vascular endothelial growth factor (VEGF); VEGF receptors (VEGFR); PDGF-B; plasminogen activator mitogen 1; angiopoietin (ANG-1 and ANG-2); ANG-1 receptor TIE-2 (tyrosine kinase with immunoglobulin-like and EGF-like domains), and some members of the matrix metalloproteinase family (MMP-2 and MMP-9) [3,16]. Although both HIF-1α and HIF-2α have the abilities to heterodimerize with HIF-1β and bind to hypoxia-inducible genes bearing hypoxia response elements motif, they show a different specificity for their transcriptional targets: HIF-1α induces the expression of glycolytic enzymes, such as lactate dehydrogenase-A and carbonic anhydrase. In contrast, HIF-2α acts more effectively on EPO gene and genes involved in iron metabolism, whereas VEGF and GLUT-1 are regulated by both HIF-1α and HIF-2α [17].

The endothelial mitogen VEGFA is the most noteworthy of all of these HIF-1/2 targets, as it is considered the master regulator of angiogenesis in tumors.

Prolyl hydroxylases catalyze HIF-1α hydroxylation by inserting one atom of the molecular oxygen into the proline (or arginine) residue of the protein, and the other into the cosubstrate α-ketoglutarate, which is thus converted in succinate [18,19]. Dimethyloxalylglycine, a competitive antagonist of α-ketoglutarate, inhibits PHD activity and induces HIF-1-dependent

transcription of angiogenic factors. Similarly, HIF-1 activity is potentiated by iron chelators and cobalt chloride ($CoCl_2$), which inhibit PHD by displacing *Fe* (II) from the catalytic center [15,20].

Hypoxia-inducible factor-1 activity is indirectly tuned by redox active molecules that modulate PHDs. Ascorbate represents a prototype of this class of molecules. Indeed, it interacts with iron in PHDs by reducing insoluble ferric iron *Fe* (III) complexes to stable, soluble, ferrous *Fe* (II) chelates. This supports a specific role played by ascorbate in both chelating and reducing PHD-bound *Fe* (III) during hydroxylation to maintain enzyme cycling [21].

In vitro studies have shown that ascorbate suppresses hypoxia- or $CoCl_2$-induced HIF-1α activity as it acts as a cofactor of PHDs in oncogenic activated cells, suggesting that it may enhance the HIF-1-hydroxylase activity of PHDs [22–24].

Ascorbate supplementation in ascorbate-deficient $Gulo^{-/-}$ mice has been shown to reduce tumor metastases and necrosis, together with the expression of the HIF-1 targets MMP-9 and VEGF, supporting the hypothesis that ascorbate may inhibit tumor progression via HIF-1 inhibition [25].

In normal conditions, cellular antioxidant systems keep ROS in a physiological range required for a correct cell signaling. Upon excessive ROS production, or as a consequence of a defective antioxidant defense, ROS levels can rise dramatically (as observed in several tumor types) and affect HIF-1 signaling. Indeed, ROS can mediate iron oxidation, thus leading to PHD inactivation and HIF-1α stabilization [15].

Besides hydroxylation, other redox post-translational modifications have been identified to modulate HIF-1α turnover. In particular, several studies reported that HIF-1α is targeted by nitric oxide (NO) via *S*-nitrosylation (SNO) at Cys533, a critical cysteine which localizes in the oxygen-dependent degradation domain of HIF-1α. HIF-1α SNO has been demonstrated to stabilize the protein and inhibit the association with VHL even under normoxia, in such a way mimicking pseudo-hypoxic conditions [26,27]. However, the activatory role of NO in HIF-1 signaling is not always direct. Indeed, it has been reported that hypoxia induces HIF-1α stabilization indirectly via the nitrosylation of *Fe*(II) located in the catalytic site of PHDs, which results in the inhibition of PHDs hydroxylase activity [28].

