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The Role of Hypoxia-Inducible Factors in Cancer Resistance

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Abstract

Diminished oxygen availability (hypoxia) is a hallmark of the tumor microenvironment. A major regulator of cellular adaptation to hypoxia is the hypoxia-inducible factor (HIF) family of transcription factors, which play key roles in many crucial aspects of cancer biology including angiogenesis, stem cell maintenance, metabolic reprogramming, resistance to apoptosis, autocrine growth factor signaling, the EMT program, invasion and metastasis.

Resistance to chemotherapy/radiotherapy is the primary cause for treatment failure in clinical oncology. Hypoxia and accumulation of hypoxia-inducible factors (HIFs) in solid tumors have been associated with resistance to treatment and poor prognosis. HIFs causes autophagy establishment to promote survival of cancer cells, and is also associated with the promotion and maintenance of cancer stem cells, a minority subpopulation within the tumor responsible for tumor recurrence and resistance to chemotherapy.

In this review, we provide a concise comparative description of the structure, regulation, transcribed genes and roles played by each HIFα subunit in coordinating the transcriptional responses to hypoxia and on the roles they play in the promotion of resistance to anti-cancer therapy.

Keywords: Hypoxia; Hypoxia-inducible factors; Cancer resistance; HIF1α; HIF2α; HIF3α; chemotherapy-resistance

Introduction

Hypoxia is a fundamental physiological stimulus that induces adaptive responses to maintain a homeostatic state. It was defined as a reduction in O_2 availability in one condition compared with another at different spatial or temporal conditions. For that reason, a constant O_2 supply, maintained by the vascular system in mammals, is critical for proper tissue development, homeostasis, and function [1].

Another condition that causes localized hypoxia is the rapid cellular division during embryonic development because hypoxia is not always a pathological entity; there is increasing evidence that it is an important component of some cellular niches, particularly those of stem and progenitor cells [2]. In addition, an abnormal condition such as the rapid tumor growth induces O_2 deprivation in intratumoral regions [3].

Hypoxia-inducible factors (HIFs) are a family of mammalian transcription factors that regulate the expression of a wide array of hypoxia-inducible genes to deal with physiologically O_2 changing concentrations. They have been characterized as α/β heterodimers of basic-helix-loop-helix DNA binding proteins of the PER-ARNT-SIM family (bHLH-PAS), in which the β-subunit is constitutive expressed and the α-subunit is regulated by oxygen levels. The genes regulated by HIFs are involved in the cellular adaptive response to hypoxia including erythropoiesis, apoptosis, angiogenesis, proliferation, and also in tumorigenesis [4,5]. In this respect, it has been demonstrated that HIFS regulate multiple steps of tumorigenesis and are typically associated in cancer cells with changes in metabolic reprogramming, neo-vascularization, invasion, metastasis, autophagy induction, drug resistance, and poor clinical outcomes [6].

Until now, three oxygen-dependent different alpha subunits encoded by different genes have been reported in human and other vertebrate species: HIF1α, first described by Semenza and colleagues in 1992 [7], HIF2α described by several groups in 1997, and HIF3α, discovered in mouse by Gu et al. in 1998 [8] and in humans by Makino et al. [9]. But while it has been clearly established that HIF1α and HIF2α function as master regulators of the response to hypoxia and are critical regulators of tumorigenesis, the roles played by HIF3α under hypoxia and in cancer biology are far less clear. The reasons of this reside not only on the initial discovery of a large array of HIF3α variants which has posed enormous challenges to study HIF3α-mediated physiological roles, but mainly on the prevailing view of HIF3α as a negative regulator of HIF1α and HIF2α actions, on the basis of the initial finding that two of its variants, lacking transactivation domains, act as negative regulators of HIF1α and HIF2α transactivating gene functions [9-11]. However, this dogma was refuted in 2014 by Zhang et al. [12], which demonstrated that HIF3α functions as a transcriptional activator in zebrafish embryos. They showed that under hypoxia, Hif3α stabilizes and binds to HREs in the promoters of its target genes and upregulates their expression. When tested in human cells, these authors found that both human HIF3α-9 and zebrafish Hif-3α were capable of upregulating target gene expression, suggesting that the function of this transcription factor is evolutionarily conserved, and providing unequivocal evidence that Hif-3a functions as an oxygen-dependent transcriptional activator *in vivo*.

The HIF1β subunits, also known as aryl hydrocarbon receptor nuclear translocators (ARNTs), are encoded by two genes *ARNT1* and *ARNT2*. Under conditions of normal oxygen tension, the alpha subunits are hydroxylated at key proline and asparagine residues, which inhibits their transactivation function and targets them for proteasomal degradation. Upon hypoxia, the HIF alpha subunits are stabilized and accumulate in the nucleus, where they dimerize with HIF1β, allowing them bind to DNA and stimulate the transcription of their target genes [13]. However, multiple oxygen-independent mechanisms can also

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lead to HIF stabilization: growth factors, deregulated oncogenes, and/or tumor suppressor-mediated signaling results in stabilized HIF alpha subunits. These signaling cascades often activate the mammalian target of rapamycin (mTOR), which in turn induces an increase in the rate of HIF translation [14,15]. Consistent with this, high expression levels of HIFs were detected in tumor cells in the absence of hypoxia due to the elevated oncogenic signaling in these cells.

HIFs have also been shown to have oxygen-independent roles in early development in mammals, frogs, fish, and invertebrates [16,17]. The mechanisms underlying these oxygen-independent roles of HIFs, however, are not well understood. In this review, we provide a concise comparative description of the structure, regulation, transcribed genes and roles played by each HIFα subunit in coordinating the transcriptional responses to hypoxia and on the roles they play in the promotion of resistance to anti-cancer therapy.

Protein Structure of the Oxygen-Labile Alpha Subunits

The HIF1α, HIF2α and HIF3α subunits conserve similarities in their protein structure, since they have several well conserved domains. As shown in Figure 1, all three subunits contain a basic helix-loop-helix (bHLH) domain that is necessary for DNA binding at a consensus hexanucleotide E box [18,19].

