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Hypoxia-inducible Factor - 1 (HIF-1)

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Abbreviations: HIF-1, hypoxia-inducible factor 1; HRE, hypoxia response element; EPO, erythropoietin; ODDD, oxygen-dependent degradation domain; IPAS, inhibitory PAS; pVHL, von Hippel-Lindau; 2-OG, 2-oxoglutarate; PHD, prolyl hydroxylase domain; ARD1, arrest-defective-1; FIH-1, factor inhibiting HIF-1; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial cell growth factor.

Abstract

Adaptation to low oxygen tension (hypoxia) in cells and tissues leads to the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival. The primary factor mediating this response is the hypoxia-inducible factor-1 (HIF-1), an oxygen-sensitive transcriptional activator. HIF-1 consists of a constitutively expressed subunit HIF-1β and an oxygen-regulated subunit HIF-1α (or its paralogs HIF-2α and HIF-3α). The stability and activity of the α subunit of HIF are regulated by its posttranslational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation. In normoxia, hydroxylation of two proline residues and acetylation of a lysine residue at the oxygen-dependent degradation domain (ODDD) of HIF-1α trigger its association with pVHL E3 ligase complex, leading to HIF-1α degradation via ubiquitin-proteasome pathway. In hypoxia, the HIF-1α subunit becomes stable and interacts with co-activators such as CBP/p300 and regulates the expression of target genes. Overexpression of HIF-1 has been found in various cancers and targeting HIF-1 could represent a novel approach to cancer therapy.

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Introduction

The transcription factor hypoxia-inducible factor-1 (HIF-1) is a key regulator responsible for the induction of genes that facilitate adaptation and survival of cells and the whole organism from normoxia (~21% O2) to hypoxia (~1% O2) (Semenza, 1998; Wang et al., 1995). Since its identification two decades ago, what we know about HIF has grown exponentially. Due to the realization that hypoxia has a strong impact, via gene expression, on cell biology and mammalian physiology, there has been enormous growing interests in the biology of the HIF-1 pathway and its role in human diseases such as cancer. Accordingly, this review considers what has been learned about HIF-1: its discovery, its regulation, its target gene, its role in development and disease, and its implication for therapy.

1. The discovery of HIF-1

1.1. HIF-1α

HIF-1 was discovered by the identification of a hypoxia response element (HRE, 5′-RCGTG-3′) in the 3’ enhancer of the gene for erythropoietin (EPO), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia-induced transcription (Goldberg et al., 1988; Semenza et al., 1991). Subsequent studies have revealed the protein that binds to the HRE under hypoxic conditions as HIF-1, a heterodimeric complex consisting of a hypoxically inducible subunit HIF-1α and a constitutively expressed subunit HIF-1β (Wang et al., 1995). HIF-1β is also known as ARNT, the aryl hydrocarbon nuclear translocator, which was originally identified as a binding partner of the aryl hydrocarbon receptor (AhR) (Reyes et al., 1992); whereas HIF-1α was newly discovered. These proteins belong to the basic-Helix-Loop-Helix–Per-ARNT-Sim

(bHLH–PAS) protein family (figure 1) (Wang et al., 1995). The bHLH and PAS motifs are required for heterodimer formation between the HIF-1α and HIF-1β subunits while the downstream basic region affords specific binding to the HRE DNA sequence (Crews, 1998). Two transactivation (stimulation of transcription) domains, N-terminal (N-TAD) and C-terminal (C-TAD), in the C-terminal half of the HIF-1α protein have been identified later (Ruas et al., 2002). The C-TAD in particular has been shown to interact with co-activators such as CBP/p300 to activate gene transcription (Lando et al., 2002b). HIF-1α also contains an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability (Pugh et al., 1997). Later work has revealed that HIF-1α is ubiquitously expressed in human and mouse tissues and has a general role in multiple physiological responses to hypoxia, such as erythropoiesis and glycolysis that quickly counteract oxygen deficiency; and angiogenesis which provides a long-term solution (Semenza, 1998).

1.2. HIF-2α

Shortly after the cloning of HIF-1α, a closely related protein, HIF-2α (also termed endothelial Per-ARNT-Sim (PAS) protein (EPAS), HIF-like factor (HLF), HIF-related factor (HRF) and member of the PAS superfamily 2 (MOP2)) was identified and cloned (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997). HIF-2α shares 48% amino acid sequence identity with HIF-1α and accordingly shares a number of structural and biochemical similarities with HIF-1α, for instance, heterodimerization with HIF-1β and binding HREs. In contrast to ubiquitously expressed HIF-1α, though, HIF-2α is predominantly expressed in the lung, endothelium, and carotid body (Ema et al., 1997; Tian et al., 1998; Tian et al., 1997).

1.3 HIF-3α

HIF-3α, which was discovered later, is also expressed in a variety of tissues, dimerizes with HIF-1β, and binds to HREs (Gu et al., 1998). Additionally, a splice variant of HIF-3α, inhibitory PAS (IPAS), which is predominantly expressed in the Purkinje cells of the cerebellum and corneal epithelium, was subsequently discovered (Makino et al., 2001). IPAS possesses no endogenous transactivation activity, but rather interacts with the amino-terminal region of HIF-1α and prevents its DNA binding, acting as a dominant-negative regulator of HIF-1 (Makino et al., 2001). However, IPAS can also be induced by hypoxia in the heart and lung, contributing to a negative feed-back loop for HIF-1 activity in these tissues (Makino et al., 2002).

HIF-1α and HIF-2α have been more extensively studied, whereas research on HIF-3α and other HIF isoforms is relatively scarce.

2. The regulation of HIF-1

While HIF-1β is constitutively expressed and its mRNA and protein are maintained at constant levels regardless of oxygen availability (Kallio et al., 1997), HIF-1α protein has a short half-life ($t_{1/2} \sim 5$ min) and is highly regulated by oxygen (Salceda and Caro, 1997) (figure 2). The transcription and synthesis of HIF-1α are constitutive and seemly not to be affected by oxygen (Kallio et al., 1997; Wang et al., 1995; Wiesener et al., 1998). However, in normoxia, the HIF-1α proteins are rapidly degraded, resulting in essentially no detectable HIF-1α protein (Wang et al., 1995). During hypoxia, HIF-1α becomes stabilized and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1β and the HIF complex formed becomes transcriptionally active (Huang et al., 1996; Kallio et al., 1997). The activated HIF complex then associates with HREs in the

regulatory regions of target genes and binds the transcriptional co-activators to induce gene expression (Lando et al., 2002b). Tight regulation of the stability and subsequent transactivational function of HIF-1α are chiefly controlled by its posttranslational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation (Brahimi-Horn et al., 2005).

