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HIF-1: master and commander of the hypoxic world
A pharmacological approach to its regulation by siRNAs

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Abstract

The hypoxia-inducible factor-1 (HIF-1) is primarily involved in the sensing and adapting of cells to changes in the O_2 level, which is essential for their viability. It is important that this critical transcription factor be tightly regulated in order for cells to respond to a wide range of O_2 concentrations. HIF-1 regulation by post-translational modification is the central theme of the scenario of O_2 homeostasis. The alpha subunit of HIF-1 is the principal actor while the supporting actors (PHDs, FIH-1, ARD1, CITED2, p300 ...) all contribute to the complexity of the grand finale. It is well established that HIF-1 expression and activation correlates with tumor progression and resistance to cancer treatments. We will introduce the different actors involved in HIF-1 regulation, and their mechanisms of action via invalidation by siRNAs and discuss therapies targeting HIF-1, to selectively kill tumor cells that adapt to low O_2 concentrations.

Keywords: Hypoxia; Angiogenesis; Hydroxylases; Factor inhibiting HIF-1; Prolyl hydroxylase domain protein; Hypoxia-inducible factor-1

1. Introduction

The hypoxia-inducible factor-1 (HIF-1), the most important factor involved in the cellular response to hypoxia, a local tissue decrease in the oxygen concentration, has been extensively studied this last decade. While the implication of HIF-1 in angiogenesis, especially in cancer progression, has been widely studied increasing interest in its role in other cellular events is developing. These include cell survival, apoptosis, cell motility, cytoskeletal structure, cell adhesion, erythropoiesis, vasculature tone, transcriptional regulation, epithelial homeostasis, drug resistance, nucleotide-, amino acid-, iron-, glucose-, energy-metabolism and finally pH regulation [1]. The broad impact of HIF-1 on cell biology is reflected in the total number of hypoxic target genes estimated to be approximately 1–2% of all human genes; over 70 genes have so far been identified as being regulated by HIF-1.

Much of what has been learned about HIF-1 comes from studies on erythropoietin synthesis, angiogenesis and the impact of HIF-1 on tumor progression. Knockout mouse models for HIF-1α [2,3], tumor xenografts [4,5], and human cancer biopsies [3–6] have rapidly contributed to a better understanding of the regulation and the biology of HIF-1. However, despite the substantial number of investigations into HIF-1, many secrets about its function remain to be revealed. The complexity of the mechanism of action of this major transcription factor will maintain the scientific community on constant alert for many years to come. In this review we will focus on topics of particular interest to our laboratory.

2. Hypoxia-inducible factor-1 (HIF-1)

HIF-1 is a heterodimer of two basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain proteins;

Abbreviations: ARD1, arrest defective-1 protein; CITED 2, CBP/p300 interacting transactivator with ED-rich tail 2; FIH, factor inhibiting HIF-1; HIF-1, hypoxia-inducible factor-1; MAPK, mitogen-activated protein kinase; PHD, prolyl hydroxylase domain protein; SUMO, small ubiquitin-like modifier; VHL, von Hippel-Lindau

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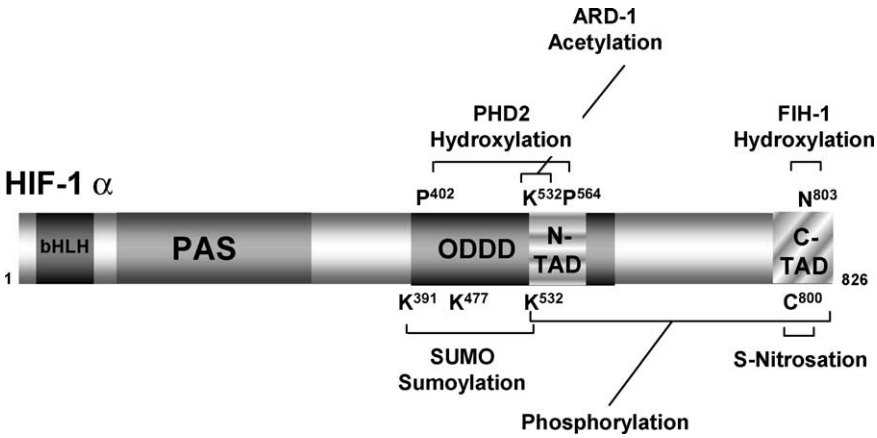

Fig. 1. Domain structure of HIF-1α and targeted residues involved in its regulation. HIF-1α possesses bHLH and PAS domains which are involved in dimerization with HIF-1β and DNA binding. The HIF-1α subunit contains two TAD, the N- and the C- TADs. The N-TAD lies within the ODDD. The ODDD regulates the stability of HIF-1α via recognition, by the von Hippel-Lindau E3 ubiquitin ligase (pVHL), of the hydroxylation state of Pro^{402} and/or Pro^{564} residues. This hydroxylation is catalyzed by the enzyme PHD (prolyl hydroxylase domain protein). Acetylation of the Lys^{532} residue by the ARD1 acetyltransferase also favors interaction with pVHL. The hydroxylation state of the Asn^{803} residue, by the enzyme FIH-1 (factor inhibiting HIF-1) inhibits binding of p300/CBP, a HIF-1α co-activator. *S*-nitrosation of Cys800, in the C-TAD, also promotes HIF-1 transcriptional activity. Three consensus sequences at Lys-^{391}, -^{477}, and -^{532} may be modified by SUMO [52]. The domain from 531 to 826 has been shown to be phosphorylated.