They also contain two repeats of Per-Arnt-Sim (PAS) domain, derived its name from the proteins found containing it: Drosophila Period (Per), the human aryl hydrocarbon receptor nuclear translocator (Arnt) and Drosophila Single-minded (Sim) [20]; the domain is itself made up of two repeats of approximately 50 amino-acid residues (known as PAS A and PAS B) separated by approximately 150 not conserved residues [21]; the function of these domains have been reported as dimerization motifs that allow binding with other PAS proteins, non-PAS proteins, and small molecules [21]. Both the bHLH and PAS domains display high sequence and functional conservation among the HIFs. In fact, while the PAS domains between HIF1α and HIF2α have approximately 70% identity, their bHLH domains share 85% identity with the basic region consisting of almost identical sequences. Comparatively, the bHLH and PAS domains of HIF3α share only 74% and 52-58% identity with HIF1α and HIF2α, respectively, revealing a more divergent nature of this paralog [13].

Another conserved domain is the PAS-associated COOH-terminal (PAC) domain, which consists of a 40–45 amino acid region located carboxy-terminal to the PAS sequence, and that likely contributes to the PAS structural domain [22,23]. All three HIF alha subunits (full lenght in the case of HIF3α) also carry N- transactivation domains (N-TAD) that are required for activation of HIF target genes. Oxygen-dependent degradation domains (ODD) within the alpha subunits confer oxygen-regulated turnover and overlap the N-TADs [24]. But whereas the alpha subunits HIF1α and HIF2α comprise and additional C-terminal transactivation domain (C-TAD) [25], HIF3α only contains the N-TAD [8,10,26]; instead of the C-TAD, the carboxy-end region of the HIF3α include a unique leucine zipper domain (LZIP) composed of four-septad leucines, and an LXXLL protein-protein interaction motif which is found immediately upstream of the ODD and LZIP domain [26] as it can be seen in (Figure 2).

The LZIP domain represents a characteristic property of a DNA binding protein [27]; and the LXXLL motif is mostly conserved in nuclear receptor co-factors [28]. The N-TAD confers the specificity to the target gene, by interacting with additional transcriptional cofactors [29]. The C-TAD contributes to the regulation of HIF target genes by binding to the co-activators CBP, p300, SRC-1, and TIF-2 [30-32]. CBP and p300 are paralogous transcriptional coactivators that are essential for linking the α-subunit and other transcription factors with co-activator complexes and with the basal transcriptional machinery, and are thus indispensable for robust transcriptional activation. However, the role of N-TAD to induce the transcription action has been reported in a subset of HIF target genes that depend exclusively on the N-TAD and are not influenced by changes in C-TAD activity [33].

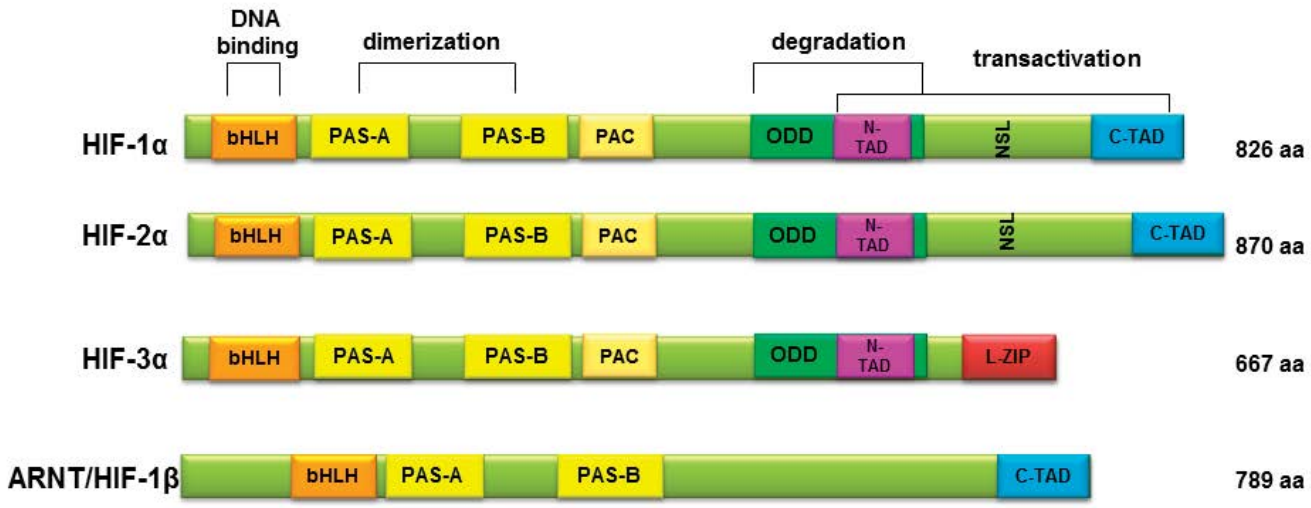

Figure 1: Hypoxia-Inducible factors. Structural alignment of HIF1α, HIF2α and HIF3α subunits. All of them contain a basic helix-loop-helix (bHLH) and two Per-ARNT-Sim (PAS-A and PAS-B) domains that mediate DNA binding and dimerization, respectively. They also contain a PAS-asociated C-terminal (PAC) domain, and an oxygen-dependent degradation domain (ODD) that is required for oxygen-dependent hydroxylation and degradation under normoxia conditions. The transactivation domains (N- and C-Terminal TADs) are responsible for the transcriptional activity of HIF1α and of HIF2α. N-TAD domain is located within the ODD domain and C-TAD domain at the C-terminal region of the protein. HIF3α lacks C-terminal transactivation domain and instead contains LZIP domain. HIF1α and HIF2α have nuclear localization signals (NLS). HIF1β is the aryl hydrocarbon receptor nuclear translocator (ARNT), which has a bHLH domain, PAS-A and PAS-B domains, and only one C-terminal transactivation domain. Modified from Pasanen et al. and Maynard et al.