Given its well-established role in tumor angiogenesis, HIF-1 has been considered an attractive therapeutic target for cancer therapy. Direct HIF-1 inhibitors, which affect the expression or function of HIF-1, and indirect HIF-1 inhibitors, which act on other molecules in related pathways, have been identified. The former class of inhibitors targets HIF via different mechanisms, for example, by inhibiting mRNA expression; protein synthesis; dimerization; DNA binding, and transcriptional activity. However, only few of them are in clinical trials, probably due to their pleiotropic (side)effects. One of these, BAY 87–2243, is a mitochondrial complex I inhibitor that reduces HIF-1α stabilization [29]; PX-478 has been reported to inhibit HIF-1 translation [30] and KCN-1 acts on the interaction between HIF-1α and its transcriptional coactivator p300 [31] (Fig. 2).

Modulators of the redox signaling

Reactive oxygen species (including among others H_2O_2 and NO) are generated as nonfutile or side products of the catalysis of many cellular enzymes, such as endoplasmic oxidoreduction 1 (ERO1), nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), the complex I and III of mitochondrial electron transport chain (ETC), and nitric oxide synthase (NOS), have been documented to exert direct effects on the levels/activity of angiogenic mediators. Therefore, in this section we will analyze the regulation and the role in angiogenesis of some ROS-generating enzymes; how their activity is correlated to HIF-1; and whether they may act as ideal target for antiangiogenic therapy in cancer.

ERO1

ERO1 is an endoplasmic reticulum (ER)-resident protein disulfide oxidase. It is a flavin adenine dinucleotide (FAD)-containing protein which takes part in disulfide bond formation of a number of client proteins. In particular, ERO1 restores the oxidized state of protein disulfide isomerase (PDI) once this has introduced disulfide bonds in new ER-resident client proteins, by coupling this reaction with two-electron reduction in O_2 to H_2O_2 [32,33]. A rough calculation estimates that, for each disulfide introduced in proteins, ERO1 generates one molecule of H_2O_2, suggesting that ERO1 is one of the main sources of H_2O_2, accounting for about the 25% generally produced in the cells [34].

Saccharomyces cerevisiae contains only ERO1p whereas, in mammals, there are two ERO1 paralogs, ERO1 α and ERO1 β. Human and yeast Ero1s modulate their oxidative activity differently: Human ERO1 α exploits Cys^{104} and Cys^{131} to regulate its overall activity, indeed the mutant of Cys^{131} is hyperactive and generates more H_2O_2 [33,35].

In vitro, ERO1 α and ERO1 β show comparable activity in accepting electrons from PDI and

