

REVIEW



HIF-1 in cancer therapy: two decade long story of a transcription factor

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ABSTRACT

Background: Oxygen (O₂) homeostasis is an indispensable requirement of eukaryotes. O₂ concentration in cellular milieu is defined as normoxia (~21% O₂), physoxia (~1–13% O₂) or hypoxia (~0.1–1% O₂). Hypoxia, a striking micro-environmental feature in tumorigenesis, is countered by tumor cells via induction of O₂ governed transcription factor, hypoxia inducible factor-1 (HIF-1). Post discovery, HIF-1 has emerged as a promising anticancer therapeutic target during the last two decades. Recent reports have highlighted that enhanced levels of HIF-1 correlate with tumor metastasis leading to poor patient prognosis.

Material and methods: A systematic search in PubMed and SciFinder for the literature on HIF-1 biology and therapeutic importance in cancer was carried out.

Results: This review highlights the initial description as well as the recent insights into HIF-1 biology and regulation. We have focused on emerging data regarding varied classes of HIF-1 target genes affecting various levels of crosstalk among tumorigenic pathways. We have emphasized on the fact that HIF-1 acts as a networking hub coordinating activities of multiple signaling molecules influencing tumorigenesis. Emerging evidences indicate role of many HIF-induced proteomic and genomic alterations in malignant progression by mediating a myriad of genes stimulating angiogenesis, anaerobic metabolism and survival of cancer cells in O₂-deficient microenvironment.

Conclusions: Better understanding of the crucial role of HIF-1 in carcinogenesis could offer promising new avenues to researchers and aid in elucidating various open issues regarding the use of HIF-1 as an anticancer therapeutic target. In spite of large efforts in this field, many questions still remain unanswered. Hence, future investigations are necessary to devise, assess and refine methods for translating previous research efforts into novel clinical practices in cancer treatment.

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Introduction

Oxygen (O₂) homeostasis is a critically important factor for the survival of vertebrates [1]. The transcriptional induction of a multitude of genes engaged in angiogenesis, glucose metabolism, cell proliferation/survival and the regulation of physiological responses occurring at low O₂ levels is brought about by hypoxia inducible factor-1 (HIF-1), an O₂-labile DNA binding transcriptional activator [1]. Hypoxia abundantly occurs in core of the tumors owing to high proliferation rates of cancer cells coupled with the presence of abnormal vasculature [2]. Studies on the molecular mechanisms of hematopoietic growth hormone erythropoietin (EPO) induction, the most salient response to hypoxia, set the course for recognition of HIF-1 which operates ubiquitously in mammalian cells [3]. It was later purified and designated as HIF-1 [4]. Since its discovery [4], biochemical purification and molecular characterization [5], there has been an exponential rise in the interest to study the importance of HIF-1 in tumor biology in the past two decades, which has been depicted in a timeline form in Figure 1.

DNA sequencing in combination with genome-wide chromatin immunoprecipitation (ChIP-seq) and microarray

analysis projected many directly transactivated HIF target genes and microRNAs (miRNAs) that promote acclimatization to acute and chronic hypoxia [6]. However, within a cell only a fragment of this vast spectrum of genes is activated, causing cell-specific hypoxic responses [7]. Apart from this, gene expression is also indirectly regulated by HIFs through transactivating genes that encode microRNAs [6] and chromatin-modulatory enzymes [8]. The target genes triggered by HIF-1 includes those involved in angiogenesis, glucose metabolism, cell proliferation/viability, invasion and metastasis [9]. Proteins encoded by HIF target genes fall into two prominent categories, proteins increasing O₂ delivery and those decreasing O₂ consumption thus making HIF-mediated pathways critical in the development, physiology and disease. Notably, majority of human tumors have an overexpression of a wide plethora of these gene products [reviewed in 9], thus emphasizing the relevance of HIF-1 based transcriptome modulations in tumor pathophysiology. In this review, our focus will be more on the recent insights into the understanding of HIF-1 expression, activation and functions during cellular responses. We will outline emerging reports on various levels of crosstalk among HIF-1 and its metabolic regulators

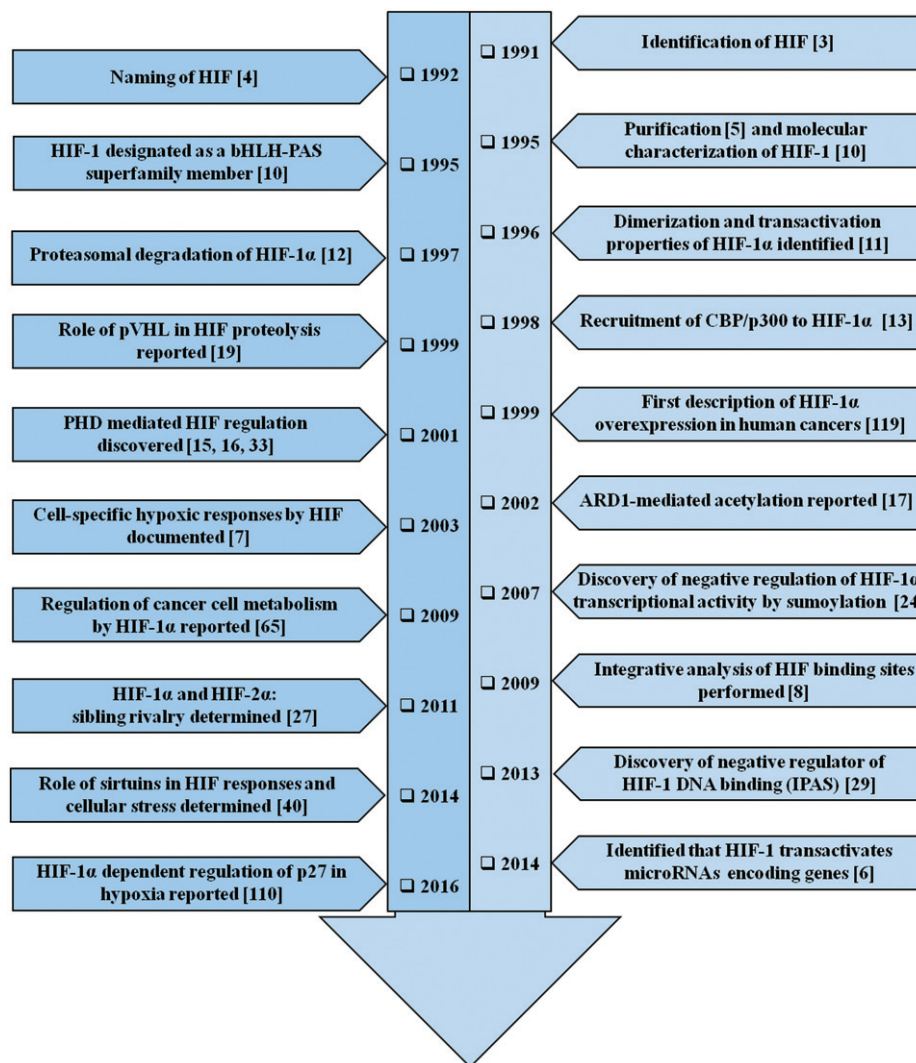


Figure 1. The timeline of HIF-1 research. The year-wise sequence of path breaking events in HIF-1 research are indicated with references. Events include the initial identification of HIF-1 up to the work being carried out in this field recently. The list is non-exhaustive and only a few major events are highlighted in the flowchart.

to summarize the role of HIF-1 downstream genes in tumorigenesis.