HIF-(1, 2 and 3)α the highly regulated subunit, and HIF-1β the constitutive subunit also called aryl hydrocarbon receptor nuclear translocator (ARNT). ARNT2 and ARNT3 could also be implicated in the formation of different putative dimers with HIF-1/2/3α increasing adaptability to reduced oxygen availability. The N-terminal half of HIF-1α contains bHLH and PAS domains that are required for dimerization and DNA binding. The C-terminal half contains domains required for degradation and transactivation: the oxygen-dependent degradation domain (ODDD) which confers oxygen-dependent instability, two independent transactivation domains (N-TAD and C-TAD) and in between, an inhibitory domain (ID) that negatively regulates the transactivation domains [7] (Fig. 1).

A number of splice variants of HIF-1α have been detected and one is lacking 127 base pairs corresponding to exon 14 of human HIF-1 [8]. The lack of this exon produces a frame shift resulting in the generation of a stop codon that corresponds to amino acid 736 in the protein product. Consequently, the entire HIF-1α protein structure is conserved except for the C-TAD. This variant can still dimerize with HIF-1β (ARNT), bind to the hypoxia-responsive element (HRE: 5′-RCGTG-3′) and transactivate genes but with less potency. The role of this alternative splicing remains unclear and is still under investigation.

HIF-1-dependent target gene expression is tightly regulated by at least two separate oxygen-dependent mechanisms: direct hydroxylation of proline residues by prolyl hydroxylases triggers the degradation of HIF-1α whereas asparagine hydroxylation inactivates the C-TAD of HIF-1α. However, other mechanisms appear to also play a critical role in the fine-tuning of stability and transactivation. In the following paragraphs, the characteristics and functions of the different players that regulate HIF-1α stability and activity, by its post-translational modification, will be presented. Subsequently, the role of the different actors in this complex, intertwined scenario of HIF-1 regulation will be described and discussed.

3. Definition of the different actors involved in regulation of HIF-1α stability

3.1. PHDs

Three prolyl hydroxylases namely prolyl-4-hydroxylase-domain proteins (PHD1, PHD2, PHD3) have been characterized recently [9]. These three homologues were originally designated, respectively, EGLN2,1,3 on the basis of protein sequence homology to EGL-9, the HIF-1 prolyl hydroxylase of *Caenorhabditis elegans* [10]. These iron-dependent enzymes convert proline into hydroxyproline, a reaction that requires oxygen, 2-oxoglutarate and ascorbate. Two proline residues in human HIF-1α, Pro^{402} and Pro^{564}, have been shown to be hydroxylated and are part of a conserved consensus sequence LXXLAP [10]. The activity of these enzymes is governed by the O_2 concentration within the cell, which defines these proteins as oxygen sensors. The requirement for iron as a cofactor explains the observed hypoxia-mimetic effects of iron chelators such as desferrioxamine (DFO) and iron antagonists such as cobalt chloride ($CoCl_2$). Besides controlling the activity of the PHDs, the O_2 concentration also controls the expression of PHD2 and PHD3 mRNAs [10–12]. Thus, in a manner similar to p53, HIF-1α governs its own stability by controlling the expression of PHD2 [11,13]. Metzen et al. [12] have examined the intracellular localization of different GFP-fusion, exogenously expressed, isoforms: PHD1 is specifically localized in the nucleus,

PHD2 is mainly localized to the cytoplasm and PHD3 seems to have no preference.

3.2. pVHL

Development of the von Hippel-Lindau syndrome, a rare autosomal dominant condition, is due to mutations in the tumor suppressor von Hippel-Lindau (*vhl*) gene [14]. This syndrome is characterized by the presence of specific benign and malignant tumors, in particular in clear-cell renal carcinomas and cerebellar hemangioblastomas, highly vascularized tumors that overproduce angiogenic peptides including VEGF [15]. The protein product of the von Hippel-Lindau gene (pVHL) is the recognition component of an E3 ubiquitin–protein ligase complex that targets proteins for ubiquitin-dependent proteolysis [16]. Furthermore, pVHL has been shown to polyubiquitinate the proline hydroxylated forms of HIF-1α [17,18]. Multiple mutations leading to pVHL inactivation result in the accumulation of HIF-1α in normally oxygenated cells [19]. pVHL localizes predominantly to the cytoplasm but undergoes nuclear-cytoplasmic shuttling [20]. The exact cellular localization of pVHL binding to HIF-1α and its subsequent ubiquitation is still not clear. One study suggested that it is nuclear [21] while another demonstrated that HIF-1α is degraded equally well by cytoplasmic and nuclear proteasomes [22]. pVHL has also been shown to recruit histone deacetylase (HDAC) and KREB-A domain protein both of which may influence HIF-1 transcriptional activity [23,24]. The oncogenic E3 ubiquitin ligase murine double minute 2 (MDM2) has also been reported to bring about ubiquitination of HIF-1α but in a p53-dependent fashion [25]. In fact p53 has been shown to interact with HIF-1α but direct interaction has been recently questioned and may occur indirectly via MDM2 [26].