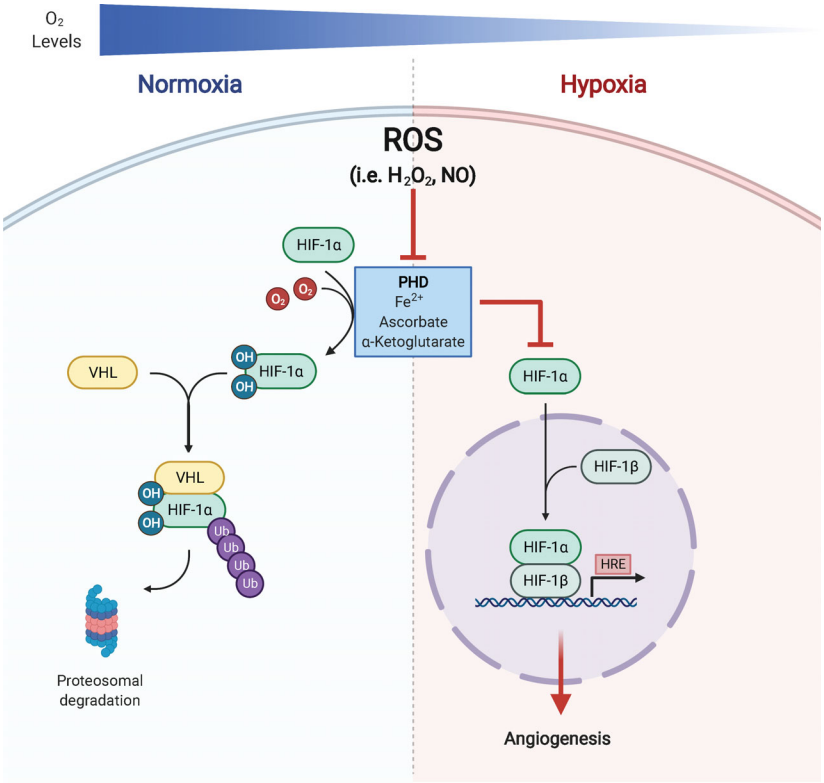

Fig. 2. HIF-1-dependent angiogenic switch in cancer cells. Schematic representation of HIF-1 signaling pathway under normoxic and hypoxic conditions. In normoxic conditions (left panel), HIF-1α is hydroxylated by PHD, which leads to the binding with VHL, part of a E3 ubiquitin ligase complex. This interaction results in the polyubiquitination and proteasomal degradation of HIF-1α. In hypoxia (right panel), the lack of oxygen and increased levels of ROS (including H_2O_2 and NO) inhibit PHD activity, preventing HIF-1α degradation. Thus, HIF-1α accumulates in the cytoplasm and subsequently translocates in the nucleus, where it binds to HIF-1β forming a transcriptionally active HIF-1 complex. The HIF-1 complex recognizes and binds to the hypoxia response element (HRE) sequence, promoting the transcription of HIF-1 targets, among which those involved in angiogenesis.

generating H_2O_2. The main difference between them is related to their expression: ERO1α is ubiquitously expressed in most cells, whereas ERO1β is selectively expressed in pancreatic cells and enterocytes. At variance with yeast (in which ERO1p deficiency is lethal) in higher eukaryotes, ERO1's protein disulfide oxidase activity is compensated by other enzymes, such as peroxiredoxin (PRDX4) and Glutathione peroxidase 8 (GPx8), that participate in the disulfide bond formation while metabolizing H_2O_2. Therefore, mice lacking (knock-out, KO) for both ERO1 isoforms show only a delay in disulfide bond formation and a subtle pathological phenotype [36–39].

ERO1α (henceforth, ERO1) is regulated by hypoxia and ER stress, a condition elicited by unfolded proteins. Given its role in protein folding, ERO1 takes part to the homeostatic unfolded protein response (UPR), a plethora of corrective measures that, through the activation of signaling pathways mediated by protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1, and activating transcription factor 6 (ATF6), promote the attenuation of protein translation, chaperone activity, and protein degradation in order to restore ER homeostasis [40].

Mounting evidence suggests that UPR, in general, and the PERK branch, in particular, exert cytoprotective effects and promote tumor progression [41]. PERK activation attenuates global protein expression through eIF2α phosphorylation, but selectively enhances ATF4 translation, which regulates the transcription of CCAAT/enhancer-binding protein homology protein and ERO1 [42]. The PERK/ATF4 arm of the UPR is also involved in the angiogenic switch in tumors, and ATF4 has been proposed to promote transcription of the VEGFA [43]. Strikingly, ERO1 is induced in hypoxia, this being distinctive of solid tumors and associated with a negative outcome [44]. Analyses of mRNA expression profiles of human breast cancers from The Cancer Genome Atlas indicate that ERO1 is highly expressed in the most aggressive basal breast cancer and less in the luminal ones. Moreover, analysis from the Metastatic Breast Cancer project indicates an inverse correlation between ERO1 levels in the primary aggressive triple negative tumor and the time at which distant metastases are detected, arguing for a pivotal role of ERO1 in conferring aggressive phenotype [45]. This evidence gives support to other observations indicating that ERO1 overexpression is associated with other different forms of cancer and poor prognosis of some of them [46–49].