The modification of HIF-1α occurs within several domains. In normoxia, hydroxylation of two proline residues and acetylation of a lysine residue in its ODDD promote interaction of HIF-1α with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Masson et al., 2001; Srinivas et al., 1999). PVHL complex tags HIF-1α with ubiquitin and thereby marks it for degradation by the 26S proteasome. In addition, hydroxylation of an asparagine residue in the C-TAD inhibits the association of HIF-1α with CBP/p300 and thus inhibits its transcriptional activity (Lando et al., 2002a).

2.1. Prolyl hydroxylation by PHDs --- signaling for polyubiquitination

De novo synthesized cytoplasmic HIF-1α is rapidly hydroxylated by a family of 2-oxoglutarate (2-OG)-dependent dioxygenases on proline 402 (P402) and 564 (P564) located within ODDD (Masson and Ratcliffe, 2003; Masson et al., 2001; Srinivas et al., 1999). The proline residues are conserved in HIF-2α (P405 and P530) and HIF-3α. They are also part of a consensus sequence LXXLAP that is conserved for the two sites of all the isoforms except for the second proline of HIF-3α, which has the sequence LXXLHP (Bruick and McKnight, 2001; Masson et al., 2001). Mutation of both proline residues disrupted the interaction of HIF-1α with pVHL and increased its stability in the presence of normal oxygen levels, whereas mutation of either proline alone only partially stabilized HIF-1α (Masson et al., 2001).

Human HIF-α dioxygenase was termed Prolyl Hydroxylase Domain (PHD), HIF-Prolyl Hydroxylase (HPH), or Egg-laying Nine (EGLN), and three isoforms have been reported: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1, and PHD3/HPH1/EGLN3 (Bruick and McKnight, 2001; Epstein et al., 2001; Huang et al., 2002). The biochemical characteristics of the PHDs are similar to the collagen prolyl-4-hydroxylases, which are also 2-OG-dependent dioxygenases and require oxygen (O_2) for hydroxylation as well as Fe^{2+}- and ascorbate as cofactors (Schofield and Zhang, 1999). The collagen prolyl-4-hydroxylases though are not able to catalyze HIF-1α/HIF-2α proline hydroxylation (Jaakkola et al., 2001). The hydroxylation process splits O_2, with one oxygen atom being transferred to the proline residue and the other reacting with 2-OG to generate succinate and CO_2 (Bruick and McKnight, 2001; Masson and Ratcliffe, 2003). Inactivation of the PHDs by 2-OG analogues can increase the half-life of HIF-1α (Ivan et al., 2002; Jaakkola et al., 2001). Fe^{2+} at the active site of the PHDs is loosely bound by two histidine residues and one aspartic acid, forming a 2-histidine-1-carboxylate coordination motif. The requirement of Fe^{2+} for PHDs was demonstrated in the observation that iron chelators and metal irons such as Co^{2+}, Ni^{2+}, and Mn^{2+}, are able to stabilize HIF-1α, probably by diminishing the availability of Fe^{2+} for the enzyme or substituting Fe^{2+} from the Fe^{2+}-binding site (Masson and Ratcliffe, 2003; Yuan et al., 2003). Ascorbate helps to maintain Fe in the ferrous (Fe^{2+}) state and is important in maintaining and achieving full activity of the PHDs (Bruick and McKnight, 2001; Epstein et al., 2001).

All three PHDs have the potential to hydroxylate HIF-α *in vitro* with their relative activities as PHD2>>PHD3>PHD1, and PHD2 was shown to be the key limiting enzyme that controls the HIF-1α turnover *in vivo* (Berra et al., 2003; Huang et al., 2002).

Knockdown of PHD2 by its specific small-interfering RNA (siRNA) is sufficient to stabilize HIF-1α levels under normoxia, whereas siRNA silencing of either PHD1 or PHD3 is unable to produce similar effects on HIF-1α (Berra et al., 2003). In addition, it was found that the mRNA and protein of PHD2 were induced during a hypoxia challenge, and the mRNA of PHD3 was upregulated as well, while that of PHD1 remained unaffected (Epstein et al., 2001; Metzen et al., 2003a). This may represent a way by which HIF-1α self-regulates its expression. Furthermore, Siah1 and 2, the specific E3 ligases of PHD1 and 3, are transcriptionally upregulated during hypoxia, thereby, elevating the degradation of these PHDs by the proteasome (Nakayama and Ronai, 2004). When overexpressed as tagged proteins in transfected cells, PHD2 was found to be localized primarily in the cytoplasm, while PHD1 localized in the nucleus and PHD3 was in both compartments (Metzen et al., 2003a). Despite its primary localization in the cytoplasm, PHD2 is able to shuttle between the cytoplasm and the nucleus, thereby contribute to HIF-1α degradation in both compartments. Although all three enzymes are widely expressed in many tissues, they exhibit tissue specific overexpression. PHD2 was abundant in adipose tissue (Oehme et al., 2002), PHD3 in the heart and placenta (Lieb et al., 2002; Oehme et al., 2002), and PHD1 in the testis (Lieb et al., 2002). The differences of the enzyme activity of PHDs, subcellular localization, and tissue distribution may enable a graded or tissue-specific response to hypoxia. Some proteins such as OS-9, the protein product of a widely expressed gene with unclear function, have been shown to promote prolyl hydroxylation by interacting with both HIF-1α and PHDs, therein, promoting the O_2-dependent degradation of HIF-1α (Baek et al., 2005). Additionally,

several second messengers have also been shown to modify the activity of PHDs (Appelhoff et al., 2004; Hirota et al., 2004; Temes et al., 2005).

In the presence of oxygen, the PHDs are active and hydroxylate the prolines of HIF-1α, constituting a recognition signal for binding of pVHL and subsequent ubiquitination, followed by degradation of HIF-1α (Ivan et al., 2001; Jaakkola et al., 2001). The absence of oxygen causes no enzyme activity, no modification of proline, and no pVHL/HIF binding, resulting in HIF-1α stabilization and accumulation in the cell. The absolute requirement of oxygen as a co-substrate suggests PHDs as the oxygen sensor in cells (Epstein et al., 2001).

