

# Molecular Mechanisms of Hypoxia-Regulated Angiogenesis

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## Abstract

Hypoxia is an important regulator of angiogenesis and it stimulates neovascularization from existing blood vessels. At the molecular level, it occurs mostly through transcriptional regulation of genes which contain a core consensus sequence called hypoxia response element (HRE) via hypoxia inducible factors (HIFs) action. The discovery of HIFs hydroxylases as a family of dioxygenases that regulate HIFs dependently on the oxygen availability have significantly

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improved our understanding of the mechanisms of hypoxia signaling. Moreover, a broad number of factors have been shown to influence HIF stability and their effects could be mediated via several possible mechanisms including nitrosylation, deacetylation, or oxidation. The induction of HIF leads to the complex regulation of the pro- and antiangiogenic factors and extensive research will be essential for thorough understanding of the role of hypoxia in disease development and will help to identify new therapeutic targets for treatment of such hypoxia-dependent disorders.

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**Keywords**

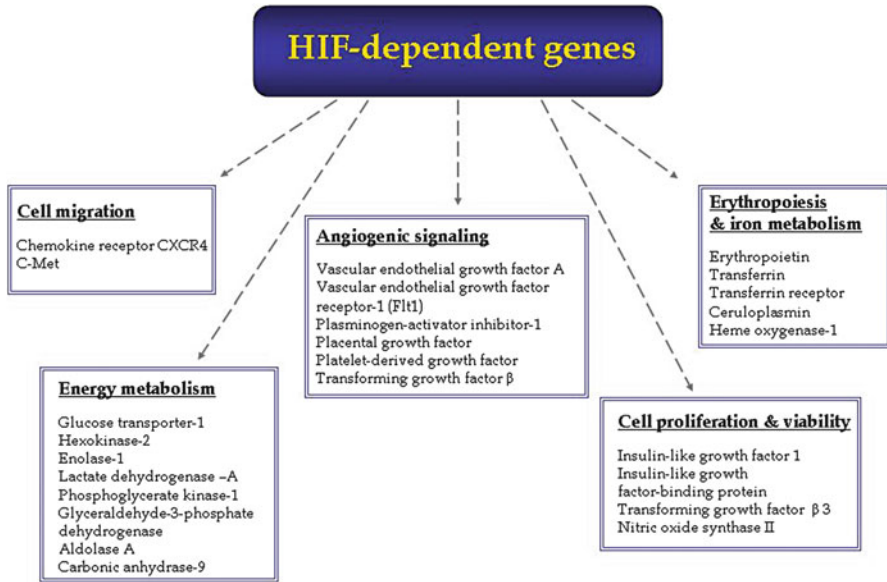
Hypoxia inducible factor • HIF-1 • HIF-2 • HRE • MicroRNA • Oxidative stress • Vascular endothelial growth factor • IL-8

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## 8.1 Introduction

Oxygen is vital for living cells and it plays a fundamental role in their metabolism. An inadequate oxygen supply to tissues and cells causes hypoxia, a state which can restrict their functions. The ability to maintain oxygen homeostasis is essential to the survival of all vertebrate species. On the other hand, several pathological conditions like stroke (cerebral ischemia) and heart infarction (myocardial ischemia) may result from the detrimental effect of hypoxia. Moreover, hypoxia has been recognized as one of the fundamentally important features of tumor growth and metastasis.

The hypoxic response is primarily mediated by the family of hypoxia inducible transcription factors (HIFs). Their activation leads to transcription of HIFs target genes—nowadays more than 150 HIFs-dependent genes controlling different processes including energy metabolism, cell growth and apoptosis, or vasomotor control was identified (Fig. 8.1). Moreover, to counteract the undesirable effects of low oxygen concentration, the new blood vessels providing oxygen to the ischemic tissues are formed. The expression of pro-angiogenic factors like vascular endothelial growth factor (VEGF) or interleukin-8 (IL-8) was thought to be strongly increased in hypoxic conditions; however, recent studies underline the complexity of angiogenic response after HIFs induction. The complication of cellular response to low oxygen concentration may be also related to context specific effects of individual HIF isoforms. Latest research underlined that HIF-1 and HIF-2 possess both overlapping and unique target genes and may also trigger specific roles: HIF-1 predominantly drives the initial response to hypoxia (<24 h), whereas HIF-2 is responsible for chronic response (>24 h).



**Fig. 8.1** Selected HIF-dependent genes. HIFs transactivate genes involved in a number of adaptive processes in response to hypoxia including angiogenesis, energy metabolism, cell migration, proliferation, etc.

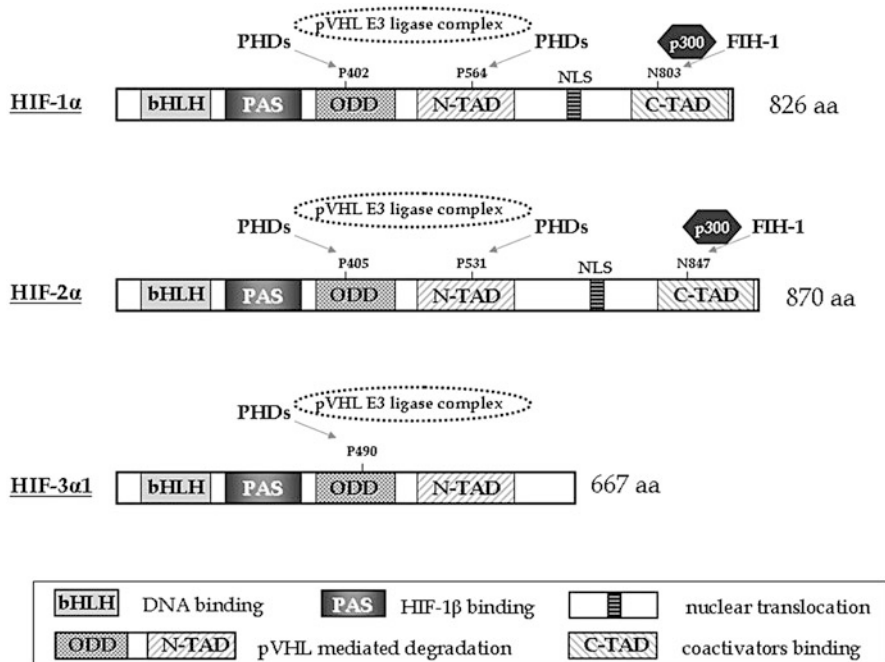
## 8.2 Stabilization of the Hypoxia Inducible Factor