3.3. ARD1

The arrest-defective-1 protein, a member of a large and diverse super-family of acetyltransferases that includes histone acetyltransferases [27,28], was first discovered in yeast and found to regulate cell cycling [29]. The acetylation of HIF-1α is the most recently characterized post-translational modification of the protein to be reported [30]. Acetylation by ARD1 of lysine^{532} located in the ODDD domain, close to hydroxylproline^{564}, appears to favor recruitment of pVHL and thus degradation by the proteasomal system [30]. Thus, ARD1 like PHDs will act as a negative regulator of HIF-1α by making it less stable. Since *N*-acetyltransferase activity is not known to be dependent on oxygen it could be expected that the modification occurs under both hypoxic and normoxic conditions. However, the ARD1 mRNA level was found to decrease slightly under hypoxic conditions, or in the presence of cobalt chloride, thus the level of acetylation of HIF-1α may decrease as the length of hypoxic exposure increases. Further investigations are required to define more precisely the normoxic versus hypoxic regulation of ARD1 activity. The ARD1 protein was detected in the cytosol indicating that ARD1 may preferentially act on HIF-1α in the cytosol [30].

3.4. NO

Nitric oxide has also been shown to decrease HIF-1α stability [31,32] though conflicting results that indicate the contrary have been reported [33]. Decreased stability was shown to result from an increase in prolyl-4-hydroxylase-dependent degradation in response to a redistribution of intracellular oxygen and thus increased availability of PHDs for oxygen [34].

4. Definition of the different actors controlling HIF-1α activity

4.1. FIH-1

First identified as factor inhibiting HIF-1 [24], FIH-1 has also been characterized more recently as an oxygen sensor involved in HIF-1α regulation [35]. FIH-1 possesses an asparaginyl hydroxylase activity that by targeting asparagine^{803} represses HIF-1α transactivation by preventing binding of the transcriptional co-activator p300/CBP to the HIF-1α C-TAD. Contrary to the PHDs, a consensus sequence for asparagine hydroxylation has not been defined but the sequence around the human asparagine^{803} is highly conserved between species. However, recently, Linke et al. [36] have demonstrated the crucial role of the adjacent residue valine^{802} in targeting asparagine^{803} hydroxylation. This Val-Asn motif seems to be primordial in maintaining the interaction between p300/CBP and the HIF-1α-C-TAD. FIH-1 does not influence HIF-1α stability but like the PHDs, is a member of the 2-oxoglutarate and iron-dependent dioxygenase family [37]. Like PHD1, FIH-1 transcription is totally independent of the oxygen concentration and seems to be constitutively expressed in all cell types so far studied [12]. Interestingly, Mahon et al. [24] reported the in vitro interaction of FIH-1 and pVHL with histone deacetylases. This recruitment could help FIH-1 modulate HIF-1α transactivation under normoxic conditions. FIH-1 appears to be mainly located in the cytoplasm, even under hypoxic conditions [12,36].

4.2. p300/CBP and other co-activators

Nuclear p300 and CBP are homologous and ubiquitously expressed proteins which possess histone acetyltransferase activity [28]. They function as transcriptional co-activators and are involved in multiple events which are central to cell growth, differentiation, transformation and apoptosis [38]. p300 binds to the C-TAD of HIF-1α through its cysteine-histidine rich domain (CH1) and increases transactivation probably by increasing histone

acetylation resulting in local remodeling of chromatin structure giving greater access of transcription factors to DNA [28]. Inhibition of the interaction of p300/CBP with HIF-1α strongly abrogates hypoxia-induced gene expression [39]. Moreover, Carrero et al. [40] showed that p300/CBP enhanced HIF-1α transactivation through both the N-TAD and C-TAD transactivation domains. Other histone acetyltransferases steroid-receptor co-activator-1 (SRC-1) and transcription intermediary factor 2 (TIF2) also interact with HIF-1α and enhance activity in a synergistic manner with p300 [40]. These authors also showed that the redox regulator protein Ref-1 potentiated transactivation. In addition, *S*-nitrosation on cysteine^{800} of HIF-1α has also been reported to increase interaction of HIF-1α with its co-activator p300 and thus increase its transactivational activity [41]. In accordance with its participation in the function of the transcriptional machinery, p300 is located in the nucleus [38].