Structure and molecular biology of HIF-1: the master regulator of hypoxia

HIF-1 transcription factor is a heterodimer with one of the three hypoxia-induced α subunits (HIF-1 α /2 α /3 α) and a stable constitutively expressing β subunit (HIF-1 β , also known as aryl hydrocarbon receptor nuclear translocator (ARNT)). HIF-1 α , a basic-helix-loop-helix (bHLH)-PER (Period)-ARNT-SIM (single minded) (PAS) superfamily member is the O₂-labile subunit and is ubiquitously expressed in hypoxia [10]. Amino (NH₂⁺) terminus half (amino acids 1-390) of HIF-1 α contains bHLH and PAS domains vital for DNA binding and dimerization. On the other hand, the carboxy (COOH⁻) terminus possesses oxygen-dependent degradation domain (ODDD), which confers O₂-dependent instability and also two autonomous transactivation domains (N-TAD and C-TAD) in addition to an inhibitory domain (ID, negative regulator of the transactivation domains) [11]. ODDD is a proline-serine-

threonine rich protein stabilization domain (PSTD) extending from amino acids 429 to 608 of HIF-1 α [12]. Nuclear localization signal (NLS) domains extend from residues 17-33 (NLS-N) and 718-721 (NLS-C) [13] (Figure 2).

During hypoxia, N-TAD stabilizes HIF-1 α , whereas C-TAD modulates transcription of HIF-1 α target genes in association with CREB-binding protein (CBP)/p300 co-activator. Low O₂ level stabilizes the α -subunit causing its nuclear translocation (where it dimerizes with HIF-1 β) and further assembly of transcriptional co-activators [13]. HIF-1 target genes contain hypoxia responsive element (HRE, containing 5'-(A/G)CGTG-3' consensus sequence) where these heterodimers bind to an enhancer domain, activating their transcription [13].

Under normoxia, two proline residues (P402 and P564 in humans) located in the LXXLAP motif [14] in ODDD of HIF-1 α are hydroxylated by prolyl-4-hydroxylases (PHDs) [15]. PHD enzymes are dioxygenases that are 2-oxoglutarate (2-OG) and O₂-dependent, requiring Fe²⁺ and ascorbate as cofactors in a reaction yielding succinate and carbon dioxide as by-products. It is widely known that the activity of PHDs depends on O₂ as a direct substrate. Hence, PHDs have been

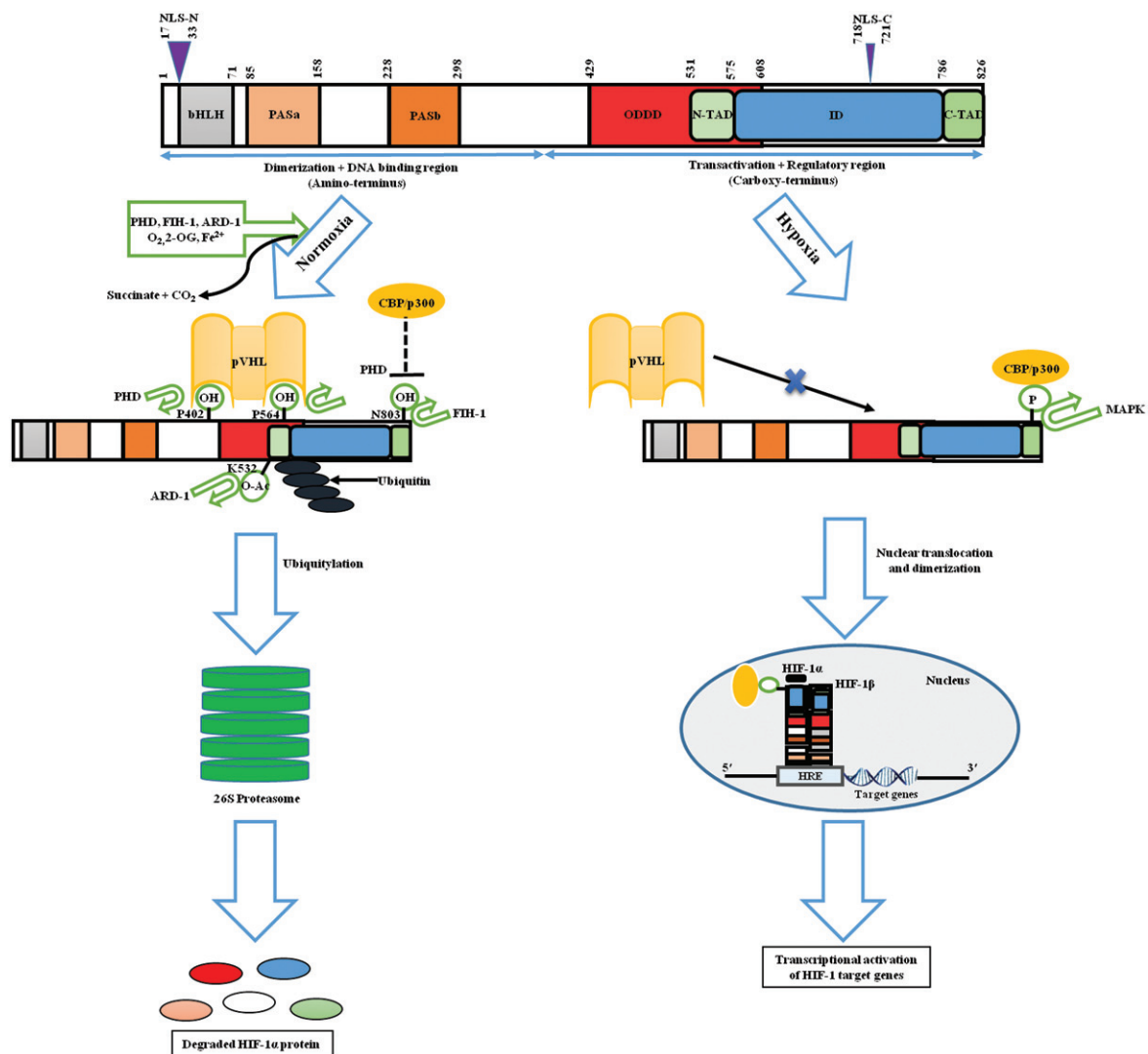


Figure 2. Structure and fate of the hypoxic master regulator. Figure presents the schematic representation of HIF-1 α structure. N-terminal includes nuclear localization signal (NLS), followed by a basic helix-loop-helix (bHLH) domain that precedes two Per-ARNT-Sim homology domains (PAS-A and PAS-B). In addition to it, inhibitory domain (ID) separates N- and C-terminal transactivation domains (N-TAD and C-TAD). Figure also portrays the fate of HIF-1 through the sequence of events that occur during normoxic and hypoxic cellular microenvironment. During normoxia, certain post translational modifications lead to HIF-1 α -pVHL binding and blocking of HIF-1 α /CBP interaction thereby causing HIF-1 α degradation leading to the inhibition of HIF-1 α -mediated transcription. During hypoxia, HIF-1 α -pVHL binding does not occur, while p300/CBP can interact with HIF-1 α , leading to transcriptional activation of target genes. Numbers highlights the amino-acid residues of various domains.