2.2. Polyubiquitination by pVHL --- signaling for degradation

Once the two proline residues of HIF-1α are converted to hydroxyproline, pVHL then captures HIF-1α. X-ray crystallographic studies of the pVHL/ HIF-1α complex have revealed that pVHL has a surface pocket into which the hydroxyproline fits accurately and the overall binding configuration is highly specific (Hon et al., 2002; Min et al., 2002). The pVHL associates with the proteins elongin C, elongin B, cullin-2, and Rbx1 to form the VCB-Cul2 E3 ligase complex (Ivan and Kaelin, 2001). Binding of HIF-1α to this multiprotein E3 complex causes polyubiquitination of HIF-1α, ultimately leading to its degradation by the proteasome (Kamura et al., 2000). However, the exact lysine residue(s) that is(are) ubiquitinated have not as yet been identified.

The pVHL was first described in von Hippel-Lindau (VHL) disease, an inherited human cancer syndrome that is characterized by the development of multiple tumors, such as clear-cell renal carcinomas, pheochromocytomas, and hemangioblastomas in the

retina and central nervous system (Ivan and Kaelin, 2001). The *VHL* gene encodes a full length (213 amino acids) and an N-terminal truncated protein (amino acids 54–213). Since both proteins show similar functions, they are often collectively referred to as pVHL. Mutations in the *VHL* gene that functions as a tumor suppressor were found in the diseases discussed above (Iliopoulos et al., 1998; Schoenfeld et al., 1998). In cells lacking wild-type pVHL, HIF-1α and HIF-2α are stable and active under normoxia, resulting in the over-production of hypoxia-inducible genes (Iliopoulos et al., 1996). Restoration of pVHL function by stable transfection reversed normoxic HIF-α protein stability and the aberrant increase of genes (Iliopoulos et al., 1996). Therefore, one mechanism by which mutations in pVHL might cause tumor formation is by permitting the stability and activity of HIF-α under normal oxygen tensions, resulting in the subsequent expression of genes encoding angiogenic factors even before cells are exposed to a hypoxic stress.

The pVHL E3 ligase complex is ubiquitously expressed in different tissues and predominantly localized to the cytoplasm. Its shuttling between the cytoplasm and nucleus enables HIF-1α degradation in both compartments (Berra et al., 2001; Groulx and Lee, 2002).

However, the pVHL-dependent pathway may not be the only pathway leading to degradation of HIF-1α. In addition to pVHL, a number of other proteins have been reported to affect HIF-1α ubiquitination and stability. For example, the oncogenic E3 ubiquitin ligase Murine Double Minute 2 (MDM2) has been suggested to bring about ubiquitination of HIF-1α in a p53-dependent fashion (Ravi et al., 2000). The protein Jab1, a transcriptional co-activator of c-Jun and Jun D, has also been shown to increase HIF-1α

levels under hypoxia, probably by competing with p53 for binding to HIF-1α (Bae et al., 2002). In addition, pVHL has also been shown to interact with other proteins involved in the HIF-1 signaling pathway and thus may regulate more than just HIF-1α stability.

2.3. Lysine Acetylation by ARD1 --- facilitating pVHL binding

Lysine residue 532 (K532) located in the ODDD domain of HIF-1α was reported to be acetylated by an acetyltransferase named arrest-defective-1 (ARD1) (Jeong et al., 2002). ARD1 was originally identified in the yeast *Saccharomyces cerevisiae* and its name came from the defective yeast mutants in the mitotic cell cycle (Whiteway and Szostak, 1985). Acetylation of K532 favors the interaction of HIF-1α with pVHL, and thus destabilizes HIF-1α (Jeong et al., 2002). Mutation of K532 to arginine resulted in increased stability of HIF-1α (Tanimoto et al., 2000). In addition, artificial maintenance or increase of the acetylated state of HIF-1α by butyric acid, a global inhibitor of deacetylases, caused decreased HIF-1α protein levels (Kim et al., 2001).

Since the activity of acetyltransferases is not influenced by oxygen, ARD1 may be active and acetylate HIF-1α regardless of oxygen conditions. But the mRNA and protein levels of ARD1 were decreased under hypoxia, which may cause less acetylated HIF-1α in hypoxia than that in normoxia (Jeong et al., 2002).

2.4. Asparagine hydroxylation by FIH-1 --- preventing CBP/p300 binding

The posttranslational modifications of HIF-1α described above regulate the stabilization of HIF-1α protein. But the stabilization alone is not sufficient for full transcriptional activation of HIF-1. The second major mechanism controlling HIF activity

is through the modulation of its transactivation domains N-TAD and C-TAD. These domains function by recruiting transcriptional co-activators such as CBP/p300, SRC-1, and TIF2 (Arany et al., 1996; Carrero et al., 2000; Ebert and Bunn, 1998; Ema et al., 1999; Kallio et al., 1998). Under normal oxygen tension, hydroxylation of the asparagines residue 803 (N803) in the C-TAD of HIF-1α (N851 in HIF-2α) by Factor Inhibiting HIF-1 (FIH-1) prevented the interaction of HIF-1α with CBP/p300 (Hewitson et al., 2002; Lando et al., 2002b; Sang et al., 2002). Hypoxia abrogated asparagine hydroxylation, which allowed the C-TAD of HIF-1α to efficiently interact with CBP/p300, activating the transcription of the respective target genes (Lando et al., 2002a). Replacement of N803 with alanine permitted co-activator binding with HIF-1α at normoxia (Lando et al., 2002b). In addition, it has been reported that FIH-1 binds pVHL, forming a ternary complex with the HIF-1α (Mahon et al., 2001). Although interaction with pVHL was not required for FIH-1 activity, histone deacetylases (HDACs) recruited by pVHL interfered with the transcription processes, therein, facilitating FIH-1 to modulate HIF-1α transactivation (Hewitson et al., 2002; Sang et al., 2002). FIH-1 is mainly located in the cytoplasm, with some fraction likely residing in the nucleus as well (Metzen et al., 2003a). The transcription of FIH-1 is independent of the oxygen concentration and it does not influence HIF-1α stability (Metzen et al., 2003a).

Like the PHDs, the asparaginyl hydroxylase FIH-1 is a 2-OG-dependent dioxygenase which also requires Fe^{2+} and ascorbate as cofactors (Lando et al., 2002a). Utilization of oxygen as a substrate allows FIH-1 to serve as a second oxygen sensor.