Previous studies report that a modest reduction in ERO1 expression is sufficient to limit VEGF secretion

[44]. More recent secretomic analyses indicate that lack of ERO1 in breast cancer cells has no major impact in normoxia, but impairs HIF-1-dependent angiogenic factors in hypoxia, suggesting some selective effect of ERO1 on angiogenesis in hypoxic conditions. Although in ERO1-deficient breast cancer cells ERO1 disulfide oxidase activity is compensated by other chaperones, the increase in VEGFA secretion was completely blunted in hypoxic conditions. Furthermore, the disulfide-containing dimer of $VEGF^{121}A$ (which contains a hand-tail intermolecular disulfide bond bridging Cys^{60} and Cys^{51}) is also the receptor-competent isoform of $VEGF^{121}A$ and was selectively impaired in the conditioned media of ERO1-deficient breast cancer cells [50]. This evidence suggests that, despite the induction of other chaperones, ERO1 activity on VEGF is not fully compensated in ERO1-deficient breast cancer cells under hypoxic conditions.

ERO1-deficient breast cancer cells exhibit low levels of VEGFA mRNA. Reasonably, this might depend on the attenuation of ATF4 signaling pathway, or the reduction in ERO1-mediated H_2O_2 fluxes that stabilize HIF-1α by inactivating PHD. However, the last hypothesis would imply that despite the abundance of the H_2O_2-metabolizing enzyme PRDX4 in the ER, H_2O_2 generated by ERO1 in the ER freely diffuses in the cytoplasm inducing HIF-1α stabilization (through inhibition of PHD) [51]. To conclude the aforementioned results on VEGF allow speculating that ERO1 loss is associated with decreased angiogenesis in breast cancer cells as effect of the general reduction in VEGFA and inability to generate a receptor-competent $VEGFA^{121}$. Consistent with this hypothesis, correlative gene expression analysis in basal breast cancer patients supports a significant positive correlation among ERO1, the PERK branch of the UPR, and VEGF, confirming the strong functional association among these three players in cancer.

In vivo, ERO1-deficient breast cancer xenografts show reduced ability to generate lung metastases, together with a decreased vasculogenesis in the primary tumors, suggesting that ERO1 inhibition might represent a good tool to selectively impair angiogenesis of solid tumors and limit metastases [45,52].

To date, three compounds have been reported to inhibit ERO1, that are: *erodoxin*, selective for the yeast isoform of ERO1, ERO1p, but with a weak activity against the protein of the mammalian orthologous, and the two functionally related molecules EN460 and QM295, identified from a high-throughput screening [53]. EN460 and QM295 interact with the reduced active form of ERO1 preventing ERO1 reoxidation and ERO1-dependent H_2O_2 production by displacing the FAD from ERO1. However, the low potency *in vivo* and the toxicity profile of both inhibitors suggest that they are not selective for ERO1, and indeed, EN460 inhibits other FAD-containing enzymes [54].

NADPH oxidases

NOX family consists of seven members, NOX1–5, and the dual oxidases (Duox) 1 and 2, with NOX1, NOX2, NOX4, and NOX5 being expressed in the vascular system. NOXs are transmembrane oxidoreductases, which share a conserved catalytic domain containing two heme groups on the N-terminal membrane side, and binding sites for prosthetic group (FAD) and substrate (NADPH) on the C-terminal cytosolic side. During the reaction catalyzed by NOXs, electrons flow from NADPH to the two heme groups via FAD and finally reach O_2 to generate H_2O_2.

NOX4 is localized mainly in the ER and mitochondria and, differently from the other isoforms, which are activated by other proteins or stimuli, is constitutively active, generating a constant flow of electrons from NADPH to FAD [55].

$NOX4^{-/-}$ mice have reduced angiogenesis, whereas endothelial-specific NOX4 transgenic mice have enhanced angiogenesis in an eNOS-dependent manner [56,57], suggesting a causal link between NOX4 and angiogenesis. NOX4 is implicated in angiogenesis induced by different cancer types, such as VHL-deficient renal carcinoma, fibrosarcoma, and gliobastoma [58–60]. NOX4 also promotes tumor angiogenesis under hypoxia through ROS-mediated stabilization of HIF-1α and subsequent expression of proangiogenic genes, such as VEGFA, glucose transporter 1 (GLUT-1), and adrenomedullin [61].