### 8.2.1 The Hydroxylation of HIF- $\alpha$ as a Major Mechanism for Its Destabilization

The discovery of the hypoxia response element (HRE), the oxygen-regulated sequence, in the erythropoietin gene and the identification of the hypoxia inducible factor-1 (HIF-1) as a master transcription factor binding this sequence [1, 2] had started the intensive research in hypoxia field. Further studies performed in other than erythropoietin-producing cells have revealed that the same regulatory element is involved in the hypoxic regulation of gene expression indicating the presence of the general oxygen-sensing system in mammalian cells.

It is now well established that the regulatory sequence, called HRE, comprises of consensus-binding site (HBS, HIF-1-binding site) with a core motif (RCGTG) [3] and HIF-1 ancillary sequence (HAS), located eight to nine nt down- or upstream of HBS, which facilitates HIF-1-mediated transcription activation [4]. The existence of HRE was confirmed in hundreds of genes regulated in hypoxic conditions.

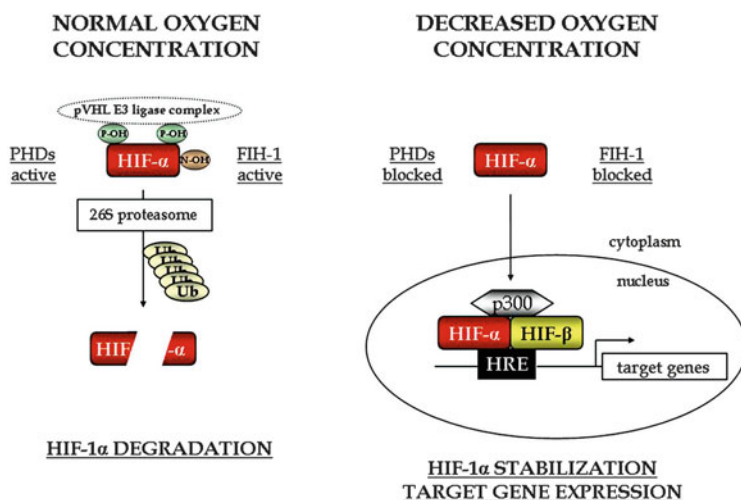
HRE is recognized by HIFs transcription factors, a heterodimers consisting of oxygen-labile  $\alpha$  (HIF- $\alpha$ ) and oxygen-independent  $\beta$  (HIF- $\beta$ , also called aryl hydrocarbon receptor nuclear translocator, ARNT) subunits. Both  $\alpha$  and  $\beta$  subunits are



**Fig. 8.2** The schematic structure of HIF- $\alpha$  isoforms. HIF-1 $\alpha$  is closely related to HIF-2 $\alpha$ , whereas the variant of HIF-3 $\alpha$ , HIF-3 $\alpha$ 1 lacks C-terminal transactivation domain (C-TAD), which is required for transcriptional activation. However all  $\alpha$  subunits are prone to oxygen-dependent degradation by the von Hippel–Lindau protein (pVHL) E3 ligase complex. The modifications of specific residues are highlighted above each protein, and the proteins that perform those modifications are shown (PHDs, FIH-1)

members of the bHLH/PAS (Basic Helix–Loop–Helix/PER-ARNT-SIM) domain family [5]. Three genes encoding distinct HIF- $\alpha$  isoforms exist in humans: *HIF1A*, encoding HIF-1 $\alpha$ ; *EPAS1*, encoding HIF-2 $\alpha$ ; and *HIF3A*, which is expressed as multiple HIF-3 $\alpha$  splice variants [5–7]. Mainly HIF-1 $\alpha$  and HIF-2 $\alpha$  mediate the hypoxia-dependent signaling, whereas HIF-3 $\alpha$  was shown to inhibit transcriptional activity of HIF-1 $\alpha$  [8]. Such difference in isoform-specific effects may be the result of their structure: HIF-3 $\alpha$  has high similarity to HIF-1 $\alpha$  and HIF-2 $\alpha$  in the bHLH and PAS domains but it lacks the C-terminal transactivation domain (C-TAD), which is required for transcriptional activation (Fig. 8.2).

Only HIF- $\beta$  subunit is constitutively expressed and not regulated by oxygen concentration. On the other hand, the expression of HIF- $\alpha$  subunits is strictly controlled at the protein level. This occurs, predominantly, through the hydroxylation of specific proline residues (Pro402 and Pro564 in HIF-1, Pro405 and Pro531 in HIF-2, Pro490 in HIF-3) within oxygen-dependent degradation domain (ODDD) present in  $\alpha$  subunits [7, 9, 10]. The hydroxylation is performed by a class of