accurately projected as O₂ sensors coupling HIF-mediated molecular responses to cellular O₂ levels [16]. Lysine-532 (K532), another ODDD residue is acetylated by an acetyl transferase enzyme, arrest defective-1 (ARD-1) [17]. Collectively, these alterations lead to 26S proteasomal degradation of HIF-1 α subunit by tethering to the β -domain of von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase at Leucine-574 (L574) [18]. Maxwell et al. [19] first demonstrated a central role of pVHL in HIF-1 α regulation in 1999, and during the next decade mechanistic specifications of pVHL-HIF pathway and regulation of proteasomal degradation and polyubiquitylation were elucidated. Further, it was found that asparagine-803 (N803) residue of HIF-1 α is hydroxylated by asparaginyl hydroxylase, factor inhibiting HIF-1 (FIH-1) in an O₂-dependent way [20] (Figure 2). This drives the blocking of interaction between C-TAD and CBP/p300 domains and further abrogation of gene transcription by HIF-1 α [20]. Similar to PHDs, FIH-1 acts as a second O₂ sensor and is a

2-OG-dependent dioxygenase, requiring ascorbate and Fe²⁺ as cofactors [21].

Furthermore, S-nitrosation of HIF-1 α on cysteine-800 (C800) has been demonstrated to enhance its transactivation by interacting with CBP/p300 [22]. Later it was reported that the domain from 531 to 826 in HIF-1 α gets phosphorylated and regulates its expression [23]. It was also revealed that sumoylation on three lysine residues (K391, K477 and K532 on HIF-1 α) negatively regulates transcriptional activity of HIF-1 α [24].

HIF-1 isoforms

Homology searches in databases and cloning efforts led to the discovery of HIF-1 isoforms, HIF-2 α {HIF-1 α like factor or endothelial PAS domain protein 1 (EPAS1)} and HIF-3 α , which are other representatives of bHLH-PAS superfamily. The influence of HIF family on gene expression may vary among

numerous cell types. Similar to HIF-1 α , HIF-2 α is also stabilized by hypoxia and dimerizes with HIF-1 β . HIF-2 α , which has a restricted expression mainly in renal, endothelial, hepatic and lung cells, has 48% amino acid sequence similarity with HIF-1 α , unlike HIF-3 α [reviewed in 25]. Though both HIF-1 α and HIF-2 α stimulate target genes by linking to same HRE, they are functionally non-redundant [26]. HIF-1 α regulates glycolytic gene expression and preferentially drives apoptotic pathways not addressed by HIF-2 α , whereas HIF-2 α encourages tumor growth and angiogenesis [27].

Co-relation between HIF-1 α and HIF-2 α levels with tumor growth, angiogenesis and metastasis has been established in both animal as well as clinical investigations [1]. It is worth mentioning that HIF-1 α plays a critical role in initial responses (2–24 h) to severe hypoxia (<0.1% O₂) whereas HIF-2 α impacts chronic hypoxia (48–72 h) [28]. Very little was known about the newest isoform HIF-3 α until the identification of its spliced variant, inhibitory PAS (IPAS) which in a dominant negative way regulates HIF-1 DNA binding ability [29]. Recent reports have indicated that HIF-3 α is hypoxia-activated and promotes a distinct transcriptional response in zebrafish embryos [30]. HIF-3 α , unlike the other two isoforms has only one TAD, a unique LXXLL motif and a leucine zipper domain [30].

To date significant developments have occurred towards deciphering HIF's roles under both pathophysiological and physiological conditions. During physiological settings, interplay of HIF-1 α with HIF-2 α and HIF-3 α would provide immense understanding of HIF(s). Likewise, the interaction of these transcription factors with other important proteins and cascades will help in setting the future trends of investigation on HIF biology and its implications.

Regulation of HIF-1 pathway

In general, HIF-1 α is synthesized and constitutively transcribed by a cascade involving a series of growth factors and signaling events (Figure 3). Normoxia leads to quick degradation of HIF-1 α transcript ($t_{1/2}$ ~ 5 min) [31]. Conversely, hypoxic condition results in stability and transcriptional activity of HIF-1 α (as discussed above), through several pathways and post-translational modifications (hydroxylation, acetylation, ubiquitylation, phosphorylation, sumoylation, S-nitrosation) [32]. Under normal O₂ tension, the PHDs become active and cause prolyl hydroxylation in HIF-1, leading to a recognition indicator for pVHL binding, ubiquitylation and subsequent degradation of HIF-1 α [33]. Hypoxia ceases enzymatic activity, terminating proline modification and pVHL-HIF binding, causing HIF-1 α stabilization, nuclear translocation and further aggregation. The important modulators of HIF-1 are summarized in Table 1.

Reports have shown that activation of mechanistic target of rapamycin (mTOR) [34] and phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K) [35] can upregulate HIF-1 α translation. In addition, some growth factors activating RAS consequently stimulates RAS/RAF/MEK/ERK kinase pathway. The co-activator CBP/p300 is phosphorylated by ERK increasing the formation of HIF-1 α /p300 complex, thus in turn stimulating its transcriptional activation trait [35]. Recently HIF-1 α

has been reported to be activated at physiological O₂ levels in a p42/44 mitogen-activated protein kinase (MAPK)-dependent manner determining increased cellular proliferation commonly found in these conditions [36]. Apart from tumor suppressor genes reviewed ahead, certain growth factors and cytokines also have reasonable effects on the HIF-1 network. These include platelet-derived growth factor (PDGF) [37], insulin [38] and insulin-like growth factors (IGF) [39], which induces HIF-1 α translation (Table 1).

Recently a stress-responsive family of histone deacetylases, sirtuins is reported to influence DNA repair, transcription metabolism and metastasis [40]. These nicotinamide adenine dinucleotide (NAD⁺)-dependent enzymes have exhibited potential to regulate HIF activity, thereby strengthening the association between HIF responses and cellular stress [40]. During hypoxia, cellular NAD⁺ is decreased which reduces sirtuin 1 (Sirt1) levels, enhances HIF-1 α acetylation, and hence promotes HIF-1 α target gene expression. Poly (ADP-ribose) polymerase 1 (PARP1) is one more NAD⁺-dependent enzyme, which was reported to interact with and promote HIF-1 α transactivation [41]. Additionally, it has been reported that elevated nitric oxide (NO) levels and enhanced expression of nitric oxide synthase (NOS) isoforms increases HIF-1 α protein stability in human oral squamous cell carcinoma [42] (Table 1).