As for ERO1, NOX4 expression is regulated by the PERK/ATF4 branch of the UPR and, in a positive feedback loop, NOX4 sustains ATF4 signaling, which regulates VEGF [62]. This evidence suggests a common regulation of ERO1 and NOX4 by the PERK branch of the UPR involved in tumor angiogenesis and argues for both these factors as promising targets for antiangiogenic tumor therapy.

To date, the most studied and best characterized NOX inhibitors are *apocyanin*, *di-2-thienyliodonium*, and *diphenyleneiodonium* (DPI). DPI directly interacts with *Fe*-heme, and it has been proposed to react with FAD as well. Therefore, due to its high reactivity with all heme-containing proteins, possible use of DPI in the clinic is very limited and not recommended. Further two compounds, *VAS2870* and *VAS3947*, are emerging as novel covalent NOX inhibitors. They target a conserved active-site cysteine in NOX, producing

covalent inhibition and affecting NOX activities in the early phases of their catalytic cycle [63]. Interestingly, the use of these two inhibitors has been reported to exert beneficial effects on endothelial dysfunctions both *in vitro* and *in vivo* systems [64].

ETC

The mitochondrial ETC is composed of four protein multimolecular complexes, that are, complexes I–IV, which reside on the inner mitochondrial membrane. They are responsible for the generation of the electrochemical proton gradient required for ATP synthase (complex V) to produce ATP, and ROS, which represent side products of uncomplete reduction in oxygen. Indeed, around 1–4% of oxygen fails to be properly reduced and superoxide is produced as a consequence at complexes I and III [65].

Mutations in complex I occur in different tumors and trigger the glycolytic switch, that is, the so called Warburg effect [66], and ROS-derived metastasis [67].

Complex III generates ROS at the external side of the mitochondrial inner membrane as well as at the matrix. This site-specific ROS topology inside mitochondria is most likely important for redox signaling [68].

Hypoxic conditions stimulate ROS production at the level of Complex III in a way that is inversely correlated to the percentage of O_2: That is, the lower is the percentage of O_2, the more are the ROS produced in the mitochondria [69].

Mitochondrial ROS (mtROS) activate the hypoxic response signaling by stabilizing HIF-1α [70]. Along this line of reasoning, the inhibition of complex III with *stigmatellin* reduces hypoxic stabilization of HIF-1α *in vitro*, confirming the contribution of this complex to mtROS and stabilization of HIF-1α [71]. *Terpestacin* also counteracts mtROS production by binding a subunit of the mitochondrial complex III, thus inhibiting HIF-1α stabilization, VEGF-induced signaling, and impairment of angiogenesis [72].

Nitric oxide synthase

Nitric oxide is a gaseous free radical which acts as intracellular second messenger in manifold biological processes. In mammalian cells, endogenous NO is produced from the enzymatic oxidation of arginine to citrulline by a family of three NADPH-dependent NO synthases (NOSs). Two of them are constitutively expressed, one predominantly in neurons (nNOS) and one in endothelial tissues (eNOS); a third isoform, the inducible one (iNOS), is mainly expressed in cells of the immune system and in other tissues during the immune response [73]. NO-induced signaling mechanisms rely on different chemical interactions that NO can establish with amino acidic residues (e.g., tyrosine and cysteine) [74,75] or prosthetic groups (e.g., heme group and *Fe–S* clusters) of several proteins [76]. *Fe*-nitrosylation is probably one of the best-documented NO-induced modifications of heme-containing proteins [77], which is able to modulate the ability to bind oxygen of hemoglobin [78], as well as the activity of several enzymes involved in cell signaling (e.g., guanylate cyclase) and metabolism (e.g., cytochrome oxidase) [79,80].

As mentioned above, NO can also react with tyrosine (nitration) or cysteine residues (*S*-nitrosation or nitrosylation), thus also producing biological effects. Tyrosine nitration is essentially an irreversible damaging modification of protein backbone, whereas SNO, which is is the covalent addition of NO moiety to reactive sulfhydryl of cysteines, is emerging as one of the most relevant NO-mediated post-translational modification with biological activity [81].

During the last few years, different lines of evidence suggest that many oncoproteins can undergo gain or loss of function upon SNO, thus highlighting the role of NO in driving tumor progression.