2.5. Phosphorylation by MAPK --- enhancing transactivation

Despite the central importance of hydroxylases in sensing oxygen tension and regulating HIF-1 activity, there are other mechanisms that contribute to the control of HIF-1. It has been well-known that phosphorylation is crucial in controlling protein activities. Direct phosphorylation of HIF-1α has been reported and the Mitogen-Activated Protein Kinase (MAPK) pathway appears to play a role (Minet et al., 2001; Richard et al., 1999; Sodhi et al., 2000). It has been shown that p42/44 and p38 kinase phosphorylated HIF-1α/HIF-2α in vitro (Richard et al., 1999; Sodhi et al., 2000). Additionally, inhibitors of p42/44 and p38 blocked HIF-1α-mediated reporter gene expression (Hur et al., 2001). Transfection with active forms of p42/44 kinase stimulated HIF-1α transcription activity without affecting HIF-1α stability. Moreover, HIF-1α/HIF-2α transaction during hypoxia required p42/44 MAPKs (Conrad et al., 1999; Hofer et al., 2001; Hur et al., 2001; Minet et al., 2000). It seems that phosphorylation does not affect stability or DNA binding of HIF-1α, but rather increases the transcriptional activity of HIF-1 (Richard et al., 1999). One explanation could be that HIF-1β binds preferentially to the phosphorylated form of HIF-1α (Suzuki et al., 2001). Although the functionally relevant phosphorylation sites remain to be identified, threonine 796 in HIF-1α and 844 in HIF-2α are candidate sites (Gradin et al., 2002).

In addition to the posttranslational modification of HIF-1α described above, SUMOylation of HIF-1α has also been reported to contribute to repressing transactivation (Brahimi-Horn et al., 2005). Moreover, S-nitrosation on cysteine 800 of HIF-1α has been shown to increase its transactivation through its interaction with CBP/p300 (Yasinska and Sumbayev, 2003).

Besides hypoxia, HIF-1 is also regulated in an oxygen-independent manner. Cytokines, growth factors, environmental stimuli, and other signaling molecules have been implicated in controlling HIF-1under non-hypoxic condition (table 1) (Feldser et al., 1999; Gorlach et al., 2001; Haddad and Land, 2001; Hellwig-Burgel et al., 1999; Li et al., 2004; Richard et al., 2000; Salnikow et al., 2000; Stiehl et al., 2002). Although complex and cell-type dependent, some have been shown to stimulate HIF-1α transactivation or synthesis by activation of the MAPK or the phosphatidylinositol 3-kinase (PI3K) signaling pathways (Li et al., 2004; Zelzer et al., 1998).

3. The target gene of HIF-1

Given that cells and organs need to adapt to changes in oxygen supply, it would not be surprising to find that a significant variety of the HIF-1 target genes are regulated in a tissue specific manner. To date, there are more than one hundred HIF-1 downstream genes identified with varying functions (table 2). HIF-1 activates the expression of these genes by binding to a 50 base pair *cis*-acting HRE located in their enhancer and promoter regions (Semenza et al., 1991). Moreover, by using DNA microarrays, it has recently been reported that more than 2% of all human genes are regulated by HIF-1 in arterial endothelial cells, directly or indirectly (Manalo et al., 2005).

3.1. Erythropoiesis/iron metabolism

In response to hypoxia, the capacity of red blood cells to transport oxygen is upregulated by the expression of genes involved in erythropoiesis and iron-metabolism. Hypoxia increases the expression of EPO, which is required for the formation of red blood cells (Semenza et al., 1991). An increase in the number of erythrocytes enhances the delivery of oxygen to tissues. Products of Iron-metabolizing genes control the major

erythropoietic rate-limiting step of heme production. Hypoxia upregulates transferrin (Tf) which transports Fe^{3+} into cells (Rolfs et al., 1997); the transferring receptor (Tfr) which binds Tf and enables cellular transferrin uptake (Bianchi et al., 1999; Lok and Ponka, 1999; Tacchini et al., 1999); and ceruloplasmin (also known as a ferroxidase) which is required to oxidize ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron (Lok and Ponka, 1999; Mukhopadhyay et al., 2000). Increasing of these genes supports iron supply to erythroid tissues (Rolfs et al., 1997).

3.2 Angiogenesis

Angiogenesis is a complex process that involves multiple gene products expressed by different cell types (Conway et al., 2001). A large number of genes involved in different steps of angiogenesis have been shown to increase by hypoxia challenge (Berra et al., 2000; Bunn and Poyton, 1996; Forsythe et al., 1996; Giordano and Johnson, 2001; Levy et al., 1995; Semenza, 2002). Among them, the vascular endothelial cell growth factor (VEGF) is the most potent endothelial-specific mitogen and it directly participates in angiogenesis by recruiting endothelial cells into hypoxic and avascular area and stimulates their proliferation (Conway et al., 2001; Josko et al., 2000; Neufeld et al., 1999). Therefore, the induction of VEGF and various other pro-agiogenetic factors leads to an increase in the vascular density and hence a decrease in the oxygen diffusion distance. In addition, HIF-1 regulates genes involved in governing the vascular tone such as nitric oxide synthase (NOS2) (Melillo et al., 1995), heme oxygenease 1 (Lee et al., 1997), endothelin 1 (ET1) (Hu et al., 1998), adrenomedulin (ADM) (Nguyen and Claycomb, 1999), and the α_{1B}-adrenergic receptor (Eckhart et al., 1997). Moreover, hypoxia induces genes involved in matrix metabolism and vessel maturation such as

matrix metalloproteinases (MMPs) (Ben-Yosef et al., 2002), plasminogen activator receptors and inhibitors (PAIs) (Kietzmann et al., 1999), and collagen prolyl hydroxylase (Takahashi et al., 2000).

3.3 Glucose metabolism

Under low-oxygen supply, cells switch their glucose metabolism pathway away from the oxygen-dependent tricarboxylic acid (TCA) cycle to the oxygen-independent glycolysis (Dang and Semenza, 1999; Seagroves et al., 2001). With only 2 ATP molecules from each glucose molecule produced by glycolysis, instead of 38 ATP provided by TCA cycle, hypoxic cells elevate their ability to generate ATP by increasing the glucose uptake. This is achieved by upregulating the expression of glycolytic enzymes and glucose transporters (Wenger, 2002). Hypoxia and HIF-1 increase virtually all the enzymes in the glycolytic pathway, as well as the glucose transporters 1 and 3 (GLU1, GLU3) (Chen et al., 2001). Furthermore, the glycolysis metabolic products, such as lactate and pyruvate, have been reported to cause HIF-1α accumulation under normoxia and regulate hypoxia-inducible gene expression, hence establishing a potential positive feedback loop (Lu et al., 2002).