HIF-1 in tumors

A proper O₂ homeostasis is essential for mammalian cells in order to fulfill their energy requirement and aerobic metabolism. However, cancer cells become hypoxic due to decreased O₂ delivery and diffusion, combined with increased O₂ consumption. Tumors have high impairment in their cellular O₂ balance due to inadequate and chaotic vascularization. Hypoxia, a prominent characteristic feature of the tumor milieu drives aggressiveness of a growing tumor [43]. About 60% of solid tumors have <1% O₂ leading to intra-tumoral hypoxia. Tumor cells counter these alterations in O₂ tension by delegating HIF-1 α for orchestrating numerous of its essential functions. Upregulation of HIF-1 α activates many crucial cancer hallmarks such as angiogenesis, glucose metabolism, cell proliferation/viability, invasion and metastasis and has a crucial role in tumor survival and progression as discussed in the following sections. Figure 4 provides a list of genes targeted by HIF-1 that regulates various tumorigenic pathways. Manalo et al. [44] documented through DNA microarray experiments that greater than 2% of all genes in humans are HIF-1 regulated (directly or indirectly). In clinical settings, immunohistochemistry (IHC) analysis of patient's biopsy specimens exhibited dramatic HIF-1 overexpression in common human cancers, which leads to increased metastasis and mortality rates (Table 2). Table 2 clearly lists the localization of HIF-1 α expression in common human cancers along with the details of antibodies used for the immunostaining.

HIF-1 and reprogramming of tumor metabolism

Increased glycolytic rate during hypoxia is a critical step in meeting cellular energy demands. Metastatic tumor cells

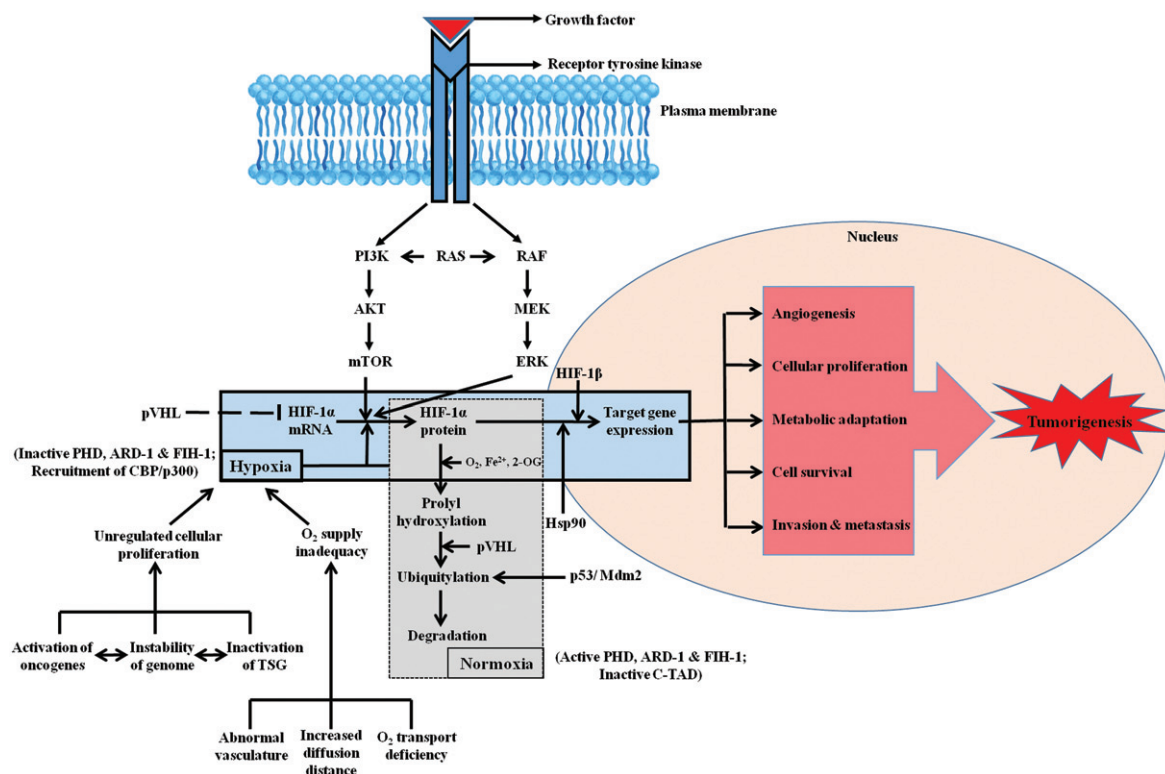


Figure 3. HIF-1 signaling cascade. Synthesis and constitutive expression of HIF-1 α by a cascade involving a series of growth factors and signaling events are indicated. The major differences among the hypoxic and normoxic signaling and sequence of events is also depicted clearly in the flowchart. Normoxia leads to HIF-1 α protein degradation whereas hypoxia leads to HIF-1 α -regulated target gene expression. The downstream sequence of events leading to tumorigenesis is also portrayed.

Table 1. Critical regulators of HIF-1 activity.

HIF-1 Regulators	Effect on HIF-1
<i>Environmental conditions</i>	
Hypoxia	Increases stability and transcriptional activity
Normoxia	Decreases stability
High temperature	Increases stability and expression
<i>Enzymes</i>	
VHL, PHD, ARD-1	Decreases stability
FIH-1, Sirtuin 1	Decreases transcriptional activity
PARP1	Increases transactivation
NOS	Increases stability
PHD and FIH-1 Inhibition	Increases stability and enhances transcription of target genes
<i>Co-factors</i>	
2-OG, Ascorbate, Fe ²⁺	Decreases stability
NO	Modulates expression
NAD ⁺	Decreases transcriptional activity
Copper	Increases expression
<i>Post-translational modifications</i>	
P402, P564 Hydroxylation	Decreases stability
K532 Acetylation	Decreases stability
N803 Hydroxylation	Decreases transcription
K391, K477, K532 Sumoylation	Negatively regulates transcriptional activity
C800 S-Nitrosation	Increases transactivation
Phosphorylation (531-826 residue)	Regulates expression
<i>Other genes</i>	
HSP90	Increases stability
mTOR, PI3K, PDGF, IGF, Insulin	Increases translation
p53, PTEN	Suppresses hypoxic induction and target activation
ERK	Increases transactivation and HIF-p300 binding
Ras	Increases expression
p44/42 MAPK	Increases stability and expression
CBP/p300	Increases transcriptional activity
IPAS	Decreases DNA binding and gene expression
HRE binding	Increases target gene expression

Major environmental, enzymatic, co-factors, post-translational and genetic regulators of HIF-1 expression, activity, translation and stability are listed.