4.3. CITED 2

CBP/p300 interacting transactivator with ED-rich tail 2, also called p35srj or MRG1, is a ubiquitously expressed nuclear protein. CITED2 came into the field of HIF-1α regulation as a negative regulator [42]. The CITED2 TAD interacts with the CH1 domain of p300/CBP with very high affinity and colocalizes in cells with p300/CBP [43]. Therefore, there exists active competition between CITED2 and HIF-1α for the same domain of p300. However, Freedman et al. [44] showed that CITED2 binds p300 with a 33-fold greater affinity than HIF-1α̇. Furthermore, it has been demonstrated that CITED2 expression is activated under hypoxic conditions through a HRE in its promoter [42]. Taken altogether, these results suggest possible negative feedback of HIF-1 transcription activity [44]. With such a level of competition for binding to p300, how is it that HIF-1 is still transactivated? The low abundance of CITED2 compared to p300/CBP, which is also sequested by binding to other transcription factors may be one explanation [45]. The balance between CITED2 and p300/CBP seems to imply that a very fine level of regulation is at play and an imbalance would have direct repercussions on HIF-1α transactivation.

4.4. MAPK

Richard et al. [46] demonstrated that, HIF-1α stabilized under hypoxic conditions is phosphorylated and that p42/44 mitogen activated protein kinase (MAPK) was capable of phosphorylating HIF-1α in vitro and in vivo. In addition, Volmat et al. [47] showed in vivo that inhibition of MAP kinase phosphatases, that maintain active ERKs in the nucleus, induces complete phosphorylation of HIF-1α. Furthermore, MAPK activation was associated with increased transcriptional activity of HIF-1 [46]. However, Sang et al. [48] showed recently that the direct phosphorylation of HIF-1α by MAPK was not correlated to its transcriptional activity but indicate that p300 was involved in MAPK signaling to HIF-1 by improving the physical interaction between p300 and the HIF-1α C-TAD.

4.5. SUMO

Small ubiquitin-related modifier, a member of the ubiquitin-like protein family shows 3D structure similarity to ubiquitin but only 18% sequence identity [49]. SUMO, a relatively small protein (about 12 kDa) is a member of a family made up of two groups, SUMO-1, -2 and -3, that can be covalently linked to proteins in an ATP-dependent manner. SUMO-1 possesses only about 50% identity compared to SUMO-2 and -3 but the latter are very similar, with 97% identity. The cascade for SUMO modification (sumoylation) is very similar to that for ubiquitin. In mammalian cells SUMO is first activated by an E1 heterodimer and transferred to an E2-conjugating Ubc9 enzyme. Three SUMO E3 ligases, ran binding protein 2 (RanBP2), protein inhibitor of activated STAT (PIAS) and Polycomb 2 (Pc2) have been identified and are thought to provide substrate specificity. As for ubiquitination the amino acid for SUMO modification is also lysine and a consensus sequence comprising the sequence ψKXE where ψ is a large hydrophobic residue (I, V, L, F), has been identified [49]. Unlike ubiquitin, SUMO does not signal proteins for degradation and may even counteract ubiquitin conjugation. Sumoylation regulates protein localisation, in particular within the nucleus, DNA repair and chromatin structure [50]. SUMO post-translational modification has also been shown to influence either negatively or positively (but more often negatively) the activity of transcription factors such as p53, heat shock transcption factor, c-Myb, GRIP1, Sp3 and AP-2 [50]. Interestingly the β subunit of HIF was shown to be sumoylated and to influence negatively its transactivation [51]. Our laboratory has preliminary evidence to suggest that the α subunit of HIF-1 is also modified by SUMO [52]. Indeed HIF-1α possesses three consensus sequences containing K^{391}, K^{477} and K^{532}. The consensus sequences are relatively conserved among species, though not for *C. elegans* or *D. melanogaster*. Two of the consensus sequences (K^{391} and K^{532}) are also conserved in HIF-2α but none are conserved in HIF-3α. Our preliminary in vitro data suggest that the SUMO E3 ligase RanBP2, which is localized in cells on the cytoplasmic face of the nuclear pore, promotes HIF-1α SUMO modification.

So the major actors involved in HIF-1α stability and activation have now been described, but under what conditions, normoxic or hypoxic (Fig. 2), do they act?

5. Normoxic regulation of HIF-1 function

The most significant recent advance into understanding the mechanism of HIF-1 regulation came from the