3.4. Cell proliferation/survival

Hypoxia and HIF-1 induce growth factors such as insulin-like growth factor-2 (IGF2) and transforming growth factor-α (TGF- α) (Feldser et al., 1999; Krishnamachary et al., 2003). Binding of such growth factors to their cognate receptors activates signal transduction pathways that lead to cell proliferation/survival and stimulates the expression of HIF-1α itself (Semenza, 2003). As mentioned above, cytokines, growth factors, as well as hypoxia in some cell types, can activate signaling pathways MAPK

and PI3K, which promote cell proliferation/survival as well as contribute to HIF-1 activity. This leads to increased HIF-1 transcriptional activity of target genes including those encoding IGF2 and TGF-α, therefore, contributing to autocrine-signaling pathways that are crucial for cancer progression (Semenza, 2003).

3.5. Apoptosis

Paradoxically, cell adaptation to hypoxia not only leads to cell proliferation/survival but also to cell death in some circumstances. Hypoxia has been shown to induce apoptosis, where HIF-1 plays a complex role (Carmeliet et al., 1998). Genetic studies using embryonic stem cells harboring a deletion of HIF-1α showed decreased apoptosis, compared to wild-type when challenged with low oxygen (Carmeliet et al., 1998). Activation of caspase-3 and Apaf-1-mediated caspase-9, and the release of cytochrome c have been reported in several cell types under hypoxic conditions (Brunelle and Chandel, 2002; McClintock et al., 2002). It has also been demonstrated that the expression of HIF-1α and HIF-1β significantly correlated with apoptosis and the pro-apoptotic factors such as caspase-3, Fas, and Fas ligand (Volm and Koomagi, 2000). Moreover, hypoxia depressed the anti-apoptotic protein Bcl-2 (Carmeliet et al., 1998), while the pro-apoptotic protein Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNip3) and its homologue Nip3-like protein X (NIX) were upregulated in a HIF-dependent manner (Bruick, 2000). Some genes involved in cell cycle control such as p53 and p21 were also found to be HIF-dependent (Carmeliet et al., 1998). In addition, p53 has been implicated in regulating hypoxia-induced apoptosis through induction of apoptosis-related genes such as *Bax*, *NOXA*, *PUMA*, and *PERP* (Schuler and Green, 2001).

In addition to the above classes of genes, HIF-1 also regulated many other target genes implicated in diverse processes such as adipogenesis (Yun et al., 2002), carotide body formation(Kline et al., 2002), B lymphocyte development (Kojima et al., 2002) and immune reactions (Hellwig-Burgel et al., 2005).

Although there are some studies showing a role of HIF-2α in the VEGF induction (Akeno et al., 2001; Compernolle et al., 2002), no *bone fide* target genes have yet been identified for HIF-2α or HIF-3α. However, a recent study using a genetic "knock-in" strategy has shown that targeted replacement of HIF-1α with HIF-2α results in expanded expression of HIF-2α-specific target genes (*i.e.* Oct-4, a transcription factor essential for maintaining stem cell pluripotency) (Covello et al., 2006).

4. The role of HIF-1 in development and diseases

Hypoxia and the HIF pathway have been linked to the embryonic development and pathophysiology of numerous human diseases. In order for solid tumors to grow, an increase of oxygen delivery to cells via angiogenesis and activation of glycolysis have been observed and named the Warburg effects (Seagroves et al., 2001). Given the importance of HIF-1 in the activation of genes essential to these processes, it is not surprising that both HIF-1α and HIF-2α have been strongly implicated in tumor progression and grade, hence conferring a selective advantage to tumor cells.

4.1. Development

Components of the HIF-1 system play essential roles in embryonic development. Knockout of either HIF-1α (Iyer et al., 1998; Kotch et al., 1999; Ryan et al., 1998), HIF-2α (Peng et al., 2000; Tian et al., 1998), or HIF-1β (Maltepe et al., 1997) resulted in abnormal vascular development and lethality in mice. HIF-1α expression increased

between embryonic day 8.5 and 9.5 (E8.5 and E9.5) in normal mouse embryos (Iyer et al., 1998). Embryos deficient in *HIF-1α* (*HIF-1α*$^{-/-}$) died by E11 as a consequence of lack of blood vessel formation, defective formation of the neural fold, and cardiovascular malformation (Iyer et al., 1998; Ryan et al., 1998). Global hypoxia was also observed. In addition, the rate of cell proliferation and the expression of hypoxia-inducible genes were decreased in *HIF-1α*$^{-/-}$ cells, compared to those of wild-type cells (Iyer et al., 1998). Although heterozygous mice carrying a single HIF-1α gene (*HIF1α*$^{+/-}$) developed normally, they displayed impaired physiological responses, when challenged by chronic hypoxia (Kline et al., 2002; Yu et al., 1999).

Targeted inactivation of HIF-2α (*HIF-2α*$^{-/-}$) in mice resulted in rather different and variable phenotypes. *HIF-2α*$^{-/-}$ mouse embryos died by E12.5-E16.5 due to inadequate blood vessel fusion and remodeling, impaired fetal lung maturation, a very slow heart rate because of insufficient catecholamine production (Peng et al., 2000; Tian et al., 1998). Thus, it appears that HIF-1α and HIF-2α have non-redundant functions in the regulation of gene expression during development, despite their close similarity in terms of amino acid sequence, domain architecture, DNA-binding capacity and hypoxic activation.

HIF-1 β$^{-/-}$ embryos died by E10.5 and showed defect in blood vessel formation, defective angiogenesis of the yolk sac and branchial arches, stunted development and embryo wasting (Maltepe et al., 1997). In addition, *HIF-1 β*$^{-/-}$ cells failed to activate genes that normally respond to hypoxia and low glucose concentration (Maltepe et al., 1997).

4.2. Cancer

Overexpression of HIF-1α and HIF-2α was found in various human cancers, probably as a consequence of intratumoral hypoxia or genetic alteration (Talks et al., 2000; Zhong et al., 1999). The interior of the tumor mass becomes progressively hypoxic as its size increases until there are adequate blood vessels obtained by tumors. Hypoxic conditions within tumors can result in increased HIF-1 stability and activity. Immunohistochemical analyses demonstrated that there are detectable levels of HIF-1α protein in benign tumors, elevated levels in primary malignant tumors, and a marked amount in tumor metastases, in contrast to its absence in normal tissues (Harris, 2002; Zhong et al., 1999). Expression of HIF target genes is generally consistent with the levels of HIF-1α. In addition, injection of HIF-1α (or HIF-1β) positive and deficient cells to immunocompromised mice revealed that HIF-1α is a positive factor to tumorigenesis (Maxwell et al., 1997; Ryan et al., 2000).