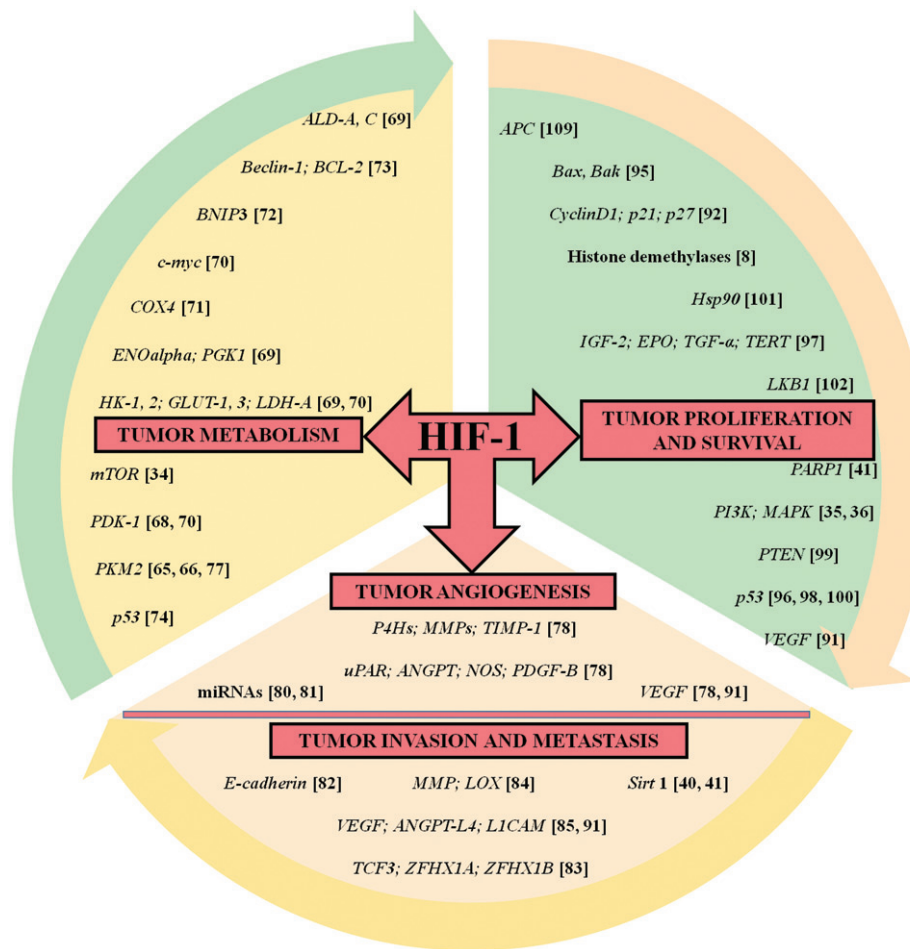


Figure 4. HIF-1 in the driver seat of tumorigenesis. The list of HIF-1 regulated genes (with corresponding references highlighted in bracket) having an impact on tumorigenic pathway is showcased. The HIF-1 target genes presented here are involved in processes such as cellular metabolism, proliferation and survival, angiogenesis, invasion and metastasis of tumor cells. The list is intended to be an illustration rather than being comprehensive.

have a remarkably increased glucose uptake rate in comparison to normal cells. This is such a reliable indication that it acts as a basis for diagnosis of tumor in patients by 18-F-fluorodeoxyglucose-positron emission tomography (FDG-PET) to ascertain metastases by imaging of FDG tracer [63]. HIF-1 serves as an important moderator of the Warburg effect, the tumor-related metabolic switch, which helps cancer cells to create energy largely by disintegration of glucose in a non-oxidative manner rather than typical oxidative phosphorylation [64]. HIF-1 stimulates the expression and activation of glycolytic enzyme isoforms differing from those found in normal cells, thereby supporting the Warburg effect by potentiating macromolecular biosynthesis and energy production pathways in human cancers [65]. For example, pyruvate kinase isoform M2 (PKM2) expression is imperative for the Warburg effect. Instead of PKM1, PKM2 physically joins HIF-1 stimulating its activity [66]. Additionally, recent reports point to mTOR and PKM2 as critical determinants of the Warburg effect, which to a certain degree can be attributed to their effect on HIF-1 α [34]. Furthermore, critical fallout of this glycolytic switch is tumor microenvironment acidosis. Collectively, the acidic milieu and metabolic transformation provides plenty of metabolic intermediates that spur progression and aggressiveness of tumors [67].

Interestingly, HIF-1 α induces glycolysis and actively restrains mitochondrial function and O₂ utilization by inducing pyruvate dehydrogenase kinase-1 (PDK-1) activity [68]. In the glycolytic pathway, HIF-1 targets include hexokinase 1 and 2 (HK1, HK2) enzymes, glucose transporters 1 and 3 (GLUT1, GLUT3), lactate dehydrogenase-A (LDH-A), aldolase A and C (ALD-A, ALD-C), phosphofructokinase liver type (PFK-L), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB-3), enolase alpha (ENOalpha) and phosphoglycerate kinase 1 (PGK1) [69]. Out of these GLUT1, LDH-A and HK2 are direct targets of oncogenic transcription factor MYC. HIF-1 α inhibits c-MYC activity under physiological conditions but during stress c-MYC works together with HIF-1 to induce expression of PDK-1 and HK2 leading to angiogenesis and aerobic glycolysis [reviewed in 70]. Also it has been seen that under hypoxic condition, HIF-1 α manipulates cytochrome c oxidase subunit 4 (COX4) switch, a homeostatic response optimizing respiration efficacy at varied O₂ concentrations [71]. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) is a HIF-1 target gene correlated to autophagy [72] through disruption of interaction between Beclin-1 (a highly conserved autophagy inducer protein) and B-cell lymphoma 2 (Bcl-2) or B-cell lymphoma-extra-large (Bcl-xL) [73]. Recent reports have exhibited that p53 is stimulated by hypoxia and acts as a negative regulator of glycolysis [74] (Figure 4).

Table 2. Human cancers exhibiting increased levels of HIF-1 α protein.

Cancer type	Antibody used	Localization of expression	References
Bladder cancer	Anti-HIF-1 α Ab-4 Mouse Monoclonal (Thermo MS1164P)	Nuclei, cytoplasm (very weak)	45
Breast cancer	Anti-HIF-1 α (BD Transduction Laboratories)	Principally in nuclei of metastasis and found in all cellular compartments	46
Cervical cancer	Anti-HIF-1 α Monoclonal (Novus Biologicals)	Exclusively nuclei	47
Colon cancer	Anti-HIF-1 α Monoclonal (Novus Biologicals)	Nuclei	48
Colorectal cancer	Anti-H1alpha67 (Abcam)	Expression restricted to cytoplasm and nuclei of metastatic cells	49
Endometrial cancer	Anti-HIF-1 α Mouse Monoclonal (BD Biosciences)	Perinecrotic	50
Esophageal squamous cell carcinoma (SCC)	Anti-HIF-1 α Monoclonal (Santa Cruz)	Weak in cytoplasm, strong in nucleus of tumor cells	51
Gastrointestinal stromal tumor	Anti-HIF-1 α	Nuclei	52
Glioma	Anti-HIF-1 α Clone 54 (BD Biosciences)	Nuclei of tumor cells surrounding necrotic areas	53
Head and neck SCC	Anti-HIF-1 α (BD Transduction Laboratories)	Perinecrotic severely hypoxic areas	54
Laryngeal cancer	Anti-HIF-1 α Monoclonal (Santa Cruz)	Mixed nuclear and cytoplasmic	55
Liver cancer	Anti-HIF-1 α Mouse Monoclonal (Novus Biologicals)	Nuclei of cancer cells near site of necrosis	56
Lung cancer	Anti-HIF-1 α Mouse Monoclonal (Novus Biologicals NB100-479)	Cytoplasmic in normoxia and nuclear in hypoxia	57
Melanoma	Anti-H1alpha67 Mouse Monoclonal (Santa Cruz sc-53546)	Cytoplasm and nucleus	58
Oligodendroglioma	Anti-H1alpha67 (Novus Biologicals)	Tumor cells	59
Ovarian cancer	Anti-HIF-1 α (BD)	Nucleus	60
Prostate cancer	Anti-HIF-1 α [EP1215Y] (Abcam ab51608)	Cytoplasm	61
Renal cancer	Anti-H1alpha67 Mouse monoclonal (Novus Biologicals)	Nuclear expression in tumor cells	62