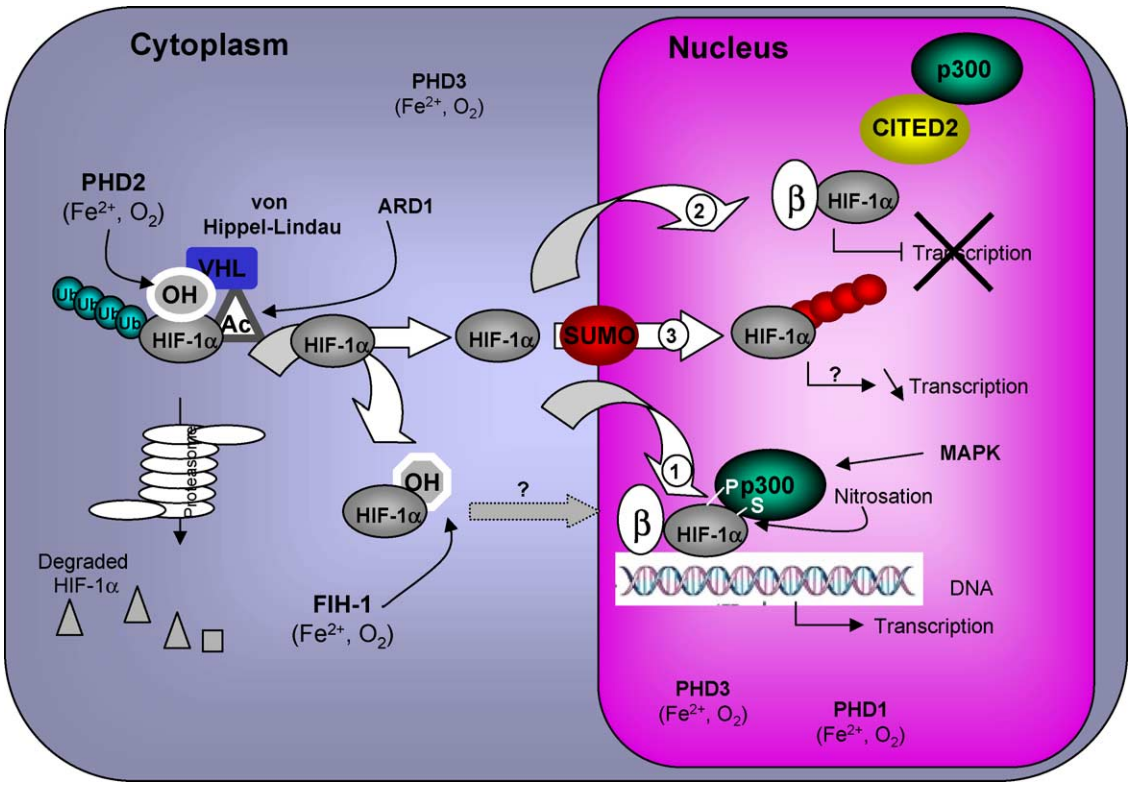

Fig. 2. Regulation of the stability/degradation and transactivation of HIF-1α. *Cytoplasm* (*under normoxic conditions*): at normal oxygen levels (normoxia), but also when HIF-1α escapes PHDs and/or FIH-1 hydroxylation (?), HIF-1α is located in the cytoplasm. The enzyme prolyl-4-hydroxylase 2 (PHD2), which requires O_2, Fe^{2+}, ascorbate and 2-oxoglutarate for activity, hydroxylates Pro^{402} and Pro^{564} on HIF-1α. This hydroxylation state is required for binding of the von Hippel-Lindau (pVHL) tumor suppressor protein, a component of an E3 ubiquitin–protein ligase predominantly located in the cytoplasm, which will then targets HIF-1α for ubiquitin-dependent proteolysis. pVHL binding is also promoted by acetylation of Lys^{532} by the ARD1 acetyltransferase. Another hydroxylase, factor inhibiting HIF-1 (FIH-1) provides a second check point for any HIF-1α that escapes degradation. FIH blocks the binding of p300/CBP and therefore inhibits HIF-1 transactivation. *Nucleus* (*under hypoxic conditions*): (1) HIF-1α is located in the nucleus allowing transcriptional activation of HIF-1 target genes. MAPK signaling will then improve the physical interaction between p300 and the HIF-1α C-TAD as does *S*-nitrosation of Cys^{800}. (2) However, CITED2 which binds p300 with higher affinity than HIF-1α negatively regulates HIF-1 transcriptional activity. (3) SUMO modifications of HIF-1α has been shown in vitro, as shown for HIF-1β, SUMO could act negatively on HIF-1 transactivation.

discovery that an enzymatic system capable of sensing changes in oxygen availability, namely the 2-oxoglutarate and iron-dependent dioxygenases, which are involved in governing the instability of HIF-1α in normoxia [10,53]. These enzymes, real oxygen sensors, were characterized as HIF prolyl hydroxylases domain proteins (PHDs). Under normoxic conditions, the HIF-1α ODDD is the critical target for HIF-1α hydroxylation. In fact, hydroxylation of the $proline^{402}$ and/or $proline^{564}$ of human HIF-1α, in response to PHDs action, provides a recognition motif for pVHL binding. Both modified prolines are contained within the conserved LXXLAP motif, also present in HIF-2α and HIF-3α [54], that can be independently recognized by the PHDs with differential efficacy of interaction between the PHDs [55]. Whereas interaction with the $proline^{564}$ site is promoted by all three enzymes, this site is only modified by two of the isoforms (PHD1 and PHD2) [10]. Recently, Berra et al. [11] demonstrated, by exploiting the small-interfering RNA (siRNA) approach, that specific silencing of PHD2 was sufficient to stabilize HIF-1α levels under normoxic conditions. Expression of HIF-1α, in normoxia, is inversely proportional to the amount of PHD2 (Fig. 3). Indeed, specific ablation of PHD2 by siRNA stabilizes HIF-1α protein to levels higher than that obtained with a 4-h hypoxic stress (1–2% O_2) (Fig. 3). Since these results were observed in all human cells analyzed, Berra et al. [11] concluded that PHD2 is the key limiting oxygen sensor controlling steady-state levels of HIF-1α in normoxia. These findings are in agreement with the study of Huang et al. [56] showing that, in vitro and among the 3 PHDs, PHD2 displays the highest activity for HIF-1α hydroxylation. Therefore, the question of the physiological role of PHD1 and PHD3 remains open. Our preliminary results suggest that these two enzymes become activated during a long hypoxic stress [11] (Ginouvès, Pouysségur and Berra, in preparation). More interestingly, PHD2 depletion in normoxia, not only stabilizes HIF-1α but induces its translocation to the nucleus, where it can activate a HRE-dependent reporter gene [11] or endogenous genes such as IGFBP3 and carbonic anhydrase 9 (CAIX) (Mazure, Dayan, Berra and Pouysségur, in preparation). However, PHD2 silencing was not sufficient to reach the levels of transactivation observed under hypoxic conditions [11] (Fig. 4). Since the C-TAD is considered to