There is a remarkable frequency of common genetic alterations in cancer cells associated with increased HIF-1α expression. As mentioned in VHL disease, for example, loss of function of VHL resulted in constitutively expressed HIF-1α (Iliopoulos et al., 1996). In addition, loss of function of wild type p53, which is inactivated in most of human cancers, increased HIF-1α levels and enhanced HIF-dependent transcription in tumors (Ravi et al., 2000). Loss of function of tumor suppressor gene PTEN in glioblastoma-derived cell line resulted in increased HIF-1α levels and HIF-1-mediated gene expression, probably via activating of the PI3K/AKT signaling cascade (Zundel et al., 2000). The transforming potential of the v-Src oncogene is thought in part due to its induction of HIF and gain of function of v-SRC increased expression of HIF-1α and HIF-dependent genes (Jiang et al., 1997). Moreover, enhanced HER2 receptor tyrosine kinase

signaling has been shown to increase the rate of synthesis of HIF-1α (Laughner et al., 2001). Increased activity of the HER2 receptor tyrosine kinase is a prevalent and important genetic alteration in breast cancer, correlating with tumor aggressiveness and decreased patient survival. Therefore, it appears that HIF-1α overexpression confers selective advantages to tumor cells. A correlation between HIF-1 overexpression and patient mortality, poor prognosis or treatment resistance has been noted in many studies (Semenza, 2003).

4.3. Ischemic disease

Activation of HIF activity has also been demonstrated in a broad range physiological response to ischemic, hypoxic and inflammatory conditions, where it plays a positive role to respond to the damage to organs or tissues. For example, the levels of HIF-1α and VEGF were increased in the myocardium when patients develop acute coronary artery occlusion (Lee et al., 2000). Effective vascular remodeling following ischemic injury depended on an integrated program of HIF-dependent gene expression. Increasing of HIF-1α expression and HIF-inducible genes was also observed in sheep and rat models of myocardial and cerebral ischaemia (Bergeron et al., 1999; Martin et al., 1998). In addition, induction of HIF-1α or HIF-2α and their target genes has been shown in the pre-eclamptic placenta (Rajakumar et al., 2003), by macrophages in rheumatoid synovia (Hollander et al., 2001), in the ischemic retina (Grimm et al., 2002; Ozaki et al., 1999), as well as from wound healing (Elson et al., 2000).

5. The implication of HIF-1 in therapy

The importance of HIF-1 as a transcription factor and the broad spectrum of processes influenced by HIF suggest that it could have important clinical implications.

The many aspects of HIF-1 regulation provide a variety of possibilities for therapeutic intervention.

5.1. Cancer therapy --- inhibiting HIF-1 activity

First, immunohistochemical analysis of HIF-1α expression in tumor biopsies may provide the prognostic information, and thereby, identify subsets of patients requiring aggressive therapy (Harris, 2002). In order to achieve high specificity to hypoxic tumor regions or cells, HREs from hypoxia-responsive genes can be used to express marker therapeutic genes that selectively expressed in tumor cells that are hypoxic and overexpress HIF-1 (Dachs et al., 1997).

It has been suggested that disruption of the HIF-1 pathway might be effective in the treatment of pancreatic cancer (Chen et al., 2003). The study demonstrated that dominant-negative HIF-1α reduced the tumorigenicity of pancreatic cancer cells through the suspension of glucose metabolism (Chen et al., 2003). It also rendered the cancer cells sensitive to apoptosis and growth inhibition induced by hypoxia (Chen et al., 2003). A HIF-1α C-TAD polypeptide that competes for CBP/p300 binding has been shown to decrease the expression of VEGF and tumor growth in mice (Kung et al., 2000).

Many novel therapeutic agents targeting signal-transduction pathways have shown to block HIF-1α function and have anti-angiogenic effects (Harris, 2002). These agents include Herceptin, Iressa, Herbinycine (inhibitors of tyrosine kinase); calphostin C (inhibitor a protein kinase c, PKC); wortmannin and LY294002 (inhibitors of PI3K); PD98095 (inhibitor of a MAPK); rapamycin (inhibitor of a FRAP/mTOR); Diphenylene iodonium (a redox signaling blocker), and mannoheptulose (inhibitor of a glucokinase). Several small molecular inhibitors of the HIF-1 transcriptional activation pathway have

also been identified and shown to decrease HIF-1α levels, inhibit the expression of VEGF and other HIF-1 target genes, impair xenograft growth and vascularization, as well as inhibit angiogenesis (Rapisarda et al., 2002). These molecules include topoisomerase I inhibitors, YC-1 (3-(5'-hydroxy-methyl-2'-furyl)-1-benzylindazole), 17-AAG (17-allyl-aminogeldanamycin, inhibitor of HSP90); inhibitors of the redox regulator thioredoxin-1; and the newly identified a-methoxyestradiol (2ME2) that disrupts tumor microtubules (MTs).

5.2 Ischaemic disease therapy --- promoting HIF-1 activity

In contrast to the inhibition of HIF-1 activation in cancer therapy, promoting its activation could be advantageous in ischaemic diseases (Elson et al., 2001; Vincent et al., 2000). Ischaemic diseases such as stroke and heart attack are caused by localized hypoxia manifested as cerebral and myocardial ischemia, respectively. Increase of the VEGF expression by HIF-1α or HIF-2α could induce the formation of new blood vessels of the target area in the brain and heart, thereby providing an increased blood flow and oxygen supply and reduce harmful response to ischemia (Semenza, 1998). Transgenic mice overexpressing HIF-1α in epidermis showed increased expression of VEGF and marked induction of hypervascularity without induction of edema, inflammation, or vascular leakage (Elson et al., 2001). The macrophage derived peptide PR39 has been shown to stabilize HIF-1α by decreasing its degradation, resulting in accelerated formation of vascular structures *in vitro* and increased myocardial vasculature in mice (Li et al., 2000). Direct induction of HIF-1 has been achieved by using N- or C-terminal of ODDD polypeptides that block VHL-mediated degradation (Maranchie et al., 2002). Targeting of

proline and asparaginyl hydroxylases could also be potential strategies for increasing HIF activity (Ivan et al., 2002).