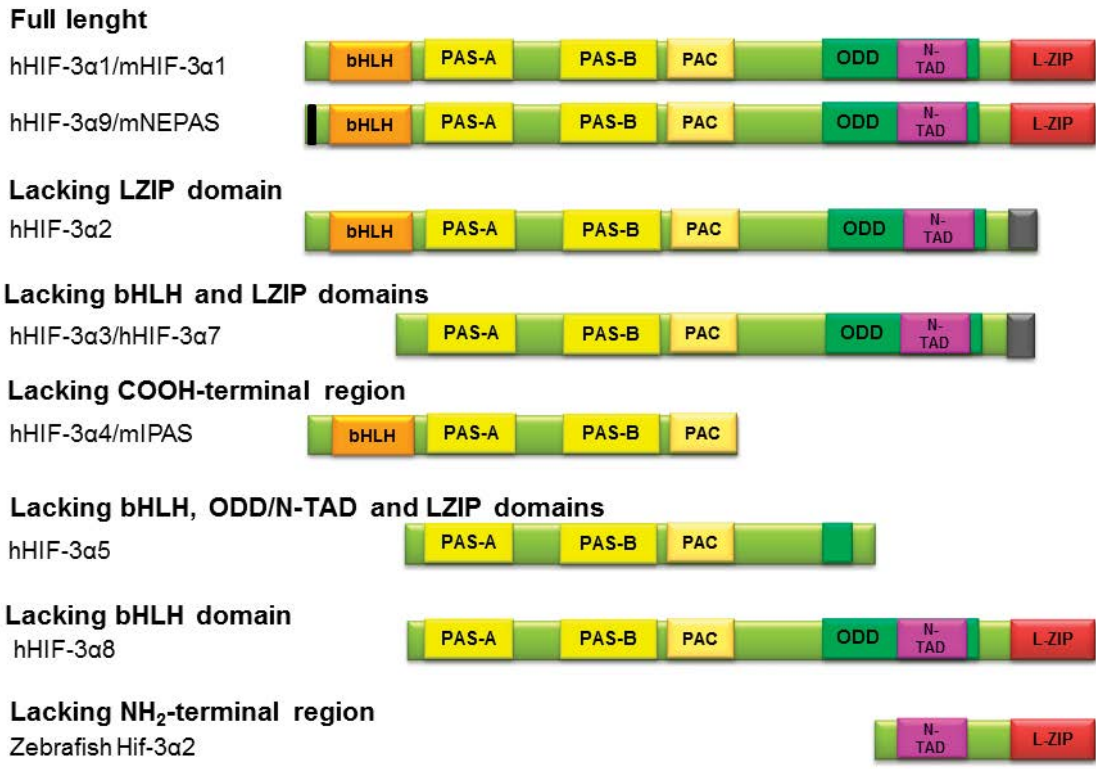

Figure 2: Hypoxia inducible factor-3α variants. Structural alignment of functional domains of HIF3α are illustrated as open boxes similar to Figure 1. HIF3α1, HIF3α2, HIF3α4, and HIF3α9 contain bHLH domains. All HIF3α variants contain PAS-A and PAS-B domains, except zebrafish Hif3α2, which lacks PAS domains. The hHIF3α4, hHIF3α5, and Hif3α2 variants lack ODD domains. N-TAD domain is present in all HIF3α variants except in HIF3α4 and HIF3α5. HIF3α9 is similar to full length HIF3α1 protein, but there are three possible variants of it because of the use of different promoters. Modified from Cunming Duan, Am J Physiol Cell Physiol 2016.

Transcriptional and Post-transcriptional Regulation of Alpha Subunits

A salient feature in both HIF2α and HIF3α subunits regulation is that, in addition to altering protein stability, hypoxia also increases their levels by inducing their gene transcription. This mechanism distinguishes the *HIF1α* gene from the *HIF2α* and *HIF3α* genes. In this respect, Lin et al. [34] reported that transcription of HIF1α and HIF2α is differentially regulated under hypoxia in neuroblastoma cell lines. They found that while transcription of HIF1α was consistently repressed by both acute and chronic hypoxia, transcription of HIF2α was consistently upregulated under the same hypoxic conditions. Their observations thus suggest that expression of HIF1α and HIF2α is differentially regulated in the hypoxic tumor microenvironment, although the underlying mechanisms remain to be fully investigated.

In the case of HIF3α, there is also experimental evidence that hypoxia induces *HIF3α* gene transcription and has been documented in human cells, mice, rats, and zebrafish. Interestingly, the hypoxic induction of *HIF3α* gene expression is mediated by HIF1α and/or HIF2α in a tissue-specific fashion [35]. The hypoxic induction of *HIF3α* gene expression may be particularly important for the HIF3α variants lacking an ODD domain.

Post-Translational Modifications

The HIF alpha subunit is subjected to post translational modifications, whose affect its stability and its gene transactivating functions. The oxygen-dependent stability and activity of the HIFα subunits are traditionally associated to post-translational modifications such as hydroxylation, acetylation, ubiquitination, and phosphorylation. The most characterized of them are the hydroxylations, which are catalyzed by 2-oxoglutarate and iron (Fe^{2+})-dependent family of dioxygenases that employ molecular oxygen as co-substrate and ascorbate as cofactor, and are called prolyl hydroxylases (PDHs) [36,37]. Proline hydroxylation allows binding of the von Hippel-Lindau (VHL) tumor suppressor protein, which in conjunction with elongins B and C functions as an E3 ubiquitin ligase complex, which poly-ubiquitinates the HIFα subunits targeting them for proteasomal degradation.

The HIF1α subunit has two specific proline residues at its ODD domain that are hydroxylated: the proline 564 [38,39] and the proline 402 [36]. These both sites are necessary for optimal targeting of the HIF1α to the proteasome and the hydroxylation order is important since Pro564 controls the efficient hydroxylation of proline 402 [40]. The HIF2α subunit is also hydroxylated by PHDs at the ODD domain, but in different positions of proline residues: Pro405 and Pro531. In the case of HIF3α, it has been demonstrated that pVHL recognizes the hydroxylated Pro490 at the ODD domain of HIF3α. So at least, one site of this α-subunit has been demonstrated to be subject to regulation by oxygen levels [26].