**Fig. 8.3** HIFs stabilization during hypoxia. Under normoxia, HIF- $\alpha$  is subjected to oxygen-dependent prolyl hydroxylation by PHDs leading to pVHL E3 ligase complex binding and ubiquitination and degradation by the 26S proteasome. Moreover, asparagine hydroxylation by FIH-1 inhibits binding of the HIF $\alpha$  coactivator p300/CBP. In hypoxic conditions, both PHDs and FIH-1 are not active and non-hydroxylated HIF-1 $\alpha$  associates with HIF $\beta$  in nucleus. HIF $\alpha$ /HIF $\beta$  heterodimer binds to HRE sequence at the promoters of HIF-responsive genes and upon binding to the coactivators p300/CBP initiates their transcription

enzymes called prolyl hydroxylases (PHDs). They require molecular  $O_2$ , 2-oxoglutarate, iron ions ( $Fe^{2+}$ ), and ascorbic acid to be fully active, and they are inhibited in hypoxic conditions [11]. From three PHDs identified so far and shown to hydroxylate HIF-1 $\alpha$  in vitro (PHD1, 2, and 3) [12], in vivo PHD2 isoform plays a major role in normoxic HIF-1 $\alpha$  regulation [13, 14]. Hydroxylated HIF-1 $\alpha$  is recognized by the  $\beta$ -domain of von Hippel–Lindau tumor suppressor protein (pVHL) and is targeted for degradation by the 26S proteasome [15–17]. This process is preceded by the ubiquitination of the pVHL $\alpha$  domain by the elongin-C/elongin-B/cullin-2 E3-ubiquitin-ligase complex. The requirement of oxygen for PHDs functioning suggests that under hypoxic condition their activity is abrogated. In fact, when the oxygen level drops down, HIF- $\alpha$  subunit escapes recognition by the pVHL ubiquitin–ligase complex and proteasomal degradation and could be transported to the nucleus. There, after dimerization with HIF-1 $\beta$  [18], the active complex binds to HRE to induce transcription of numerous hypoxia-responsive genes (Fig. 8.3).

Importantly, there are also other mechanisms responsible for the controlling of the HIF-mediated transcription. To form an active HIF- $\alpha/\beta$  complex able to transactivate numerous genes, the binding of specific coactivators, like p300/CREB-binding protein (CBP) is necessary. Other than PHDs, oxygen-regulated hydroxylase-domain protein termed factor-inhibiting HIF-1 (FIH-1) regulates the binding of p300/CBP. FIH-1 modifies asparagine residue (Asn803 in human

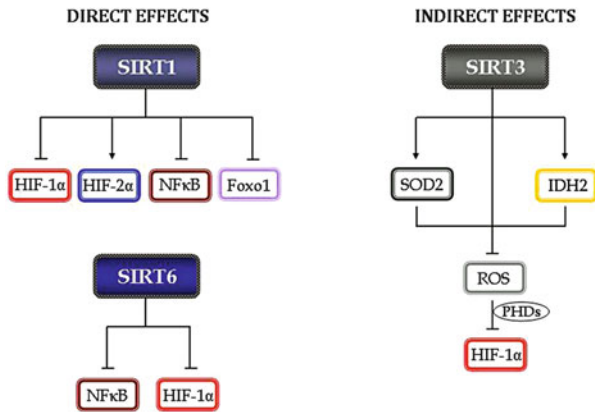
HIF-1 $\alpha$ , Asn851 in HIF-2 $\alpha$ ) in the C-TAD in the presence of oxygen, what leads to blockage of recruitment of p300/CBP coactivator to HIF- $\alpha$  [19]. The regulation of HIF- $\alpha$  by FIH-1 leads to the inhibition of interactions between HIF- $\alpha$  and transcriptional coactivators via sterical hindrance caused by Asn hydroxylation. Similarly to PHDs, FIH-1 activity is inhibited under hypoxic conditions, allowing the binding of p300/CBP to HIF-1/2 $\alpha$ , thus increasing HIF transactivation (Fig. 8.3).

### 8.2.2 Not Only Hydroxylation: ROS-Mediated HIFs Stabilization

In the past, reactive oxygen species (ROS) have been considered only as toxic by-products of metabolic processes. It is also believed now that abnormally high levels of ROS contribute to the oxidative stress-mediated damage leading to the development of many diseases. However, recent studies underline the importance of the low levels of ROS as a part of homeostatic signaling pathway. One of the ROS-regulated pathway is the HIF signaling.

A numerous studies have presented so-called ROS hypothesis and underlined the significance of ROS for HIF-1 $\alpha$  stabilization [20–22]. It was proposed that hypoxia-mediated production of superoxide at complex III of the mitochondrial electron transport chain and its conversion to H<sub>2</sub>O<sub>2</sub> by the superoxide dismutase (SOD) directly inhibits PHDs enzymes by oxidizing the essential nonheme-bound iron. However, recently Masson and colleagues have shown that PHDs have low sensitivity to inhibition by H<sub>2</sub>O<sub>2</sub>. In contrast, the other HIF-hydroxylated enzyme, FIH-1, was much more susceptible to H<sub>2</sub>O<sub>2</sub>-dependent inactivation [23]. These results suggest that molecular mechanisms responsible for the interaction between ROS and HIFs still have to be investigated.

On the other hand, there are other observations linking ROS and PHDs/HIF. Sirtuins are NAD-dependent deacetylases involved in metabolism, stress response, and longevity [24]. It was recently reported that the member of sirtuins family, SIRT3, destabilizes HIF-1 $\alpha$  by inhibiting ROS production and keeping PHDs in the active state, thereby leading to HIF-1 $\alpha$  degradation. In SIRT3 null cells, ROS level is increased and it contributed to increased HIF-1 $\alpha$  stabilization/activity and finally to increased glycolysis and cellular growth [25]. Additionally, Bell et al. [26] proved that SIRT3 modulates the progression of tumors in ROS- and HIF-dependent way. The absence of SIRT3 led to increased ROS level and to activation of pro-tumorigenic HIF-1 $\alpha$  in normoxia and its hyperactivation in hypoxia. After injection of HCT116 colorectal carcinoma cells with stable knockdown of SIRT3 to immunodeficient Nu/Nu mice, the growing tumors were bigger than tumors derived from control HCT116 cells. Additionally, when injected mice were subjected to potent antioxidant, N-acetylcysteine (NAC), the rate of tumor growth and its size was comparable to control conditions [26]. Moreover, there are other studies suggesting the effect of SIRT3 on ROS production. SIRT3 can directly target isocitrate dehydrogenase 2 (IDH2), a major source of NADPH in the mitochondria [27] and SOD2, activating mitochondrial ROS scavenging [28] and consequently influence cellular redox status and affect HIF signaling.