Conclusion

Despite recent rapid advance in understanding the molecular mechanisms of the HIF pathway in response to hypoxia, there are many important questions that yet remain to be answered. For example, the distinct role of enzymes modifying HIF-1α posttranslationally, the interplay among the HIF-1α posttranslational modifications, the identity of additional target genes of HIF-1, the function of the paralogs of HIF-1α such as HIF-2α and HIF-3α, the link between HIF-1 activation and other oncogenic or tumor suppressor pathways, the mechanism by which the HIF-1 pathway contribute to tumor growth and other pathological responses. Unravel of such questions should provide new insights into cellular adaptation to hypoxia and aid to discover new therapeutic approaches to diverse human diseases.

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Figure legends

Figure 1. Domain structure of human HIF-α and HIF-1β.

HIF-α (HIF-1α, HIF-2α, HIF-3α, IPAS) and HIF-1β belong to the basic-Helix-Loop-Helix (bHLH) and Per-ARNT-Sim (PAS) protein family. HIF-α contains an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability through the hydroxylation of two proline (P) residues and the acetylation of a lysine (K). The proline residues are conserved in HIF-2α and HIF-3α. HIF-1α and HIF-2α also contain two transaction domains (C-TAD and N-TAD), while HIF-1β has only one TAD. The total number of amino acids of each subunit is marked at the end of the domain structure.

Figure 2. Oxygen-dependent regulation of HIF-1 stabilization and transactivation.

In normoxia (left), two proline residues of HIF-1α (P^{402} and P^{564}) and asparagine (N^{803}) are hydroxylated by PHDs and FIH-1, respectively, in an O_2, 2-oxoglutarate (2-OG), and Fe^{2+}-dependent manner. Hydroxylated HIF-1α proteins bind to the E3 ubiquitin ligaseVHL complex, leading to its degradation by the proteasome. Acetylation of lysine (K^{532}) by ARD1 favors the interaction of HIF-1α with VHL. Hydroxylated N^{803} blocks the recruitment of transcriptional coactivator CBP/p300.

In hypoxia (right), the activities of PHDs and FIH-1 are inhibited due to lack of O_2, resulted in no proline and asparagine hydroxylation. Consequently, there is no VHL binding and HIF-1α is stabilized. Stabilized HIF-1α proteins translocate to the nucleus and bind to HIF-1β. HIF-1β may bind preferentially to the MAPK-induced phosphorylated form of HIF-1α. Non-hydroxylated N^{803} of HIF-1α allows CBP/p300

recruitment to the target genes, resulting in gene transcription. In addition, the expression of ARD1 is decreased under hypoxia, causing less acetylated HIF-1α.

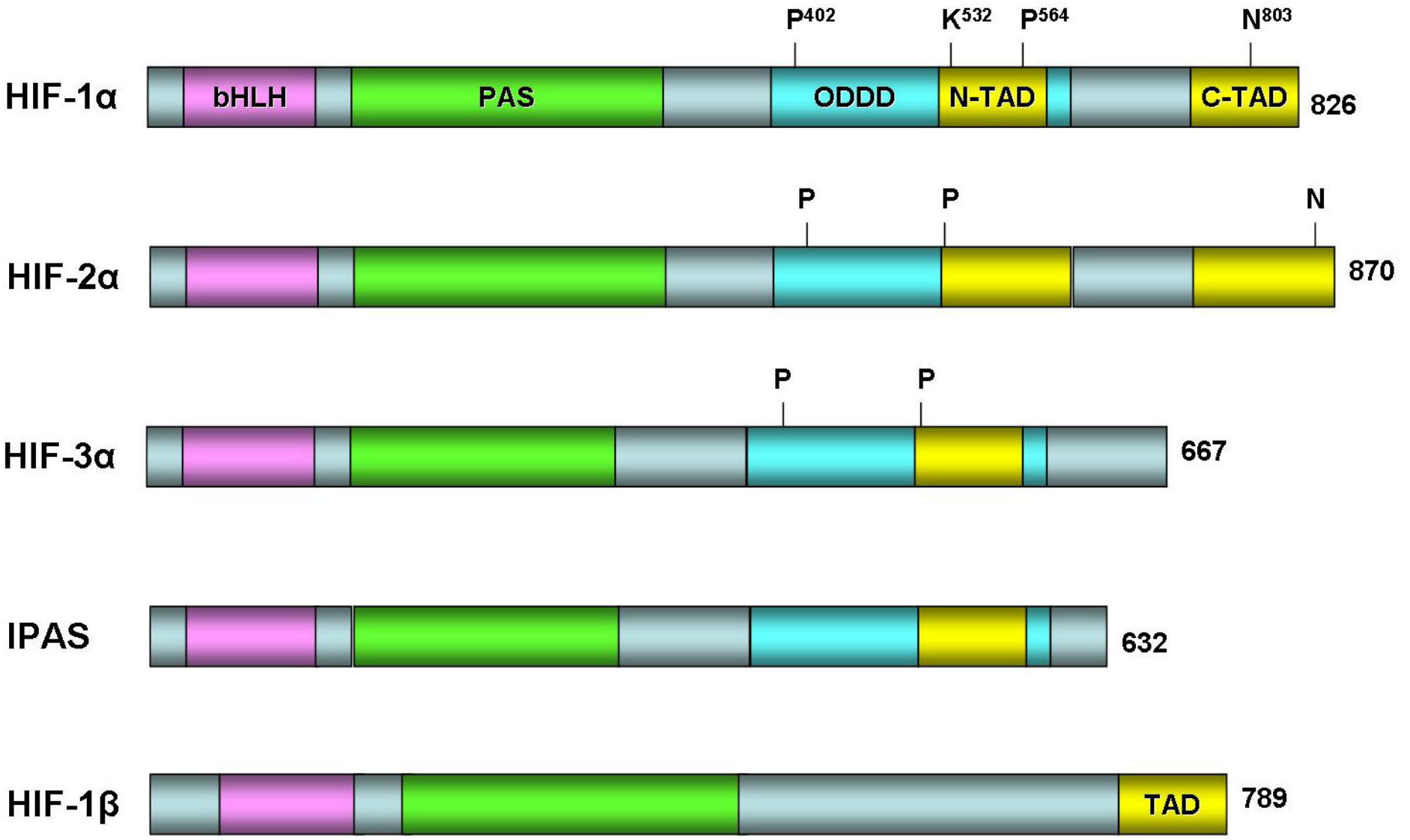
Table 1. Environmental regulator of HIF-1.