Whereas PHDs mediate the hydroxylation of conserved ODD domain prolyl residues, the activity of the C-TAD of HIF1α and HIF2α is additionally regulated by hydroxylation of a single conserved asparaginyl residue (in HIF1α: Asn803 and in HIF2α: Asn851) to prevent its physical interaction with the transcriptional coactivator

CBP/p300, resulting in silencing of HIFs transcriptional ability. This hydroxylation is catalyzed by another member of the 2-oxoglutarate and iron-dependent family of dioxygenases, called factor-inhibiting HIF (FIH-1) [41]. Despite FIH and PDHs are members of the same dioxygenases family, the estimated Km values of FIH-1 and the PHDs for molecular oxygen are different [42]. FIH has a lower Km for oxygen than the PHDs, meaning that the PHDs would be inactivated first, while FIH-1 would require more severe hypoxia to lose activity. FIH-1 can still exert a catalytic effect at 0.2% oxygen concentration, whereas the PHDs are inactive under the same conditions [43]. Nevertheless, the oxygen-dependent sensitivity of FIH-1 apparently fluctuates between different cell types independently of the PHDs [44]. Another interesting data is that the FIH action appears to require the association with VHL [41]. Since HIF3α lacks the C-TAD, the FIH-mediated hydroxylation mechanism is unlikely involved in HIF3α regulation.

Under hypoxic conditions, the PHD enzyme cannot hydroxylate HIF, and therefore HIFs are not recognized by pVHL. As mentioned before, In addition to oxygen, PHDs require Fe^{2+}, 2-oxoglutarate, and ascorbate for prolyl-hydroxylase activity. Thus, their action can be inhibited by nitric oxide, several metabolic intermediates of the tricarboxylic acid (TCA) cycle such as succinate and fumarate [45], and also by the reactive oxygen species (ROS) presumably by oxidizing PHD-bound Fe^{2+} [46]. As a result of dioxygenases inhibition, HIFα subunits accumulate in the cell, dimerize with HIF1β, translocate into the nucleus, recruit coactivators such as P300/CBP, and bind to hypoxia response elements (HRE: [A/G]CGTG) in the promoter regions of the target genes [47].

Phosphorylation of HIF1α and HIF2α subunits has been demonstrated to enhance transactivation of target genes by either disrupting interaction with VHL and thereby stabilizing HIFs, or by increasing the affinity of HIFs for transcriptional coactivators [13]. In this regard, it has been reported that casein kinase II phosphorylates both HIF1α and HIF2α at conserved theonine residues in their C-TADs and that the mutation of these residues diminished their activity [48,49]. In addition, it has also been reported that ERK1 directly phosphorylates the C-terminal domain of HIF1α (at Ser-641 and Ser-643) and that these modifications mask a nuclear export signal leading to increased nuclear accumulation and transcriptional activity [50]. There are also examples in which the phosphorylation of HIFs decreases HIF stability or activity. For example, the phosphorylation of HIF1α within the PAS-B domain at Ser-247 by casein kinase 1, inhibits its association with HIF1β, decreasing HIF1α -induced gene expression [51,52]. Glycogen synthase -3β (GSK-3β) phosphorylates HIF1α at several serine residues within its ODD domain inducing its degradation *via* proteasome in a VHL-independent manner [53].

HIFs are also regulated by lysine acetylation both positively and negatively, depending on the location of the modified lysine. Acetylation of amino-terminal HIF1α lysines negatively affects HIF1α stability and impairs activation of its target genes [54]. However, the acetylation of lysines located at the carboxy-terminal region of HIF1α increases HIF1α protein levels and enhances target gene activation [55,56]. In addition, it has been reported opposing effects of SIRT1 on HIF1α and HIF2α transcriptional activities: whereas SIRT1 forms a complex with HIF2α and deacetylates conserved lysine residues in the N-TAD which enhances HIF2α transcriptional activity *in vitro* and *in vivo*, SIRT1 was reported to deacetylate lysine residues in HIF1α resulting in HIF1α transcriptional repression [57].

Post-Transcriptional Modification by Alternative Splicing

The *HIF3α* gene has been found subjected to complex regulation producing a large number of mRNA variants due to the utilization of different promoters, different transcription initiation sites, and alternative splicing in mammals. However, as pointed by Duan C in 2016 [35], the existence of multiple variants may not be unique to the *HIF3α* gene or restricted to vertebrates, since human *HIF1α* and *HIF2α* genes can both give rise to several variants by the same mechanisms. According to NCBI and Ensemble databases, there may be several different human HIF1 and HIF2 transcripts, which have not been explored.

The human *HIF3α* gene has 10 predicted variants, as depicted in Figure 2. The gene spans 43 kb and contains 17 exons. It has 3 different promoters so HIF3α can be transcribed with an amino-terminal a, b or c type. Furthermore, different mRNAs can be produced by alternative splicing [26], as shown in Figure 2. The HIF3α-1 contains the NH_2-terminal type c, and presents the exons 1 to 17, so once translated, possesses 667 aminoacid residues. HIF3α-9 contains 669 residues and differs from HIF-3α-1 only by several amino acids of the very NH_2-terminal end. This HIF3α-9 is considered as full-length canonical protein because it has all of the six characteristic domains. HIF3α-2 has 632 residues and contains the bHLH, PAS-A and -B, PAC, ODD, and N-TAD domains, but lacks the LZIP domain and the second LXXLL motif. HIF3α-3 lacks the bHLH and LZIP domains. HIF3α-4, which presents 363 aminoacid residues, is similar to the mouse IPAS in structure and it has the bHLH, PAS-A, and PAS-B domain, but lacks the ODD and LZIP domains. HIF3α-5 only has the PAS-A, PAS-B, and PAC domains. HIF3α-6 should present 237 residues and was thought to be an artifact. HIF3α-7, with 607 aminoacid residues, lacks the bHLH domain and it also has a different sequence after the ODD/N-TAD domain due to the utilization of a unique exon 15. HIF3α-8 lacks only the bHLH domain and has 613 aminoacid residues. HIF3α-10 retains only the intron 1, so it encodes a 7 aminoacid peptide [26,58,59].