**Fig. 8.4** The regulation of angiogenic factors by sirtuins. SIRT1 and SIRT6 have direct, complex effect on several factors involved in the angiogenic response, as they inhibit pro-angiogenic HIF-1 $\alpha$ , NF $\kappa$ B, as well as anti-angiogenic Foxo1 with concomitant upregulation of HIF-2 $\alpha$ . SIRT3 indirectly, via inhibition of ROS, through several mechanisms including deacetylation of SOD2 and IDH2 proteins, activates PHDs leading to inhibition of HIF-1 $\alpha$

### 8.2.3 Not Only Hydroxylation: The Role of (De)acetylation in HIFs (De)stabilization

As mentioned above, SIRT3, a member of deacetylases family may regulate HIF stabilization through ROS- and PHD-dependent signaling. However, the action of SIRT3 and other members of sirtuins family may also rely on the direct deacetylation of HIFs protein (Fig. 8.4).

Interestingly, it was shown that specific members of sirtuins family may exert different effects on HIF transcription factors. Deacetylation of HIF-1 $\alpha$  at Lys674 by SIRT1 led to blocking p300 recruitment and consequently this repressed HIF-1 target genes [29]. On the other hand, the activity of HIF-2 $\alpha$  increases after deacetylation by the same nuclear SIRT1 [30]. Moreover, the expression of HIF-2 $\alpha$ -dependent genes, namely, VEGF, SOD2, and erythropoietin was also induced in response to SIRT1 action [30]. SIRT1 was also able to downregulate PHD2 protein level through deacetylase activity [31]. Importantly, SIRT1 is regulated by hypoxia in HIF-1 $\alpha$ - and HIF-2 $\alpha$ -dependent manner what suggests both positive and negative feedback loops between SIRT1 and HIFs [32]. Moreover, SIRT6 was also shown to be a negative regulator of HIF-1 $\alpha$  stability and protein as SIRT6-deficient cells exhibit increased HIF- $\alpha$  activity [33].

Additionally, the direct involvement of sirtuins in the controlling angiogenic response was underlined. SIRT1 was found to be highly expressed in the vasculature during blood vessel growth, where via deacetylation of the forkhead transcription factor Foxo1, a crucial negative regulator of blood vessel development inhibits its anti-angiogenic activity [34]. Moreover, the knockout of SIRT1 led to dysregulation of number of genes essential for vascular growth, maturation, and

remodeling resulting in the defective blood vessel formation and blunted ischemia-induced neovascularization [34].

Sirtuins represent class III histone deacetylases (HDACs) enzymes and also other members of HDACs have been reported to interact with and influence HIF-1 activity. HDAC4 was found to modulate HIF-1 $\alpha$  protein N-terminal lysine acetylation level, stability, and HIF-1 activity [35]. On the other hand, inhibitors of class I/II HDACs (HDACIs), like valproic acid or trichostatin A, have been shown to downregulate HIF signaling by either reducing functional HIF-1 $\alpha$  levels or repressing HIF- $\alpha$  transactivation activity (reviewed in [36]).

The aberrant expression of HDACs was implicated in the cancer development and the extensive number of HDAC inhibitors have been shown to exert the antitumor effects. The molecular mechanism of the observed action may involve HIFs regulation, however, as deacetylases play a role in numerous processes like transcriptional regulation, epigenetic programming, chromosomal remodeling and they regulate plethora of factors, HIFs modification represents only one possibility.

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### 8.3 The Cross Talk Between HIFs and NF $\kappa$ B: The Implications for Inflammatory Angiogenesis

Inflammatory reaction is not only characterized by the excessive expression of inflammatory cytokines like TNF- $\alpha$  or IL-1 $\beta$  but is also associated with reduced oxygen tension [37]. It was shown that various inflammatory stimuli like lipopolysaccharide (LPS) [38] or pro-inflammatory cytokines [39–41] are able to induce HIF system under normoxic conditions. Such situation may be especially important during tumor progression as inflammation can facilitate the development of cancer [42, 43]. The cross talk between HIFs and inflammatory machinery may lead to the upregulation of the expression of pro-angiogenic factors, resulting in the increased vascular leakage, tumor vascularization, and finally metastasis. Moreover, a growing body of evidence indicates the presence and activation of HIF signaling in various inflammatory diseases, like rheumatoid arthritis [44], asthma [45], or atherosclerosis [46].

The regulation of HIF-1 $\alpha$  by inflammatory cytokines was shown to occur both at the transcriptional and translational level. Especially important seems to be the interaction of HIFs and nuclear factor  $\kappa$ B (NF $\kappa$ B) as both transcription factors are induced by hypoxia and inflammation and influence each other. Importantly, not only hypoxia may regulate HIF-1 $\alpha$  mRNA level through NF $\kappa$ B [38, 47] but also in normoxic conditions NF $\kappa$ B is a direct modulator of HIF-1 $\alpha$  expression [48]. In fact, the functional NF $\kappa$ B response element is present in the HIF-1 $\alpha$  promoter at  $-197/-188$  base pairs upstream of the transcriptional start site [48]. First indication that an active NF $\kappa$ B-binding site is present in HIF-1 $\alpha$  promoter was shown in 2006, when Frede and colleagues studied the effect of LPS on HIF-1 $\alpha$  mRNA in human monocytes and macrophages [38]. Additionally, during hypoxic induction of HIF-1 $\alpha$  in pulmonary artery smooth muscle cells, the binding of the NF $\kappa$ B subunits p50 and p65 to the HIF-1 $\alpha$  promoter have been shown [47]. Finally, direct interaction



(although with different affinity) of all NF $\kappa$ B family members—p65, RelB, c-Rel, p52, and p50 with HIF-1 $\alpha$  gene/promoter at an NF $\kappa$ B consensus site has been reported, whereas a truncated version of the HIF-1 $\alpha$  promoter construct which lacks the NF $\kappa$ B site was not activated by the NF $\kappa$ B subunits [48].