The increased levels of HIF-1 protein were determined by IHC analysis of patient's tumor biopsies (the numbers mentioned in the last column depict references for the same). The cellular localization of HIF-1 α expression and the type of antibody used for immunostaining is also listed.

HIF-1-regulated switch in tumor angiogenesis

The angiogenic switch in hypoxic tumor microenvironment may be attributed to enhanced O₂ consumption and curtailed O₂ delivery as a result of increased diffusion distance. The discovery that hypoxia induces vascular endothelial growth factor (VEGF), served as a crucial link between hypoxia and angiogenesis [75]. Lately, it was reported that copper induces HIF-1 α and VEGF expression in hepatic and breast cancer cells [76]. Recently, it has come to light that in pancreatic cancer, PKM2 interferes in the activation of HIF-1 α that further triggers secretion of VEGF and subsequent formation of blood vessels [77]. Angiogenesis an intricate multistep and ordered process is essential for tumor formation and involves numerous genes, regulators and pathways [78]. HIF-1 α has been described to portray a vital part in angiogenesis, thus, silencing/inhibiting this pathway might result in reduced ability of cells to muster a strong vasculature and colonize at distant sites, even in the presence of an invasive phenotype.

The angiogenic induction results in an increased vascular density and decreased diffusion distance of O₂. However, local flow of blood under pathophysiological setting is regulated by vascular tone modulation through HIF-1 α -mediated direct induction of various genes encoding angiogenic growth regulators. This includes urokinase type plasminogen activator receptor (uPAR) that modulates matrix metabolism, angiopoietin 1 and 2 (ANGPT), vascular tone governing NOS, VEGF and PDGF-B (Figure 4). Additionally, HIF-1 α signaling pathways regulate factors such as collagen prolyl-4-hydroxylases (P4Hs), matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMP-1) [78]. *In vivo*

investigations revealed that targeting HIF-1 function significantly inhibits tumor vascularization [79]. Previous studies have demonstrated number of miRNAs induced during hypoxia and they play critical roles in angiogenesis [80]. miR-429 is induced during early stage of hypoxia in tumor cells and regulates HIF-1 mRNA levels [81]. Therefore, it is evident that HIF-1 antes up angiogenesis by a largely complex mechanisms rather than straightforward VEGF induction.

HIF-1 at the nexus of tumor invasion and metastasis

Metastasis, the primary reason of cancer-related mortality, takes place in a series of well-defined events. These encompass local invasion of tumor cells followed by intravasation into the blood stream, survival of circulating tumor cells, extravasation to far-off sites and finally proliferation that leads to colonization. The invasive and metastasizing property of tumor cells is unleashed by hypoxia. Stimulation of genes regulated by HIF has been related with enhanced metastasis in multiple tumors [9]. HIF isoforms have been reported to show divergent effects on invasion and metastasis [27]. Many studies have pointed towards the detection of HIFs in multiple cancers but their role in regulating extracellular matrix (ECM) degeneration, glucose metabolism, invasion and metastasis remains largely unexplored.

One of the most striking hallmarks of invasive cells is epithelial-to-mesenchymal transition (EMT), which is symbolized by impairment of epithelial cell-to-cell contact and the attainment of mesenchymal traits and motility. HIF-1 directs the expression of many EMT regulators thereby acting as one of

the contributing factor in tumor metastasis. Many reports have revealed that HIF-1 α expression in cancer increases invasion and induces the loss of E-cadherin [82]. In addition to this, HIF-1 drives transcriptional activation of genes encoding repressors of E-cadherin expression such as TCF3, ZFH1A and ZFH1B as well as other such proteins that are involved in providing a rigid cytoskeleton and cell-to-cell adhesion [83].

HIF-1 is engaged in the hypoxia-intervened modulation of many genes engrossed intricately in tumor growth and advancement [9]. The extent of tumor invasiveness and metastasis depends on HIF-1 regulated transcriptional activation of MMP and lysyl oxidase (LOX) that degrades and remodels ECM, respectively [84]. Further, HIF-1 target genes include permeability factors such as VEGF that promote cancer cells intravasation into blood vessels. In addition to this, secreted ANGPT-L4 and cell surface L1 cell adhesion molecule (L1CAM) proteins cause extravasation of malignant cells into metastatic sites [85] (Figure 4). Furthermore, silencing of HIF-2 α was found to be more effective in decreasing ECM degradation and reducing metastasis under hypoxia [27].

Experimental investigations on mouse models revealed that foundation of a premetastatic niche is needed at the metastatic site before the arrival of cancer cells. Tumor-secreted factors are the initiators of this process and act by mobilizing the bone-marrow-derived cells and their subsequent recruitment to the metastatic sites. These factors are further responsible for the modification of ECM thereby generating a niche required for extravasation and colonization of cancer cells [86]. A small subset of cancer cells capable of unlimited self-renewal are called cancer stem cells (CSCs) commonly known as tumor initiating cells. Many HIF inhibitors have been reported to target CSCs in an attempt to improve the effects of chemotherapy and angiogenesis inhibitors in *in vivo* models. CSCs offer unlimited proliferation potential and are responsible for metastatic tumors [87].

Many recent reports suggested the role of HIF-1 in CSCs maintenance in various cancer types [88]. Hypoxic exposure to cancer cell lines induces a HIF-1-dependent expression of certain genes that promote pluripotency and represses differentiation [88]. Breast cancer mouse model having knockdown of HIF-1 α led to a reduction in the percentage of breast cancer stem cells [89]. A major cause that limits the efficacy of cytotoxic chemotherapy in certain tumors is the enrichment of CSCs following treatment. CSCs have been reported to enhance the expression of genes that increase neoplastic cell survival by mediating cellular efflux of chemotherapeutic drugs [90]. It is quite possible that a strong positive selection occurs for severe O₂ gradient generating cancer cells due to the linked hypoxia-induced proliferation of CSCs. In a nutshell, these reports validate the role of intratumoral hypoxia and HIF-1 as the critical regulators of CSCs maintenance by transactivating genes promoting CSC phenotype.