The HIF3α isoforms are often expressed in different tissues, at different developmental stages, and are differentially regulated. They have distinct or even opposite functions when tested by overexpression approaches [35]. For instance, while the full-length human HIF3α-1 can stimulate HRE-dependent reporter construct activity and up-regulate unique target genes [8,12], human HIF3α-4 isoform, a shorter isoform that lacks the TAD domain, inhibits the activity of HIF-1α and HIF-2α [11,60] in a similar manner as mouse IPAS was shown to inhibit HIF-1α activity [9].

Recently, Zhang et al. [17] have identified a novel zebrafish Hif3α spliced variant, termed Hif3α-2 isoform, as an oxygen-insensitive nuclear protein. Despite its lack of the bHLH and PAS domains, Hif3α-2 has HRE-dependent transcriptional activity. They investigated the *in vivo* role of Hif3α-2 using transgenesis and CRISPR/Cas9-mediated gene editing and showed that Hif3α-2 inhibits canonical Wnt signaling by binding to β-catenin and destabilizing the nuclear β-catenin complex, in a manner independent of its HRE-dependent transcriptional activity [17].

Genes Regulated By Hif Alpha Subunits

The three HIFα subunits are regulated in a similar fashion by hypoxia, bind to HIFβ and to same HREs, and share many overlapping genes and functions. However, HIFs are non-redundant and regulate both overlapping and unique downstream target genes as it can be seen

in Tables 1-5. With the generation of knockout mice, tissue specific conditional mice, and other experimental approaches, it has been demonstrated the functions they are involved in, and which genes they regulate.

There are distinct groups of HIFα -regulated genes: (1) those that are up-regulated only by each HIFα subunit, (2) those that are regulated by both HIF1α and HIF2α, or by HIF1α and HIF3α with similar potencies, and (3) those that are regulated by both HIF1α and HIF3α but with different potencies. Importantly, it has been showed that the transcriptional activity is conserved between species, since for example, zebrafish Hif3α and human HIF3α-1 and HIF3α-9 isoforms up-regulate similar target genes [12].

The genes regulated by each HIFα subunit, and the reported functions for each one are shown in (Tables 1-3). The reported genes regulated by both HIF1α and HIF2α, or by both HIF1α and HIF3α, are shown in (Tables 4 and 5), respectively. Although a highly structural homology exists between HIF1α and HIF2α, these transcription factors may also be differently regulated and transactivate common and unique target gene products in a cancer cell-dependent manner under normoxic and hypoxic conditions. As it can be seen in (Tables 1 and 2), in general, HIF1α may specifically induce the enhanced expression of glycolytic enzymes such as hexokinase-2, aldolase A, phosphoglycerate kinase 1 (PGK1) and pyruvate kinase M (PKM), whereas HIF2α appears to preferentially up-regulate the gene products including TGF-α, cyclin D1 and embryonic stem cell-like markers such

GENE	CELL FUNTION	CELL TYPE
HK-II	Metabolism and Glucose metabolism	RCC [91]
Glucose-6-Phosphate Isomerase	Metabolism and Immune System	RCC [91]
PFK1	Metabolism and Glucose metabolism	RCC [91]
Aldoase A	Metabolism and Immune System	RCC [91]
Aldolase C	Metabolism and Immune System	RCC [91]
Triosephosphate isomerase	Metabolism and Glucose metabolism	RCC [91]
GAPDH	Metabolism and Glucose metabolism	RCC [91]
PGK-1	Metabolism and Glucose metabolism	RCC [91]
PGM-1	Metabolism and Immune System	RCC [91]
Enolase 1	Metabolism and Glucose metabolism, and poly(A) RNA binding and transcription corepressor activity	RCC [91]
LDHA	Development Ligand-independent activation of ESR1 and ESR2 and glycolytic metabolism	RCC [91]
HK1	Glycolysis	Mouse ES [92, 93]
HK2	Glycolysis	RCC [91], mouse ES [92,93]
PKM	Glycolysis	RCC [91], mouse ES [92,93]
ALDA	Glycolysis	RCC [91], mouse ES [92,93]
ALKBH5	DNA Damage Reversal, DNA Double-Strand Break Repair and RNA Demethylation	MCF7, U2OS IMR32 cells [94]
L1CAM	cell adhesion molecule	MDA-MB-231 [95]
RHOA	Movility	MDA-MB-231 cells [96]
ROCK1	Movility	MDA-MB-231 cells [96]

HK-II: Hexokinase 2; **PFK1**: Phosphofructokinase; **GAPDH**: Glyceraldehyde-3-Phosphate Dehydrogenase; **PGK-1**: Phosphoglycerate Kinase 1; **PGM-1**: Phosphoglucomutase 1; **Enolase 1** (C-Myc Promoter-Binding Protein); **LDHA**: Lactate Dehydrogenase A; **HK1**: Hexokinase 1; **HK2**: Hexokinase 2; **PKM**: Pyruvate kinase M; **ALDA**: aldehyde dehydrogenase 1; **ALKBH5**: AlkB Homolog 5 (RNA Demethylase); **L1CAM**: L1 Cell Adhesion Molecule; **RHOA**: Ras Homolog Family Member A; **ROCK1**: Rho Associated Coiled-Coil Containing Protein Kinase 1.

Table 1: Representative genes only regulated by *HIF1α*.