Of note, the regulation of HIF by NF $\kappa$ B is evolutionary conserved—NF $\kappa$ B-binding element was found to be conserved across different species [47]. Moreover, recent work by van Uden et al. showed that even in *Drosophila*, NF $\kappa$ B regulates HIF-1 $\beta$  (tango) and HIF- $\alpha$  (sima) levels and activity both in normoxia and hypoxia [49].

As discussed earlier, sirtuins have been shown to associate, deacetylate, and regulate the activity of HIF-1 $\alpha$ . Similarly, NF $\kappa$ B acetylation status and transcriptional activity could be modified by sirtuins. Sirtuins, although with different mechanisms of action, act as a negative regulators of NF $\kappa$ B activity (reviewed in [50]) (Fig. 8.4).

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## 8.4 Nitric Oxide: Important Regulator of Hypoxia-Induced Angiogenesis

As discussed above, the activation of HIF transcription factors has been increasingly implicated in inflammatory diseases. A common hallmark of inflammatory disorders is the increased synthesis of nitric oxide (NO) by induction and activation of inducible nitric oxide synthase (iNOS). The implication of NO in the regulation of angiogenic mediators as well as HIF transcription factor has been suggested in a great number of papers.

In the past, several studies have shown the discrepant data concerning the effect of NO on HIF-1 $\alpha$  accumulation—both stabilization [51, 52] and destabilization [53, 54] have been reported. Such confusing results have been nicely explained by Mateo and colleagues who have shown that NO may exert biphasic effect on HIF- $\alpha$  stabilization, dependently on the concentration used. At low concentration, up to 400 nM diminishment in HIF-1 $\alpha$  stability was observed, whereas higher NO concentrations (above 1  $\mu$ M) increased HIF-1 $\alpha$  stability [55].

Moreover, the discrepant data about NO-mediated effects could be the result of various NO donors used in the experiments. Different factors which release NO are used, mostly SNAP (S-nitroso-*N*-acetylpenicillamine), SIN-1 (S-morpholinonydnonimine), GSNO (S-nitrosoglutathione), and DETA (diethylenetriamine NONOate). Unfortunately, sodium nitroprusside (SNP), a complex compound which releases NO together with iron ions and cyanides, was also used in many studies. SNP was shown to inhibit HIF-1 accumulation in human glioblastoma A-172 cells, whereas in the same experiment SNAP or GSNO increased HIF-1 $\alpha$  accumulation [56]. Similarly, in the human bladder cancer and in the human prostate cancer cell lines SNP decreased hypoxia-induced HIF-1 $\alpha$  protein level [57]. As mentioned earlier, SNP, after releasing NO, is converted to ferrocyanide and ferricyanide. Cyanides, inhibitors of cytochrome c oxidase, are cytotoxic molecules as well as iron which could be released from SNP is a

possible toxic factor generating highly reactive radicals, such as hydroxyl radicals via the Fenton reaction.

Importantly, NO-dependent accumulation of HIF- $\alpha$  might be a possible mechanism responsible for increase in HIF-dependent gene expression in tumors. Both increased NOS expression and NO level led to stabilization of HIF-1 $\alpha$  protein in human oral squamous cell carcinoma [58]. Similarly, in prostate cancer, Nanni and colleagues have identified the nuclear co-localization between endothelial NOS, estrogen receptor  $\beta$ , HIF-1 $\alpha$ , and HIF-2 $\alpha$  and they found that these proteins cooperate to activate transcription of protumorigenic genes [59].

The induction of HIF by NO may lead to the increased transcription of angiogenic gene expression, and nowadays it is well proven that NO is an important mediator of angiogenesis. We [60, 61] and others [62] have shown that NO is significant regulator of VEGF signaling both *in vitro* and *in vivo*. In rat vascular smooth muscle cells, IL-1 $\beta$ -induced NO production, NO donors, or plasmid delivery of NOS led to the upregulation of VEGF synthesis, which was abolished when inhibitor of NOS, like L-NAME was added [60, 61]. In rat ischemic hindlimb injected with eNOS cDNA increase in peripheral blood flow in ischemic tissue, better vascularization (increased number of CD31-positive cells in muscles), and increased VEGF level have been detected [62].

Similarly to discrepant data about HIF-1 accumulation, also there are results showing inhibition of VEGF expression after SNP treatment [56, 63] again indicating the importance of the appropriate NO donor usage. In our hands, direct comparison of SNAP, SIN-1, DETA, GSNO, and SNP on VEGF production in rat or human vascular smooth muscle cells showed that all donors, but not SNP, potently upregulate VEGF release [61, 64]. These results strongly indicate that NO increases VEGF level, but SNP cannot be used as a NO donor.

In summary, it seems that different effects of NO on HIF-1 or VEGF level described in the literature may be the result of various methodological approaches including different NO donors and various concentrations used.

The mechanism responsible for NO-mediated HIF modification relies mostly on S-nitrosylation of critical cysteine residues of several key proteins involved in HIF regulation, including HIF-1 $\alpha$  itself but not on cGMP involvement. For example, NO was shown to have inhibitory effect on PHDs 1–3 *in vitro*. In human embryonic kidney cells stimulated with exogenous NO donor (GSNO) dose dependently inhibition of PHD activity and reduction of HIF-1 $\alpha$  hydroxylation leading to its stabilization was observed [65]. Moreover, SNAP was shown to inhibit FIH-1 leading to enhanced HIF-1 $\alpha$  C-TAD activity. Additionally, NO may S-nitrosylate the VHL blocking its ubiquitination activity [66].