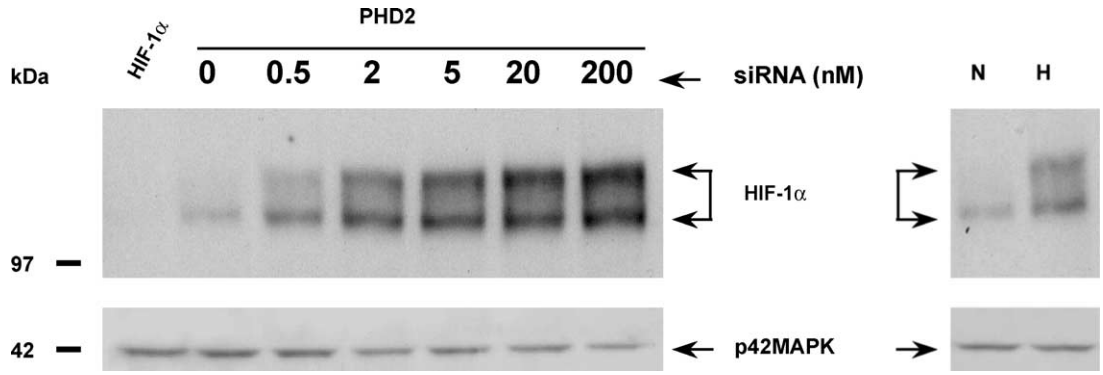

Fig. 3. Small-interfering RNA to PHD2 up-regulate HIF-1α in a dose-dependent manner under normoxic conditions, from [11]. HIF-1α expression was analyzed by Western blotting after transfection of HeLa cells with increasing doses of siRNA directed against PHD2. Total extracts of cells incubated in normoxia (N; 20% O_2) or hypoxia (H; 1–2% O_2) were used as a control for HIF-1 expression. Detection of p42/p44MAPK was used as a control for total protein loading.

be blocked under normoxic conditions by FIH-1 hydroxylation this result suggests that the observed transactivation was the result of N-TAD activation. This result also suggests that the difference in HIF-1-dependent activation between PHD2 depletion and hypoxia represents the contribution of the activation of the C-TAD.

Hydroxylation is not the only post-translational modification that induces HIF-1α destabilization. ARD1 the HIF-1α acetyltransferase acetylates HIF-1α that has escaped PHD2 hydroxylation and subsequently stimulates pVHL-mediated ubiquitination of HIF-1α [30].

FIH-1, through its ability to hydroxylate the HIF-1α C-TAD at asparagine^{803} abolishes interaction with its co-activator p300/CBP [35]. We postulate that if HIF-1α escapes the degradation process mediated by the PHD2/VHL/proteasome pathway, hydroxylation of Asn^{803} by FIH-1 should abolish residual transactivation of HIF-1 in normoxia. To test this hypothesis we invalidated FIH-1 in normoxia. Interestingly, we obtained a five- to six-fold increase in HIF-1-mediated HRE reporter activity (Fig. 4; Mazure, Dayan, Berra and Pouysségur, in preparation). This simple experiment, conducted in HeLa cells, indicates that indeed, in normoxia, FIH-1 exerts a second level of negative control on the residual HIF-1α that has escaped proteasomal degradation. This finding is in agreement with reports describing invalidation of HIF-1α [57–59].