GENE	CELL FUNTION	CELL TYPE
ARG1	Inhibitor of NO production	Macrophages [97]
EPO	Erythropoiesis	Kidney [98-100], liver [100, 101]
OCT4	pluripotency	Mouse ES [84], hESC [99]
SCGB3A1	Secretoglobin 3A1	NSCLC [103]
TGFα	Growth Factor	RCC [83, 104], HCC (HepG2 [105], Huh7 [12] cells)
CCND1	Cell cycle progression	RCC [83], HCC (HepG2 cells [105])
DLL4	NOTCH signaling, EC branching	Mouse ECS [106]
ANG2	Blood vessel remodeling	Mouse ECS [106]
NANOG	pluripotency	hESC [102]
SOX2	pluripotency	hESC [102]
MMP9	invasion	RCC [107]
PAI-1	fibrinolysis	RCC [108], LN229 [109]
uPAR	invasion	RCC [108]
DMT-1	Iron metabolism and Lysosome	Mouse intestine [110, 111]
FBN1	Iron transporter	Mouse intestine [110, 111]

ARG1: Arginase 1; **EPO**: Erythropoietin; **OCT4**: Octamer-Binding Protein 4; **SCGB3A1**: Secretoglobin Family 3A Member 1; **TGFα**: Transforming Growth Factor Alpha; **CCND1**: Cyclin D1; **DLL4**: Delta Like Canonical Notch Ligand 4; **ANG2**: Angiopoietin 2; **NANOG** (Homeobox Transcription Factor Nanog); **SOX2**: SRY-Related HMG-Box Gene 2; **MMP9**: matrix metalloproteinases-9; **PAI-1**: plasminogen activator inhibitor-1; **uPAR**: urokinase-type plasminogen activator receptor; **DMT-1** (SLC11A2: Solute Carrier Family 11 Member 2); **FBN1**: Fibrillin 1; **hESC**: human embryonic stem cell.

Table 2: Representative genes only regulated By *HIF 2α*.

GENE	CELL FUNTION	CELL TYPE
REDD1	Involved in mTOR pathway	HEK293 cells [12]
LC3c	Autophagy	HEK293 cells [12]
SQRDL	Metabolism	HEK293 cells [12]
zp3v2	Embryogenesis	Zebrafish [12]
*isg15**	Chemotactic activity towards neutrophils, direction of ligated target proteins to intermediate filaments, cell-to-cell signaling, and antiviral activity during viral infections	Zebrafish [12]
sqrdl	Metabolism: catalyze the conversion of sulfide to persulfides, thereby decreasing toxic concencrations of sulfide	Zebrafish [12]
EH546362		Zebrafish [12]
*gcnt7**	Metabolism and O-linked glycosylation	Zebrafish [12]
*mcl1b**	BCL2 Family Apoptosis Regulator	Zebrafish [12]
*casp8**	Signaling pathways of apoptosis, necrosis and inflammation	Zebrafish [12]

REDD1: DNA Damage Inducible Transcript 4**; LC3c** (Autophagy-Related Protein LC3 C)**; SQRDL:** Sulfide Quinone Reductase-Like (Yeast)**; zp3v2:** zona pellucida glycoprotein 3d tandem duplicate 2**; isg15:** Interferon-Stimulated Protein, 15 KDa**. *; sqrdl :** Sulfide Quinone Reductase-Like**; EH546362** (cystatin 14b, tandem duplicate 2)**; gcnt7:** Glucosaminyl (N-Acetyl) Transferase Family Member 7***; mcl1b:** myeloid cell leukemia 1b***; casp8:** Caspase 8, Apoptosis-Related Cysteine Peptidase***. (*)** Exist a human ortholog gene**.**

Table 3: Genes only regulated by *HIF 3α*.

GENE	CELL FUNTION	CELL TYPE
NDRG-1	Stress responses, hormone responses, cell growth, and differentiation	RCC [91]
DMXL-1	Regulatory functions	RCC [91]
GLUT1	Glucose transport	RCC [91, 92, 93]
ADRP	Lipid metabolism	RCC [91]
CAXII	pH homeostasis	RCC [91]
FILAG	Cytoskeletal structure	RCC [91]
IL-6	Inmmune cytokine	RCC [91]
ADM1	Angiogenesis	RCC [91]
VEGF	Angiogenesis	RCC [91, 83, 29] Hep3B [91, 83, 29] HepG2 [105]
LOX	cross-linking of extracellular matrix	HEAK293T [112]
PFKFB4	Metabolism	HEAK293T [112]
BNIP3	Autophagy, apoptosis	HEAK293T; RCC [112, 83]
RAB20	Autophagy	HEAK293T [112]
ANGPTL4	Erythropoiesis	MDA-MB-231 cells [95]
HIF3α	Transcription factor	RCC [59]

NDRG-1: N-myc downstream-regulated gene 1 protein**; DMXL-1:** Dmx-like 1**; GLUT1:** Glucose transporter 1**; ADRP:** Adipose Differentiation-Related Protein**; CAXII:** Carbonic anhydrase XII**; FILAG:** Filaggrin**; IL-6:** Interleukin 6**; ADM1:** Adrenomedullin**; VEGF:** vascular endothelial growth factor**; LOX:** Lysyl Oxidase**; PFKFB4:** 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4**; BNIP3:** BCL2 Interacting Protein 3**; RAB20 (**Member RAS Oncogene Family)**; ANGPTL4:** angiopoietin-like 4**; HIF3α:** Hipoxia Inducible Factor 3.

Table 4: Representative genes regulated by both *HIF1α* and *HIF2α*.

GENE	CELL FUNTION	CELL TYPE
*myhz2**	Cytoskeleton organization	Zebrafish [12]
susd5	Hyaluronic acid binding	Zebrafish [12]
*atxn1b **	Involved in transcriptional repression and to regulate developmental processes.	Zebrafish [12]
*mlp3c **	Autophagy	Zebrafish [12]
zgc:153723	Sulfotransferase activity	Zebrafish [12]
*zgc:153126**	Cytoskeleton organization	Zebrafish [12]
fmn2b	Segmentation, central nervous system	Zebrafish [12]
htra1a	Development	Zebrafish [12]
wu:fc34e06		Zebrafish [12]
*wsb1**	Probable substrate-recognition component of a SCF-like ECS (Elongin-Cullin-SOCS-box protein) E3 ubiquitin ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins	Zebrafish [12]
casp6l2	cysteine-type endopeptidase activity	Zebrafish [12]
zgc:162126 (ppp1r15a):	Larval:Protruding-mouth	Zebrafish [12]
cox5b2	Cytochrome-c oxidase activity	Zebrafish [12]
slc2a1b	D-glucose transmembrane transporter activity; sprouting angiogenesis	Zebrafish [12]