Interesting findings linking NO and mitochondrial respiratory chain were published recently [67]. The authors based on the information that NO could be produced in the mitochondria in the reaction catalyzed by cytochrome c oxidase (Cco/NO) [68]. To find if NO produced in mitochondria could be responsible for HIF- $\alpha$  stabilization, HEK 293.7 cells without NOS activity have been used. Interestingly, the cells used Cco/NO activity to generate NO and further for the stabilization of HIF-1 $\alpha$  [67].

## 8.5 microRNA and Hypoxia-Induced Angiogenesis

Identified in 1993 as small, noncoding RNA molecules which bind to the 3' UTRs of target mRNAs to negatively regulate gene expression [69], microRNAs have been shown to be implicated in many different processes including angiogenesis. To date, more than 1,500 human miRNAs have been reported (<http://www.mirbase.org>) and it is suggested that they could regulate more than one-third of the mRNAs produced.

In 2010, Chan and Loscalzo have introduced the term “hypoxamirs” to stress the existence of a number of hypoxia-regulated miRNAs [70]. Several HIF-related miRNAs have been identified till now, with the most prominent miR-210. Moreover, miR-20a, miR-20b, miR-199a, miR-424, miR-130a, miR-130b, and miR-155 have been shown to affect HIF expression, although it seems that only miR-20a (encoded by miR-17-92 cluster), miR-20b, and miR-199a directly target the 3'UTR of HIF-1 $\alpha$  [31, 71, 72].

From well-described hypoxamirs, miR-210 was shown to be regulated by both HIF-1 and HIF-2 [73] and is able to target several angiogenic factors. Overexpression of miR-210 in human umbilical vein endothelial cells increases the expression of VEGF and its receptor, VEGFR-2 leading to increased renal angiogenesis [74]. The receptor tyrosine kinase ligand Ephrin-A3, an important regulator of endothelial cells survival, migration, and differentiation is another example of angiogenic gene regulated by miR-210 [75]. Moreover, Notch signaling pathway was suggested to be activated by miR-210 in a model of cerebral ischemia [76].

Importantly, the overexpression of miR-210 has been detected in a variety of cardiovascular diseases and solid tumors. Particularly, its high level characterizes the renal clear cell carcinomas (RCCs), in which also HIF is overexpressed as a consequence of pVHL inactivation. Its important role in tumor development may be related to the fact that in response to hypoxia this miRNA regulates a wide spectrum of genes involved not only in angiogenesis but also in the mitochondrial metabolism, DNA repair, or cell survival.

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## 8.6 Hypoxia and Angiogenic Regulators

### 8.6.1 Regulation of VEGF by HIFs: A Classic Example of Pro-angiogenic Gene Regulation in Hypoxic Conditions

Angiogenesis is controlled through the equilibrium of pro- and anti-angiogenic factors. However, under special conditions, e.g., during tumor development, the level of pro-angiogenic factors increases and this facilitates the blood vessel formation.

HIFs regulate the expression of more than 150 genes, including several, involved in angiogenesis (Fig. 8.1), like VEGF, the major pro-angiogenic factor which is known to enhance proliferation, survival, and tube formation by endothelial cells.

The mechanism of VEGF regulation in hypoxic conditions is well known. It was shown already in 1995 that the promoter of the VEGF gene contains HRE and binding of HIF to this region increases the transcription of VEGF [77]. Moreover, the stability of VEGF mRNA, which is very low under normoxia is dramatically increased in hypoxia [78]. It is interesting, that whereas the average half-life of eukaryotic mRNAs is 10–12 h, the half-life of VEGF mRNA is less than 1 h, e.g., in PC12 rat pheochromocytoma cells it is about 40 min [79].

Post-transcriptional VEGF regulation may be controlled at least in part by RNA-binding proteins (RBPs) and miRNAs. The *VEGF* 3'UTR contains several important cis-acting elements including CA-rich element (CARE) or AU-rich element (ARE) and is regulated by its RBPs such as AUF1, tristetraprolin (TTP), heterogeneous nuclear ribonucleoprotein (hnRNP L), and HuR [80–83]. One of the recently discovered factor with strong binding preference for the VEGF mRNA 5'UTR is the DEAD-box RNA helicase DDX6 [84]. Some proteins like TTP and AUF destabilize VEGF mRNA in macrophages and tumor cells, respectively [81, 82]. In contrast, the hypoxia-induced inhibition of DDX6 positively affects VEGF expression [84].

The stabilization of labile VEGF mRNA relies largely on the activity of specific RNA-binding protein HuR, a member of the Elav family of proteins found in *Drosophila*. HuR binds to ARE in the VEGF 3'UTR, forming an RNA–protein complex in a hypoxia-inducible fashion [83]. Till now, the molecular details of how HuR regulates VEGF expression are not well understood. It was suggested that the mechanism of HuR action may be related to its protective effect against endonucleases as a 40 bp region adjacent to the HuR-binding site in the VEGF stability region has been identified to be susceptible to ribonucleases in the absence of HuR [85].

Data accumulated over the last years have highlighted the significant roles for miRNAs, small noncoding RNAs, for the regulation of VEGF signaling. Not only VEGF but also its receptors and components of the intracellular signaling pathway are controlled by different miRNAs, including miR-15, -16, -23, -27, -93, -200b, -221, or miR-424 (reviewed in [86]). Recently, numerous studies indicate also the cross talk between RBPs and miRNAs in the post-transcriptional regulation of gene expression, like the interplay between HuR and miRNAs which associate with the HuR-regulated mRNAs. Chang et al. indicated that HuR suppressed miR-200b expression and antagonizes its anti-angiogenic effects, leading to increase in VEGF level [87].