Several observations suggest that endogenous nitric oxide formation provokes HIF-1α stabilization, HIF-1 DNA-binding and activation of downstream target gene expression [33]. However, paradoxical results have been obtained in different types of cells [60]. This is supported by the recent study of Wang et al. [61] who proposed that

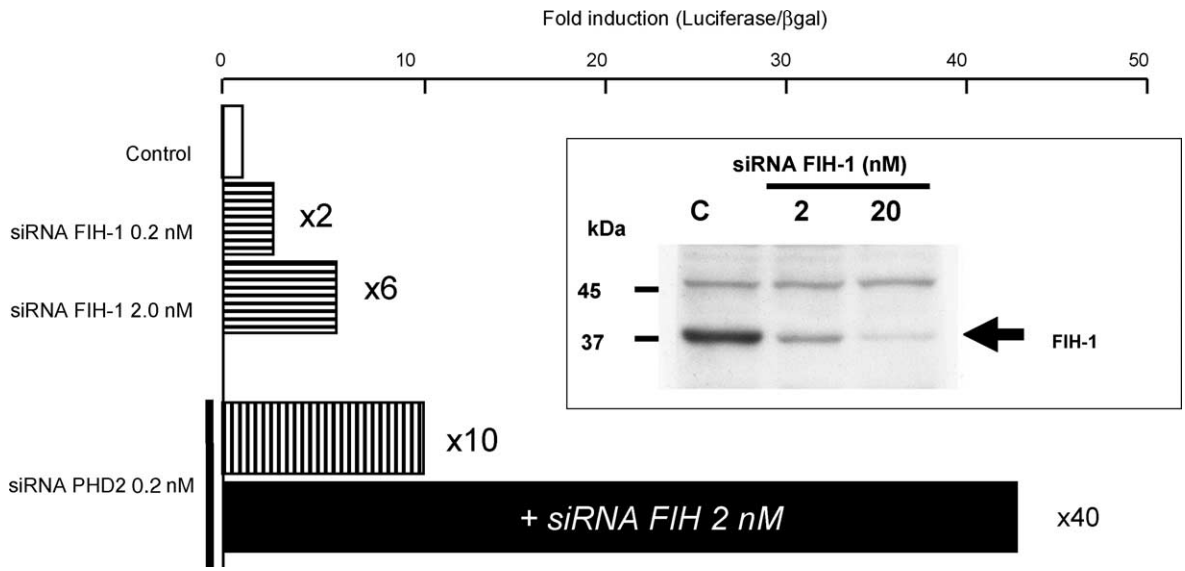

Fig. 4. Small-interfering RNA targeting PHD2 and FIH-1 synergize in increasing the transcriptional activity of HIF-1α under normoxic conditions. Specific silencing of FIH-1 using siRNA. Western blot showing proteins from HeLa cells after transfection with the indicated concentrations of siRNA (2 and 20 nM). HeLa cells were transfected with the indicated siRNA (0.2 and 2 nM for FIH-1 −0.2 nM for PHD2) together with the pHRE-δptk-LUC reporter vector that contains three copies of the HRE from the erythropoïetin gene.

NO could directly affect HIF-prolyl hydroxylase. Furthermore, Sumbayev et al. [33] showed that *S*-nitrosation is yet another form of post-translational modification to affect HIF-1α. *S*-nitrosation of Cys^{800} of HIF-1α was also shown to be involved in the increase of HIF-1 transcriptional activity via stimulation of recruitment of the p300 co-activator protein [41].

Finally, phosphorylation, another form of post-translational modification of HIF-1α has been shown to influence its activity and occurs under normoxic conditions as observed when HIF-1α is overexpressed but also under hypoxic conditions [46].

6. Hypoxic regulation of HIF-1 function

Since one of the most important post-translational modifications of HIF-1α is dependent on oxygen levels, reduced cellular O_2 tension, or hypoxia, which occurs in a number of physiological and pathological situations, will modify HIF-1α function. Cells not only perceive radical modulation in the O_2 concentration but also subtle changes. PHDs and FIH-1 have been characterized as effective oxygen sensors [10,35], though they do not have the same K_m values for O_2 and inhibition by 2-oxoglutarate analogs [54,62]. Koivunen et al. [62] reported that the K_m of FIH-1 for 2-oxoglutarate and for O_2 are systematically lower than that of the HIF prolyl-4-hydroxylases. Interestingly, these data suggest that the hypoxic nutritional stress that affects the availability of O_2 and/or glucose will first affect the activities of the PHDs and that a larger decrease would be required to reduce FIH-1 activity. Thus, in response to a minor decline in the O_2 concentration, HIF-1α levels will increase but with maintenance of the inhibition of transactivation by FIH-1. A further decrease in the O_2 concentration would be required to reduce FIH-1 activity and allow maximal p300 binding and full stimulation of HIF-1 activity. These results confirm the role of FIH-1 as a second control point in HIF-1α regulation but also as a modulator in HIF-1-dependent target gene expression. Under severe hypoxic conditions, both PHDs and FIH-1 activities would be repressed and consequently hydroxylation of Pro^{402}, Pro^{564} and Asn^{803} in human HIF-1α would be blocked. Interaction of HIF-1α with pVHL will not occur and HIF-1α will escape degradation by the proteasome. This in turn results in nuclear accumulation of HIF-1α and allows its dimerization with HIF-1β. Finally, HIF-1 recognizes specific HRE in target genes and will recruit p300/CBP via the unmasked HIF-1α C-TAD. In conclusion, a decrease in the O_2 gradient in tissues will induce an opposite gradient of HIF-1α and therefore a HIF-1 gradient with partial transcriptional activity. This activity will be progressively revealed with the concomitent and progressive inactivation of FIH-1.