Increase in NOS expression is usually observed in different human breast cancers and this correlates with malignancy [82]. Indeed, it has been reported that NO produced in the proximity of tumor (by both cancer or noncancer cells) can promote aggressiveness and development of a chemotherapy-resistant phenotype [83]. Several studies have also been reported about the implication of NO in HIF-1α and angiogenesis regulation in both physiological and pathological conditions [84]. The first biological function identified for NO was the control of vascular tone and permeability. This aspect is particularly relevant in cancer, where it is well known that SNO is involved in angiogenesis and tumor cell adhesion to endothelium, as well as in contributing to cancer cell spreading [85]. Increase in NO production in cancer cells correlates with upregulation of VEGF, leading to the increase in cancer metastasis. This is also essential to enhance vascular permeability induced by VEGF. In particular, stimulation of endothelial cells with VEGF induces NOS3-dependent SNO of β-catenin at Cys^{619}. This leads to dissociation of β-catenin from VE-cadherin and full disassembly of adherens junction complexes [86]. Importantly, NOS3-deficient mice are unable to induce vascular permeabilization upon VEGF treatment [86], suggesting the use of NOS3 inhibitors in cancers that rely on epithelium permeability to metastasize.

HIF-1 drives new vessel formation and, as mentioned above, NO fluxes stabilize HIF-1α even in normoxia. However, the regulation of HIF-1α by NO is still debated and seems to depend on the concentration and exposure time. In particular, recent observations indicate that short-term exposure to NO stabilize, while chronic fluxes destabilize HIF-1α [87–89]. Furthermore, there is also evidence that NO can exert different effects on HIF-1α stability in hypoxia vs normoxia. Some lines of evidence, indeed, indicate that NO donors act as pseudo-hypoxia mimickers, thereby inducing HIF-1 transcriptional activity even under normal oxygen tension [90, 91]. By contrast, other studies suggest the occurrence of opposite effects (i.e., decrease stability of HIF-1α) under hypoxia [26,87,92]. In regard to this apparent controversy, it has been reported that, besides Cys^{533}—whose effects of SNO have been well established—HIF-1α also contains another reactive cysteine (i.e., Cys^{800}) which is susceptible to SNO, but still not characterized in terms of the effects produced. A number of evidence reports that Cys^{800} SNO results in increased HIF-1 transcriptional activity, whereas other papers show that this modification reduces interaction of HIF-1 with its coactivator p300 which, in turn, abolishes HIF-1-mediated gene transcription [93, 94].

As mentioned above, NO can also directly interact with the oxygen sensor enzymes PHDs [27]. So far, the molecular mechanisms by which NO inhibits PHDs are not clearly described.

Finally, NO can also modulate HIF-1 pathway by affecting oxygen availability for the mitochondrial respiratory chain. Low NO concentrations, indeed, have been reported to reduce mitochondria-mediated HIF-1α accumulation under hypoxia in a ROS-independent manner, as antioxidants do not recover HIF-1 activation [95,96]. In particular, it has been reported that low NO concentrations can displace O_2 from the cytochrome *c* oxidase, making it available for PHDs [95,97].

Besides the well-known role of mitochondria in the energy production, it has been found that NO-mediated change on mitochondria can play an important role also in the regulation of other intracellular signaling, for example the ER stress response [98]. In NO-generating cells, the inhibition of respiratory rate correlates with mitochondrial Ca^{2+} fluxes, which upregulates the ER stress mediator and Ca^{2+}-binding chaperone GRP78 (binding immunoglobulin protein, BIP) activating its cytoprotective properties [98]. NO can also modulate HIF-1α-signaling by suppressing the activation of NOX. Indeed, DETA-NONOate, sodium nitroprusside, and sodium acetylpenicillamine, three different NO-donors, were shown to inhibit the production of superoxide by NOX in a concentration- and time-dependent manner through the SNO of p47phox (NoxO1) [99].