The transcriptional regulation of VEGF by HIFs can be modulated by other transcription factors. Interestingly, the complex regulation is achieved by the members of E2F transcription factors family. This family consists of eight members which may have the opposite effect on VEGF transcription. E2F7 and E2F8 were shown to stimulate angiogenesis via transcriptional induction of VEGF [88]. The key event in this regulation is the formation of an E2F7/8–HIF-1 $\alpha$  transcriptional complex that directly binds and stimulates the activity of VEGF promoter. The positive regulation of VEGF transcription by E2F7/8 is quite surprising as these factors are classified as repressors, and activation of VEGF transcription is unique mechanism. However, the significance of these factors for angiogenic process is

also provided by *in vivo* studies using E2F7<sup>-/-</sup>E2F8<sup>-/-</sup> mice. Deletion of the E2F7 and E2F8 genes causes serious vascular defects and lethality around embryonic day 10.5 [89]. In contrast, another member of E2F family, E2F1 negatively regulates hypoxia-induced VEGF expression [90]. In hypoxic conditions, E2F1 associates with p53 and downregulates VEGF expression exclusively, without affecting other hypoxia-inducible genes. Importantly, E2F1<sup>-/-</sup> mice display enhanced angiogenesis, endothelial cell proliferation, and reperfusion in a hindlimb ischemia model, resulting from enhanced VEGF expression [90].

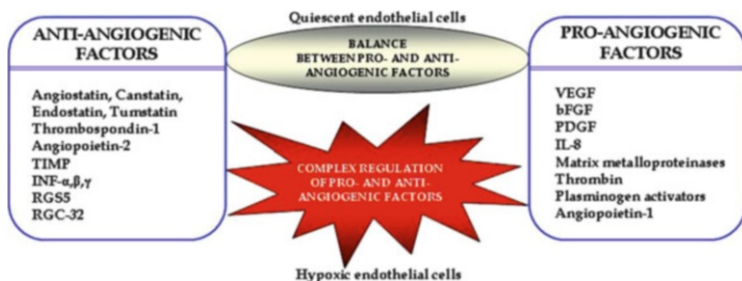
Additional transcription factors play important role in the hypoxic regulation of VEGF-driven angiogenesis. Related transcription enhancer factor-1 (RTEF-1) and early growth response 1 (EGR-1) can both target VEGF to enhance angiogenesis [91, 92]. RTEF-1 was shown to regulate HIF-1 $\alpha$  both in normoxic and hypoxic conditions and to increase capillary density and blood recovery after the hindlimb ischemia [93]. Not only VEGF (VEGF-A) is a target gene of RTEF-1. Several studies have shown that other genes involved in the regulation of vascular growth, like fibroblast growth factor receptor-1, FGFR-1 [94], or VEGF-B [95] are regulated by RTEF-1. Similarly, EGR-1 acts not only on VEGF. This transcription factor was suggested to be a master regulator of angiogenic, inflammatory, procoagulant, and permeability-related genes [92].

Finally, translation of VEGF mRNA is increased under low oxygen tension. It was shown that even in such unfavorable stress conditions like hypoxia, when cap-dependent initiation of translation is compromised, the synthesis of VEGF protein is maintained at high level. This is because VEGF mRNA contains the internal ribosomal entry site (IRES) in the 5'UTR sequence and when cap-dependent translation is suppressed under hypoxia this IRES-dependent translation predominates [96].

### 8.6.2 Complex Effect of Hypoxia on Angiogenesis Stimulators: Not Only Induction Is Observed

The hypoxia-induced upregulation of VEGF expression at mRNA and protein level was shown in the multiple cell lines, including cancer cells. However, although very important, VEGF is not the sole-acting angiogenesis factor. Among others, there are platelet-derived growth factor (PDGF), placental growth factor (PIGF), transforming growth factor- $\beta$  (TGF $\beta$ ), fibroblast growth factor (FGF), angiopoietins (Ang), matrix metalloproteinases (MMP), or interleukin-8 (IL-8) (Fig. 8.5).

A lot of studies have found the correlation between HIF expression and intratumoral microvascular density (reviewed in [97]). Since hypoxia is one of the major inducers of tumor vasculature, HIFs and VEGF were suggested to be the attractive targets for anticancer therapy. However, clinical data suggest that the benefit of anti-angiogenic therapies with anti-VEGF antibodies does not last long, as many patients encounter progression of cancers. This can be explained by the recurrence of tumor angiogenesis through the compensatory production of



**Fig. 8.5** Angiogenesis regulation—the balance between pro- and anti-angiogenic factors. The balance between pro- and antiangiogenic factors is important for the quiescent endothelial cells. Generally, the expression of proangiogenic factors increases whereas inhibitors of angiogenesis decreases in hypoxic conditions; however, recent reports suggest the complex regulation of factors regulating angiogenesis

mediators other than VEGF, indicating for the complex regulation of angiogenic factors in response to hypoxia.

In relation to the above observations we demonstrated that 24 h incubation of human microvascular endothelial cell line (HMEC-1) in the atmosphere of 1 % O<sub>2</sub> led to decrease of PIGF expression, the stimulator of blood vessel growth [98]. Moreover, we have also shown that the expression of another pro-angiogenic agent, IL-8 was downregulated by hypoxia/HIF-1 in endothelial cells [98, 99]. Importantly, in such conditions VEGF was induced, showing opposite effect of hypoxia conditions on the expression of different pro-angiogenic factors. Next we reported that in contrast to HIF-1, overexpression of HIF-2 isoform resulted in increased expression of IL-8 [100]. The mechanism of the opposite effect of HIF-1 and HIF-2 on IL-8 expression involves the recruitment of different transcription factors—IL-8 inhibition by HIF-1 is mediated via the down-regulation of the NF-E2-related factor 2 (Nrf2) transcription factor [99], whereas stimulatory effect of HIF-2 on IL-8 relies on the induction of Sp-1 [100]. Moreover, c-Myc transcription factor plays a crucial role in the regulation of HIF-1/HIF-2 mediated IL-8 expression. We have shown that HIF-1 $\alpha$  not only downregulates the level of c-Myc but concomitantly it also increases the production of Mxi-1, c-Myc antagonist. On the other hand, HIF-2 $\alpha$  increases c-Myc activity [100].