In addition, the protein Jab1, a transcriptional co-activator of c-Jun and Jun D, has been shown to interact with HIF-1α and may compete with p53 and thereby increase HIF-1α stability under hypoxic conditions [63]. The molecular chaperone heat shock protein Hsp90 interacts with both HIF-1α and β and has been suggested to protect HIF-1α from pVHL-independent proteosomal degradation [64]. In addition, HIF-1β promotes HIF-1α stability possibly by competing with Hsp90 for binding [65].

Even under low levels of O_2, HIF-1α stability may be compromised when in the presence of NO, which has been shown to destabilize hypoxia-induced HIF-1α by activating PHDs, [61]. In addition, Hagen et al. [34] confirmed recently that the destabilization of HIF-1α via NO and other inhibitors of mitochondrial respiration in hypoxia is dependent on the PHD activity where NO probably increased the availability of non-respiratory O_2 to the enzyme.

Acetylation of HIF-1α at $lysine^{532}$, by ARD1 has been reported to contribute to the down-regulation of the protein level of HIF-1α under both normoxic and hypoxic conditions since transfection with an antisense ARD1 resulted in stabilization of the protein [30]. However, it was also reported that the mRNA levels of ARD1 decreased under hypoxic conditions thereby suggesting that lower amounts of ARD1 would be available for acetylation of HIF-1α in hypoxia.

Stabilized HIF-1α can undergo further activation of its transactivation function. Phosphorylation via MAPK was reported to enhance transactivation [46]. However, it appears that MAPK signaling facilitates HIF-1 activation through increased transcriptional activity of p300 possibly via phosphorylation of p300 transactivation domain, under normoxic and hypoxic conditions, and that this interaction does not require HIF-1α C-TAD phosphorylation [48].

7. Conclusion

If p53 is recognized as the guardian of the genome, HIF-1 could be considered as the master and commander of the hypoxic world, the guardian against nutritional stress. HIF-1α shows substantial molecular complexity where its regulation is tightly controlled through post-translational mechanisms, in particular hydroxylation. The presence or absence of this modification under, respectively, atmospheric (21% O_2) conditions and drastic hypoxia that correlate with instability/inactivation and stability/activation are well defined. However, the intermediary situation of partial normoxic-hypoxic conditions that for sure prevails in the whole organism is less well understood. The multiple control points that regulate HIF-1α may depend on the cellular environment at a particular time within different physiological or pathological situations.

It is now well established that overexpression of HIF-1α is linked to poor prognosis in human cancer and is associated with treatment failure and increased mortality [1]. In tumors, overexpression of HIF-1α can either simply reflect

the depletion of O_2 and glucose in rapidly growing cells, or activation of HIF-1α protein synthesis or genetic alterations in the HIF-1α degradation pathway.

Angiogenesis, a crucial step in tumor growth, is activated under hypoxic conditions via the expression of the key regulators: vascular endothelial growth factor (VEGF) and angiopoïetin 2. The *vegf* gene, but also that of erythropoïetin, are major targets of HIF-1, and have been extensively studied. Expression of HIF-1 correlates fully with hypoxia-induced angiogenesis, especially in solid tumors. The involvement of HIF-1 in tumor progression makes this transcription factor a perfect target for cancer therapy. Knowledge of the mechanisms of action of all the actors in the hypoxic pathway is thus becoming a priority in identifying new agents capable of specifically targeting HIF-1. Strategies based on screening of small-molecules as inhibitors of HIF-1 are being investigated [1]. A number have been identified and target microtubule polymerization, topoisomerase, Hsp90 or thioredoxin 1. Such inhibitors decrease the level of HIF-1α and prevent the angiogenic activity within hypoxic regions of pathological tissues [66]. However, these drugs do not specifically target HIF-1 thus further investigations are required to obtain more specific inhibitors.

Specific targeting of HIF-1 will be of crucial importance as complete inhibition of its expression will certainly have repercussions on progression of tumor angiogenesis. Furthermore, inhibitors of HIF-1, which may become important therapeutic agents, could also be used in the treatment of diabetic retinopathy or pulmonary hypertension since VEGF up-regulation is also involved in these disorders. However, evidence for paradoxal effects of HIF-1 on tumor growth, such as growth acceleration of HIF-$1\alpha^{-/-}$ ES-derived tumors, has been observed [57]. Cell type, oncogenic mutations and microenvironment may influence HIF-1 behavior. Activators of the HIF-1 pathway may also show potential in treatment of disorders such as myocardial ischemia, diabetes and wound healing where VEGF is down-regulated.

As illustrated in this review, gene-silencing using siRNA has recently been shown to be a powerful approach for understanding gene function. This technique is based on sequence-specific post-transcriptional gene-silencing by short double-stranded RNA (dsRNA), a mechanism very well conserved throughout a variety of organisms from worms, flies, plants and mammals [67]. The potential application of this new technology to the clinic remains to be investigated. Nevertheless, considering the exquisite specificity of the effect, considerable effort is going to be devoted to this question in the future.

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