HIF-1: the playmaker in tumor proliferation and survival

The first differentiating change between neoplastic and normal cells is the enhanced cellular proliferation rate and a

diminished cell death rate caused due to increased expression of survival and growth factors. In the HIF pathway, VEGF is a noteworthy transcription target with involvement in cellular proliferation and metastasis in tumors [91]. Emerging evidences document the potential role of HIF-1 in preventing cell death, stimulating cellular proliferation or even induction of apoptosis [92] as discussed below. The putative target genes of HIFs that can modify cell-cycle progression are cyclin D1, p21 and p27 [reviewed in 92]. The part that HIF pathway portrays in cell death is dubious and it depends on the O₂ concentration to determine whether apoptosis will occur or not. 0–0.5% O₂ level in cells induces apoptosis whereas if O₂ levels are in 1–3% range apoptosis does not occur [93].

The key determinant of apoptosis in cells is ATP; during hypoxia abundant glycolytic ATP leads to apoptosis [94]. Moreover, O₂ deprivation leads to inhibition of electron transport chain thereby causing a reduction in membrane potential at the mitochondria. This sequence of events causes release of cytochrome C into the cytosol of hypoxic cells through activation of BCL2-associated X protein (Bax) or BCL2-antagonist/killer (Bak), leading to activation of caspase-9-mediated apoptosis [95]. In addition to it, p53, an important apoptosis regulator, can induce Bak and Bax proteins thereby stimulating the apoptosis cascade through cytochrome C [96]. The growth/survival agents encoded by genes regulated by HIF in various tumors include VEGF, EPO, IGF-2 and transforming growth factor- α (TGF- α). These genes are the controlling hub of all the important tumor progression pathways, including angiogenesis, proliferation, invasion and colonization of far-off sites. Additionally, the expression of telomerase reverse transcriptase (TERT) causes the immortalization of tumor cells [reviewed in 97] (Figure 4).

Tumor suppressor genes and HIF-1

Tumor suppressor genes (TSGs) p53 [98] and phosphatase and tensin homolog (PTEN) [99] influence HIF-1 α by suppressing its hypoxic induction and target activation, probably via AKT modulation (Table 1). Literature suggests association of p53 loss of function with enhanced levels of HIF-1 α in certain tumors. In normoxia, HIF-1 α interacts with p53 allowing mouse double minute 2 homolog (Mdm2) governed HIF-1 α ubiquitylation and proteasomal degradation [100]. The chaperone heat shock protein 90 (Hsp90) interacts with HIF-1 α directly and is shown to induce certain conformational changes making it fit to couple with HIF-1 β , hence initiating transactivation pathway [101]. Interestingly, exposure to elevated temperature strongly upregulates HIF-1 α in mice model, suggesting a unique mechanism that stabilizes HIF-1 α under normoxia [101]. Many of the predominant driver mutations observed in malignant cells alter tumor metabolism as part of their mode of action. Recent data implicate the tumor suppressor gene liver kinase B1 (LKB1) as a central regulator of tumor metabolism and growth control through the regulation of HIF-1 α -dependent metabolic reprogramming [102]. Similarly, loss of PTEN can promote increased glucose uptake through elevated PI3K/Akt/mTOR signaling [103] while loss of

the VHL also promotes a similar metabolic phenotype through stabilization of the HIF-1 α [104].

Experimental data suggests that HIF-1 α can itself serve a tumor suppressor role in certain solid tumors [105]. In clear renal cell carcinoma, where VHL loss of function leads to an accumulation of HIF-1/2 α , HIF-1 α is lost during tumor progression, indicating its tumor suppressor role in the later stages of tumorigenesis [106]. The importance of HIF-1 α in leukemia has been investigated in different experimental models, which proposes that HIF-1 α has vital role in self-renewal and proliferation of leukemic cells. HIF1 α deletion impairs the proliferation of chronic myeloid leukemia by inhibiting progression of cell-cycle and induction of apoptosis in leukemia stem cells [107]. Similar observations have been made for the requirement of HIF-1/2 α in human AML using echinomycin inhibition [108]. Deletion of HIF-1 α resulted in faster development of the disease and an enhanced leukemia phenotype in some of the investigated models in acute myelogenous leukemia (AML) thus validating the tumor suppressor role of HIF-1 α [105]. Furthermore, hypoxia promotes a reduction in adenomatous polyposis coli (APC) mRNAs and protein in colorectal tumor via HIF-1 α -dependent mechanism, suggesting that suppression of APC by hypoxia can contribute to increased survival in hypoxic tumors [109]. It was also observed that loss of p53 in certain type of tumors is associated with elevated levels of HIF-1 α . In hypoxic tumors, loss or mutations in p53 revokes any chances for Mdm2-mediated degradation of HIF-1 α [100]. The role of HIF-1 α in regulating events that are involved in cell cycle arrest has been unclear. However, it is seen that hypoxia could cause HIF-1 α dependent increase in the expression of p27 leading to cell cycle arrest in G0/G1 phase [110].

Targeting HIF-1 for therapy: preclinical strategies

It is quite evident till now that HIF-1 regulation is an exceedingly convoluted network comprising several overlapping mechanisms and signaling cascades. Each and every step of this complex process has offered immense potential for research and therapeutic benefits. There has been huge curiosity in unraveling the intricate biology and processes of HIF-1 pathways. HIF-1 has shown profound impact on cancer progression, thus, there has been great enthusiasm in developing therapeutics that could target HIF-1. But, the utter complexity of HIF-1 pathway regulation has made this a very challenging task.

In an effort to discern the physiological importance of HIF (a key stress-responsive transcription factor), many recent studies have been performed on HIF-1 α knockout models both *in vitro* and *in vivo*. Recently, HIF-1 α ^{-/-} in mouse embryos has been reported to be lethal due to vascular regression and cardiac malformations. Also, HIF-1 α $\pm$ mice have been shown to develop normally but have impaired hypoxic and ischemic responses. Furthermore, studies were performed on HIF-1 α ^{-/-} mice confirming that HIF-1 α maintains O₂ homeostasis [111]. Various reports also suggested that PHD inhibition stabilizes HIF and augments gene expression which along with FIH inhibition induces a constellation

of target genes [reviewed in 112]. HIF-1 α loss-of-function mutation has been shown to modulate longevity and stress tolerance in *Caenorhabditis elegans* by different pathways. It has advanced our knowledge about the regulatory networks linking oxygen homeostasis and ageing [113]. In a recent study, HIF-1 α deletion led to enhanced leukemia phenotype in mice model [105]. Knockdown of HIF-1 α expression has been reported to cause complete inhibition of the hypoxic induction in breast cancer stem cells [89].

Targeting HIF-1 for therapy: current clinical settings

To date, inhibitors of HIF-1 have been classified by their inhibitory mechanisms yet none of them appears to inhibit the HIF-1 pathway as their specific target [114]. Hence, design of selective HIF-1 targeting molecules is highly likely to be the scope of future research. The regulation of HIF-1 α comprises very complex cascades hence designing the selective and rational inhibitors of HIF-1 α becomes highly challenging task. It is evident that the success of this research importantly depends on the sensitive and specific screening methods available. Targeting HIF-1 has become focus of cancer therapy research and quite a few HIF-1 suppressing agents are in the discovery pipeline.