There are a growing number of studies underlying the complexity of the regulation of hypoxia-dependent angiogenesis regulators. Interestingly, in HIF-1-deficient colon cancer cells, the hypoxic induction of VEGF was only partially blocked, whereas NF $\kappa$ B-dependent increase in IL-8 expression was observed. What is more, HIF-1 inhibition did not influence vascularization of tumors—the microvessel density was identical in tumors that had wild-type or knock-down HIF-1 $\alpha$  [101]. The unexpected and surprising results have been recently presented by Yu and Hales [102]. In lung cancer tumor, long-term exposure to hypoxia repressed the growth of tumor and decreased microvessel density in the tumor tissues. Importantly, the same conditions led to acceleration of tumor progression and microvasculature in the colon cancer. The mechanism responsible for the opposite effect of hypoxia on angiogenesis and

tumorigenesis in different tumor types observed in this study was not investigated in details, however, the differences in the expression of  $\text{Na}^+-\text{K}^+$  ATPase, a versatile signal transducer, between these two tumor types was underlined. The inhibition of the sodium pump expression was observed in lung cancer tumors but not in colon cancer tumors. It is possible that the basal and hypoxia-induced expression of pro-angiogenic mediators was different in these two models, but it was not checked in this study. In another work [103], in the transgenic polyomavirus middle T breast cancer mouse model, the exposure of mice to acute cyclic hypoxia did not influence the primary tumor growth as well as lung metastasis. Interestingly, the number of CD31-positive cells in hypoxic tumors was lower than in control tumors.

Importantly, several studies tried to correlate HIF-1 expression with the survival rate of cancer patients. Although the general idea indicates that HIF-1 promotes angiogenic-dependent tumor progression, there are also studies presenting opposite results.

Volm and Koomagi have analyzed 96 paraffin-embedded sections obtained from patients with non-small cell lung carcinomas (NSCLCs) [104] and reported no relationship between HIF-1 $\alpha$  or HIF-1 $\beta$  and proliferation. On the other hand, significant correlation between HIF-1 expression and apoptotic markers was detected. Surprisingly, patients with HIF-positive carcinomas had significantly longer median survival times than patients with HIF-negative carcinomas. These interesting results prompted the authors to check the expression of oncogene and tumor suppressor products and proliferative, apoptotic, and angiogenic factors in 216 patients with long-term surviving NSCLCs patients. Again, from several factors differentially expressed between control and studied patients, high level of HIF-1 $\beta$  was observed in carcinomas of long-term survivors [105]. Finally, recent data suggest also that activation of HIFs due to the inhibition of PHD2, can paradoxically improve the effectiveness of antitumor therapy due to tumor vessel normalization, increasing the delivery of chemotherapeutics [106].

### 8.6.3 Hypoxia-Regulated Angiogenic Inhibitors

As mentioned before, the regulation of pro-angiogenic factors by hypoxia/HIFs is complicated and still not fully discovered process. Moreover, not only activators but also inhibitors of angiogenesis (Fig. 8.5) could be regulated in HIF-dependent manner. Generally, the results of different studies indicate the inhibitory effect of HIFs activation on the regulation of angiogenic inhibitors. Thrombospondin-1 (TSP-1), a glycoprotein with major roles in cellular adhesion and vascular smooth muscle proliferation and migration, was shown to be downregulated in hypoxic conditions in different *in vitro* and *in vivo* models [98, 107, 108]. In the human microvascular endothelial cells and pericytes, the expression of other potent anti-angiogenic factor, endostatin was shown to be inhibited by hypoxia [109]. Additionally, the expression of angiostatin was decreased in a swine model of neonatal hypoxia [110]. In contrast to the above studies, several work underline the importance of hypoxia-induced expression of anti-angiogenic and anti-apoptotic factors.

One of the newly recognized hypoxia-overexpressed angiogenic inhibitor is regulator of G protein signaling 5 (RGS5) [111]. The acceleration of RGS5 by hypoxia is HIF-1 dependent and occurs also after treatment with cobalt chloride (CoCl<sub>2</sub>), hypoxia-mimicking compound, stabilizing HIF as well as with prolyl hydroxylase inhibitors, dimethyloxalylglycine (DMOG), or ethyl-3,4-dihydroxybenzoate (DHB). Importantly, RGS5 attenuates growth and induces apoptosis of endothelial cells *in vitro* and hinders angiogenesis *in vivo* [111].

An and colleagues [112] reported hypoxic induction of response gene to complement-32 (RGC-32), important modulator of cell proliferation. Increase in RGC-32 expression is mediated by HIF-1, both at the transcriptional and posttranscriptional levels, and moreover, also VEGF stimulates RGC-32 expression. Notably, the overexpression of RGC-32 in endothelial cells inhibits cell proliferation and migration via downregulation of another major angiogenic protein, FGF-2. *In vivo*, anti-angiogenic effect of RGC-32 was tested in two different models: this factor suppressed SW480 melanoma tumor growth as well as attenuated the blood flow recovery after hindlimb ischemia. Additionally, the reduction of CD-31-positive endothelial cells was observed in both experimental models.

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### Conclusions

Although extensive studies on the identifying molecular mechanisms of hypoxia-regulated angiogenesis are performed in many laboratories worldwide, still much work remains to be done to discover fully the mechanisms of hypoxia/HIF-regulated expression of angiogenic factors and to transfer this knowledge to translational medicine. Based on this information, optimal HIF-acting therapeutic agents could be developed what will result in an improved clinical outcome.

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