regulator	Function/pathway involved	consequence	reference
Nickel (Ni^{2+})	Decreases cellular Fe level Inhibits PHDs	Increased HIF-1α	(Davidson et al., 2005)
	Down-regulates the expression of FIH-1 and ARD1		(Ke et al., 2005)
	Depletes intracellular ascorbate		(Salnikow et al., 2004)
	PI3K/Akt		(Li et al., 2004)
Cobalt (Co^{2+})	Replaces Fe	Increased HIF-1α	(Yuan et al., 2003)
	Down-regulates the expression of FIH-1 and ARD1		(Ke et al., 2005)
	Depletes intracellular ascorbate		(Salnikow et al., 2004)
Arsenite	PI3K	Increased HIF-1α	(Gao et al., 2004)
	ROS		(Duyndam et al., 2001)
	p38 MAPK		(Duyndam et al., 2003)
Chromium	p38 MAPK, ROS	Increased HIF-1α	(Gao et al., 2004)
Vanadate	PI3K/Akt, ROS	Increased HIF-1α	(Gao et al., 2002)
	AMP-activated protein kinase (AMPK)		(Hwang et al., 2004)
Desferrioxamine (DFO)	Fe chelator	Increased HIF-1α	(Wang and Semenza, 1993)
Insulin	PI3K	Increased HIF-1α	(Stiehl et al., 2002)
Interleukin-1(IL-1) insulin-like growth factor (IGF)-1, IGF-2			(Feldser et al., 1999)
Fetal calf serum	ROS	Increased HIF-1α	(Richard et al., 2000)
Angiotensin II (Ang II)			(Gorlach et al., 2001)
Thrombin, Platelet-derived growth factor (PDGF)			
Nitric oxide (NO)	MAPK, PI3K	Increased HIF-1α	(Kasuno et al., 2004)
	Inhibits PHDs		(Metzen et al., 2003b)

Nitric oxide (NO) under hypoxia	Inhibits mitochondrial O_2 consumption	Decreased HIF-1α	(Hagen et al., 2003)
	Increases intracellular Fe and PHDs activity		(Callapina et al., 2005)
	ROS	Increased HIF-1α translocation and activity	(Haddad and Land, 2001)
TGF- α		Increased DNA binding	(Hellwig-Burgel et al., 1999)

Table 2. HIF-1 target genes.

Function	Gene (abbreviation)	Reference
Erythropoiesis/ iron metabolism	Erythropoietin (EPO)	(Semenza et al., 1991)
	Transferrin (Tf)	(Rolfs et al., 1997)
	Transferrin receptor (Tfr)	(Bianchi et al., 1999)
	Ceruloplasmin	(Lok and Ponka, 1999)
Angiogenesis	Vascular endothelial growth factor (VEGF)	(Levy et al., 1995)
	Endocrine-gland-derived VEGF (EG-VEGF)	(LeCouter et al., 2001)
	Leptin (LEP)	(Grosfeld et al., 2002)
	Transforming growth factor-beta3 (TGF- β3)	(Scheid et al., 2002)
Vascular tone	Nitric oxide synthase (NOS2)	(Melillo et al., 1995)
	Heme oxygenease 1	(Lee et al., 1997)
	Endothelin 1 (ET1)	(Hu et al., 1998)
	Adrenomedulin (ADM)	(Nguyen and Claycomb, 1999)
	α1B-adrenergic receptor	(Eckhart et al., 1997)
Matrix metabolism	Matrix metalloproteinases (MMPs)	(Ben-Yosef et al., 2002)
	Plasminogen activator receptors and inhibitors (PAIs)	(Kietzmann et al., 1999)
	Collagen prolyl hydroxylase	(Takahashi et al., 2000)
Glucose metabolism	Adenylate kinase-3	(O'Rourke et al., 1996)
	Aldolase-A,C (ALDA,C)	(Semenza et al., 1996)
	Carbonic anhydrase-9	(Wykoff et al., 2000)
	Enolase-1 (ENO1)	(Semenza et al., 1996)
	Glucose transporter-1,3 (GLU1,3)	(Chen et al., 2001)
	Glyceraldehyde phosphate dehydrogenase (GAPDH)	(Graven et al., 1999)
	Hexokinase 1,2 (HK1,2)	(Mathupala et al., 2001)
	Lactate dehydrogenase-A (LDHA)	(Semenza et al., 1996)
	Pyruvate kinase M (PKM)	(Semenza et al., 1994)
	Phosphofructokinase L (PFKL)	(Semenza et al., 1994)
	Phosphoglycerate kinase 1 (PGK1)	(Semenza et al., 1994)
	6-phosphofructo-2-kinase/gructose-2,6-bisphosphate-3 (PFKFB3)	(Minchenko et al., 2002)
Cell proliferation/ survival	Insulin-like growth factor-2 (IGF2)	(Feldser et al., 1999)
	Transforming growth factor-α (TGF- α)	(Krishnamachary et al., 2003)
	Adrenomedullin (ADM)	(Cormier-Regard et al., 1998)
Apoptosis	Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNip3)	(Carrero et al., 2000)
	Nip3-like protein X (NIX)	(Bruick, 2000)


P402
K532
P564
N803
HIF-1α
bHLH
PAS
ODDD
N-TAD
C-TAD
826
P
P
N
HIF-2α
870
P
P
HIF-3α
667
IPAS
632
HIF-1β
TAD
789

Normoxia

Hypoxia

P^{402} K^{532} P^{564}

HIF-1 α

N^{803}

DFO, Co^{2+}, Ni^{2+}

CO_2 Succinate

O_2 2-OG

Fe^{2+}

PHD FIH ARD

VHL

OH Ac OH
P^{402} K^{532} P^{564}

HIF-1 α

N^{803} OH

OH Ac OH
P^{402} K^{532} P^{564}

VHL

HIF-1 α

Ub Ub Ub

N^{803} OH

CBP/p300

H I F - 1 α

ARD FIH PHD

MAPK

VHL

P^{402} K^{532} P^{564}

P

HIF-1 α

N^{803}

P^{402} K^{532} P^{564}

P

HIF-1 α

CBP/p300

N^{803}

P

CBP/p300

HIF-1 α HIF-1 β

HRE

target gene