Compounds with anti-HIF-1 activity are mainly classified on their modes of action as direct or indirect HIF-1 inhibitors. Direct HIF-1 inhibitors prevent transactivation, DNA binding and transcriptional activity of HIF-1 α , on the other hand indirect HIF-1 inhibitors work by blocking the HIF-1 α transcription or translation or by promoting HIF-1 α protein degradation [25] (Table 3). Inhibiting HIF-1 offers a novel avenue for modulating the tumor niche and may have promising clinical outcomes. Currently available HIF-1 inhibitors suffer a huge bottleneck due to their nonspecific mode of action. Where on one hand, majority of HIF-1 inhibitors reported thus far show antitumor action by HIF-1 α inhibitory mechanism, which includes HIF-1 but is not limited to it. While on the other hand, some HIF-1 inhibitors have a complex HIF-1 inhibition mechanism which possibly involves blocking several points in the HIF-1 pathway unselectively.

Many small molecules have been reported as HIF-1 α inhibitors [114]. Till date, no clinically approved selective HIF-1 α inhibitor has been reported, which may be partially due to the need of specifically targeting protein-protein interactions while escaping other pathways. The chemical inhibitors of HIF-1 can be classified as inhibitors of HIF-1 α transcriptional activity, protein translation, protein degradation and protein and nucleic acid inhibitors of HIF-1 [reviewed in 115]. The major chemical inhibitors of HIF-1 activity along with their modes of action have been listed in Table 3.

It is worth mentioning that majority of the potential HIF-1 targeting therapeutics were primarily discovered to target other endogenous molecules. Better understanding of the HIF-1 in consortium with empirical testing later led to the identification of the HIF-1 inhibiting activity of these molecules [114]. This could be the reason that currently there are no specific HIF-1 targeting potential therapeutic drugs in the market. This poses a great challenge in hypoxic tumor

Table 3. Major chemical inhibitors of HIF-1 activity.

Mechanism of inhibition	Agents	Target
<i>Inhibitors of HIF-1α transcriptional activity</i>		
HIF-1 α /HIF-1 β dimerization inhibitors	Acridavine	HIF-1 α /HIF-2 α PAS-B domain
HIF-1 α DNA binding inhibitors	Echinomycin, polyamides	HRE
HIF-1 α transcriptional activity inhibitors	Chetomin	p300 recruitment
	Bortezomib	Proteasome
	Amphotericin B	FIH-1
Regulate at multiple levels	YC-1 and analogs	FIH-1
<i>Inhibitors of HIF-1α protein translation</i>		
PI3K inhibitors	Wortmannin, LY294002	PI3K
mTOR inhibitors	Temsirolimus, rapamycin	mTOR
Topoisomerase I and II inhibitors	Camptothecin, topotecan	Topoisomerase I
Microtubule disrupting agents	2-Methoxyestradiol	Microtubule depolymerization
<i>Inhibitors of HIF-1α protein degradation</i>		
Hsp90 inhibitors	Galdanamyacin	Hsp90
Histone deacetylase (HDAC) inhibitors	Vorinostat, Trichostatin A	HDAC
Other classes	Wondonin	HIF-1 α -pVHL interaction
	Cardiac glycosides	Unknown
	Resveratrol	ERK 1/2 and AKT activation
<i>Protein and nucleic acid inhibitors</i>		
HIF-1 α stability inhibitors	Metallothioneins	Zinc deprivation
Transcriptional activity inhibitors	Herceptin	VEGF induction
mRNA and protein expression inhibitors	Small interfering RNA	HIF-1 α expression
Dimerization of HIF-1 α /HIF-1 β inhibitors	Nanobodies	HIF-1 α ODDD

The major therapeutic molecules that inhibit HIF-1 α or target HIF-1 α pathway are listed. The list of inhibitors mentioned here is just a depiction and is not comprehensive..

settings that demands for specific HIF-1 targeting drugs, which are currently being administered at lower doses so as to maintain prolonged inhibition of HIF activity [97]. Furthermore, it was found that combination treatment using HIF inhibitors and anti-angiogenic agents might improve outcome efficacy, reduce the likelihood of side-effects and effectively lower drug doses as shown in mouse models [116,117]. HIF targeting molecules possess the potential to combat cancer as well as cancer-associated inflammation and hence can be efficient future therapeutics. Traditional chemotherapy drugs might be more proficient when administered along with an HIF inhibitor. Interestingly, the therapeutic effects of HIF-1 inhibition by small molecules have already been exhibited in preclinical mouse models [118]. More competent understanding of structure and molecular biology of HIF-1 domains will unquestionably result in unearthing therapeutic compounds, which further could be explored for pre-clinical trials. In a nutshell, the development of specific HIF-1 inhibitors is of great importance.

Conclusion and future prospects

Seventeen years have passed since first description of HIF-1 α overexpression in human cancers [119], and during this time a large amount of experimental data has emerged delineating the molecular mechanisms and outcomes of enhanced HIF-1 expression in tumorigenesis (Table 2). Despite numerous recent advances in discerning molecular mechanisms of HIF pathways in response to hypoxic condition, many critical questions still remain unanswered. Investigating such queries could yield novel insights into interplay between various HIF-1 post-translational modifications (PTMs), better understanding of HIF-1 paralogs' functionality and the linkage among HIF-1 and other oncogenic pathways. The main challenge in front of the research fraternity today is the identification of

malignancies in which HIF-1 portrays a crucial part in disease pathogenesis in patients. Close association of laboratory investigators and clinical oncologists is necessary to devise, assess and refine methods for translating investigative outcomes into effective and safer cancer therapies.

In the present review, physiological adaptations of HIF pathways along with its importance have been discussed. It has been highlighted that the role of HIF varies under diverse settings. Various studies have showcased that HIFs are upregulated in tumor cells. HIF-1 portrays a pleiotropic part by boosting various molecular processes linked with tumorigenesis. Apart from this, HIFs have been reported to orchestrate integral roles in several crucial aspects of tumor biology such as microRNAs and epigenetic alterations. These findings accentuate the need to explore the feasibility of targeting HIF-1 directly or indirectly to clarify the enigma behind tumorigenesis. Therapeutic effects of HIF-1 inhibition have been determined *in vivo*. In a nutshell, this research area assures momentous advances in the days to come.

Future research should attempt to unearth the missing links between HIF and cancer metabolism. With the rapid advancement of molecular biology and emerging strategies in efficiently disrupting protein-protein interactions, it is very promising that selective HIF-1 inhibitors can be developed in the future. Future investigations should be aimed toward better understanding of therapeutic modulation of HIF-1 by deciphering molecular structure of domains and mechanisms mediating critical HIF-1 functions. Considering the rapid advancements in the field of molecular biology and presently emerging strategies it would soon be possible to efficiently study protein-protein interactions that are the cornerstones in understanding HIF-1 regulation. This will not only present supplementary research avenues, but will also help to answer complex questions that will undoubtedly boost our appreciation for therapeutic applicability of HIF pathways in

malignancy. Finally, HIF-1, the play maker in the hypoxic world could be the next big player in cancer research and be the much needed answer to the enigma of tumorigenesis.

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