Myhz2: myosin, heavy polypeptide 2*; **susd5**: Sushi Domain Containing 5; **atxn1b**: Spinocerebellar Ataxia Type 1 Protein*; **mlp3c** : Microtubule Associated Protein 1 Light Chain 3 Gamma* (ortholog of the yeast autophagosome protein Atg8); **zgc:153723** (sulfotransferase family 5A, member 1); **zgc:153126** (Formin Homology 2 domain containing 3*); **fmn2b**: formin 2b; **htra1a**: HtrA serine peptidase 1a; **wu:fc34e06** (integral component of membrane: EST); **wsb1**: WD Repeat And SOCS Box Containing 1*; **casp6l2**: caspase 6, apoptosis-related cysteine peptidase, like 2; **zgc:162126** (ppp1r15a: protein phosphatase 1, regulatory subunit 15A); **cox5b2**: cytochrome c oxidase subunit Vb 2; **slc2a1b**: solute carrier family 2 (facilitated glucose transporter), member 1b. **(*)** Exist a human ortholog gene.

Table 5: Representative genes regulated by both *HIF3α* and *HIF1α*.

as Oct-3/4, Sox-2 and/or Nanog in cancer cells under normoxic and hypoxic conditions. Expression of HIF3α- regulated genes are involved in nitrogen metabolism, methane metabolism, the Jak-STAT signaling pathway, and NOD-like receptor signaling. Interestingly, genes involved in erythropoiesis an angiogenesis induction are regulated by both HIF1α and HIF2α, but not by HIF3α subunit.

The Involvement of HIFs in Cancer Treatment Resistance

The inherent or developed resistance of many cancer cells to chemotherapy, targeted types of therapy, and irradiation, is the primary cause for treatment failure in clinical oncology. This problem represents a complex and multifactorial phenomenon related to tumor microenvironment, such as hypoxia, acidosis, nutrient starvation and inflammation [61]. HIFs overexpression is associated with therapeutic resistance or decreased survival in many cancer types and it has been demonstrated that they contribute to resistance *via* multiple mechanisms. Some of these mechanisms are well documented, such as the induction of efflux pumps expression, apoptosis inhibition, cell survival promotion, autophagy induction, stemness promotion and also inducing epigenetic changes to regulate gene expression in cancer cells.

HIFs Induce Chemoresistance by Efflux Pump Expression

Prevention of toxin absorption by efflux pump mechanisms is very effective and important to induce chemical resistance in cells. Several studies in different cancer cell types have demonstrated that the multidrug resistance gene (*MDR1*), encoding the transmembrane P-glycoprotein (Pgp), which belongs to the ATP-binding cassette superfamily of transport proteins, is induced by hypoxia [62-64]. Consistent with this, HIF1α expression inhibition by antisense oligonucleotides resulted in significant hypoxia-inducible *MDR1* expression suppression and a nearly complete loss of basal *MDR1* expression. Ding et al. as has been observed the relationship between HIF-1α and MDR1/P-glycoprotein in human colon carcinoma tissues. The expression of both HIF1α protein and P-gp were significantly higher in tissue samples classified as Dukes' stages C or D, involving lymph node metastasis, than in samples classified as Dukes' stages A or B, indicating that HIF-1α was involved in tumor invasion and metastasis. They also used cultured human colon carcinoma cells (HCT-116, HT-29, LoVo, and SW480), and observed that HIF-1α expression was significantly associated with MDR1/P-gp expression in human colon carcinoma cells as well [65]. In addition, the expression of the multidrug resistance- associated protein 1 (MRP1), another ABC transporter, is also induced by hypoxia, and have been reported in the majority of brain tumors, including glioblastomas [66].

HIF-Mediated Survival after Ionizing Radiation Therapy

Ionizing radiation, such as that used in radiotherapy, kills cells by producing DNA damage, particularly DNA double strand breaks. This damage results from ionizations in or very close to the DNA that produces a radical on the DNA. It has been demonstrated that the more hypoxic tumors are more radio-resistant than the less hypoxic tumors [67].

Hypoxic tumor cells are more resistant to radiotherapy as a consequence of the interference of hypoxia with the fixation of free radical–induced DNA damage. HIF1α pathway is involved in the tumor-protective response to radiotherapy both *via* vascular protection post-irradiation, and *via* enhancing the tumor antioxidant capacity through initiating a glycolytic tumor metabolism. Targeting HIF1α and tumor glucose metabolism affects the tumor microenvironment, induces metabolic alterations, and sensitizes various solid tumors to irradiation (reviewed by Meijer et al. [68]). HIF2α also contributes to tumor cell survival after ionizing radiation treatment by reducing ROS levels generated from normal metabolic processes as well as irradiation treatment, promoting tumor cell survival. HIF2α deficiency promotes p53 phosphorylation in ccRCC and lung carcinoma cells in culture, which enhances ccRCC cell death by disrupting cellular redox balance, thereby promoting DNA damage. HIF2α also affected p53 in the absence of irradiation, suggesting that HIF2α inhibition may be effective alone or in combination with therapies other than radiation [69].

HIFs and Cell Death Pathways: Apoptosis and Autophagy

In the vast majority of transformed cells, HIF1α functions as a robust suppressor of apoptosis and functional interference with HIF1α results in enhanced cell death upon treatment with chemotherapeutic agents in tumors of different origins [70]. The molecular nature of this phenomenon was mostly explained by HIF1α anti-apoptotic target gene-induction (Bak, Bax, Bcl-xL, Bcl-2, Bid, Mcl-1, NF-κB, p53 and survivin), but also by suppression of p53 activation in response to chemotherapeutic agents [71], as it can be seen in Figure 3. HIF2α was recently shown to act in a similar manner, namely suppressing p53 activation and apoptosis in response to radiation-induced DNA damage in clear cell kidney cancer cells [69]. Interestingly, hypoxia selects for tumor cells that have lost sensitivity to p53-mediated apoptosis or that are deficient in DNA mismatch repair resulting in apoptosis resistance and genomic instability [72,73].