Given the variety of mechanisms by which NO modulates HIF-1 signaling and the consequences of excessive SNO on angiogenesis, the efficacy of NOS inhibitors has been tested in cancer therapy. So far, preclinical studies show that NOS2 and NOS3 knocking down, or

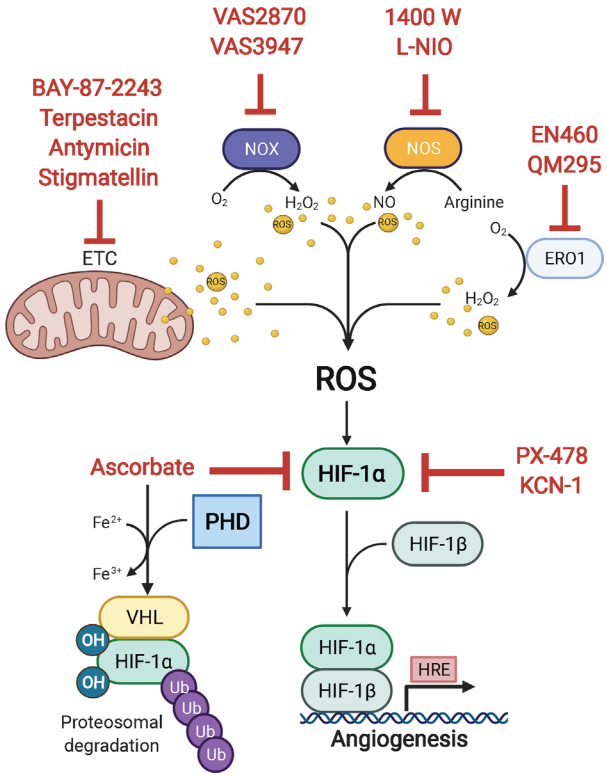

Fig. 3. Redox modulation of the HIF-1 signaling pathway in angiogenesis. Schematic representation of direct HIF-1 inhibitors and inhibitors of HIF-1 redox modulators which impair angiogenesis. Ascorbate impairs HIF-1α stabilization by acting as cofactor of PHD and thus promoting its catalytic activity, which results in increased HIF-1α degradation. PX-478 inhibit HIF-1 complex by attenuating HIF-1 translation whereas KCN-1 blocks HIF-1α interaction with its transcriptional coactivator p300. Impairment of ROS levels, that activate HIF-1α, can also be achieved via inhibition of their producers. ETC complex I can be inhibited by BAY 87-2243, while ETC complex III is inhibited by stigmatellin, antimycin, and terpestacin. VAS2870 and VAS3947 covalently bind and inhibit all NOX isoforms, preventing the formation of hydrogen peroxide. ERO1 activity can be inhibited by QM295 and EN460, which trap ERO1 in its reduced isoform suppressing H_2O_2 and protein disulfide bond formation. NOS inhibitors 1400 W and the N^5-(1-Iminoethyl)-L-ornithine (L-NIO) suppress angiogenic pathway by reducing NO and enfeebling HIF-1 signaling.

treatments with the NOS inhibitors 1400 W and the N^5-(1-Iminoethyl)-L-ornithine suppress angiogenic pathway leading to the reduction in colorectal cancer cell growth and migration (Fig. 3) [100].

Conclusion

It is nowadays commonly accepted that HIF-1 signaling is regulated by redox mechanisms and players, such as ROS- and NO-generating enzymes. These stabilize HIF-1 α, leading to VEGF transcription and consequent *de novo* tumor angiogenesis. Growing knowledge of the last few years has been setting the stage for the development of new lines of research aimed at targeting these enzymes for antiangiogenic therapy in cancer treatment. ERO1 is emerging as one of the most interesting and versatile prototypes due to multiple (indirectly transcriptional and directly post-transcriptional) effects it produces on the expression and correct assembly of VEGF and other HIF-1-dependent angiogenic factors.

The still low specificity of these new antiangiogenic strategies is slowing down preclinical validation of many new potential drugs. However, results so far obtained are very promising, and further studies deserve to be done to pave the way for new lines of intervention in cancer treatment.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

EZ and GF conceptualized the work. VM, CP, GF, and EZ wrote the manuscript.

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