Besides apoptosis, the process of autophagy is increasingly recognized as an important regulator of cellular viability under stressful conditions. Autophagy is a highly conserved catabolic process whereby long-lived or damaged proteins and organelles are engulfed in double-membrane structures called autophagosomes and targeted to the lysosomes for degradation for energy production. In addition, autophagy plays other key cellular functions such as adaptation to nutrient depletion, extension of lifespan, cellular development and anti-aging [74].

Autophagy seems to play a role at multiple levels of tumor development and it has been reported that have a protective role in carcinogenesis. Autophagy is also a consequence of cytotoxic drug treatment, and more recently, has been appreciated as a means by which cells might survive the stress of cellular insults, and so become resistant to treatment.

Enhanced autophagy has been associated with the elevated level of HIF1α in several cancer types. In this regard, it has been observed that hypoxia-mediated failure of cytotoxic treatment *in vitro* can be conferred *via* HIF1α-dependent induction of autophagy [75]. This process is mediated by the atypical BH3-only proteins such as the Bcl-2/E1B 19 kDa-interacting protein 3 (BNIP3/BNIP3L (NIX)) that are induced by HIF1α [74]. These mitochondrial associated BNIP proteins also mediate mitophagy, a metabolic adaptation for survival that is able to control reactive oxygen species (ROS) production and DNA damage [74]. However, it has been established that cells lacking expression of two HIF isoforms, HIF1α or HIF2α, elicited a lower autophagic response under hypoxic conditions. Therefore, HIF1α and HIF2α appear to be evenly matched in their capacity to induce autophagy.

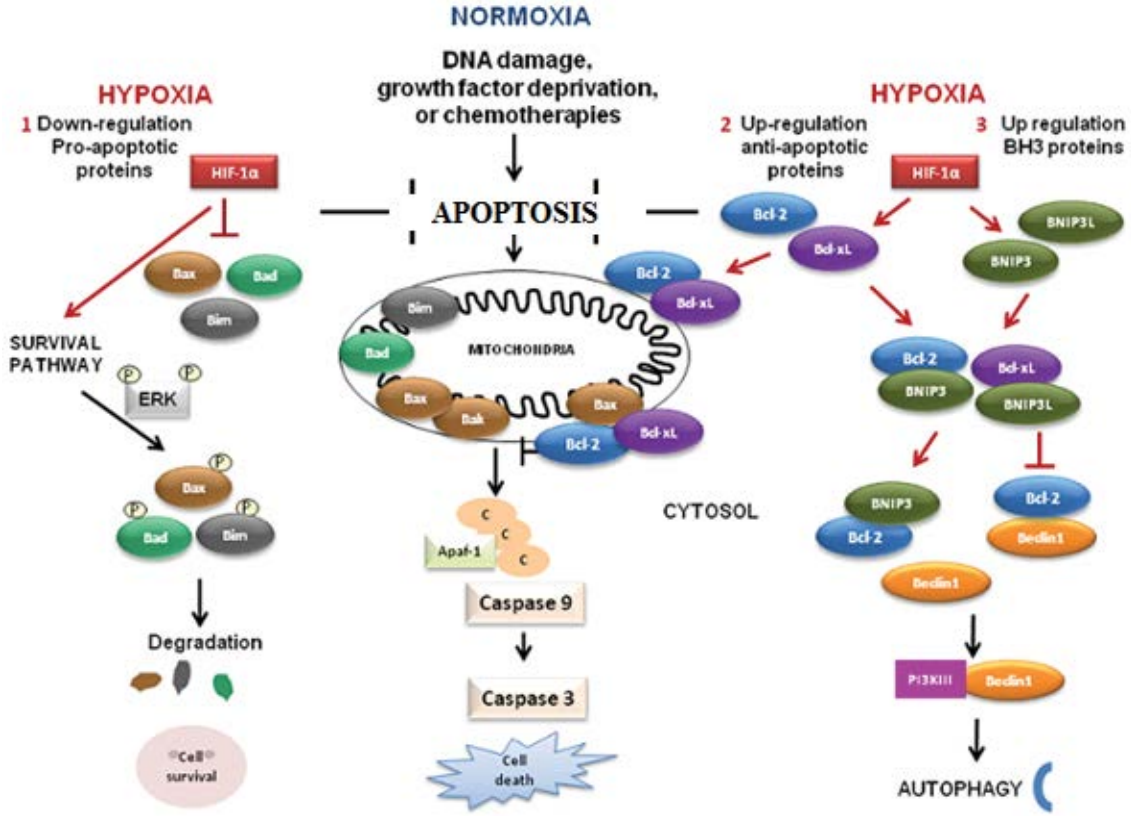

Figure 3: HIFs induce cell survival and block apoptosis. DNA damage, growth factor deprivation or chemotherapy induce intrinsic apoptotic pathway under normoxia conditions. In cancer cells, HIFs promote apoptosis resistance by the following mechanisms: 1) downregulating the expression of pro-apoptotic proteins (Bim, Bad, Bax) and activating survival signaling pathways; 2) they block apoptosis by inducing up-regulation of anti-apoptotic proteins (Bcl-2/Bcl-xL), thereby preventing Bax/Bak oligomeryzation, and cytochrome C release; 3) HIFs also induce the transcription of BH3 proteins such as BNIP/BNIP3L. These proteins bind to Bcl-2/Bcl-xL and thus Beclin1/Bcl-2 complex is dissociated and Beclin1 can bind to PI3KIII complex to induce autophagy pathway.

HIFs Promote Stemness and Cancer Aggressiveness

A growing body of experimental evidence has indicated that cancer- and metastasis-initiating cells with stem cell-like features typically display a higher resistance than the bulk mass of differentiated cancer cells to radiation therapy and chemotherapy, and thereby they can be responsible for disease relapse [76,77]. Cancer stem cells (CSC) are a small subpopulation of cells within various tumor types, which have the dual properties of self-renewal and differentiation by giving rise to both daughter cancer stem cells and bulk (non-stem) cancer cells, It has been demonstrated that CSCs are involved in initiating and sustaining tumor growth in many cancer types.

Hypoxia is associated with the promotion and maintenance of cancer stem cells. Many recent studies have shown that hypoxia inhibits differentiation of embryonic stem cells, progenitor cells [78], and mesenchymal stem/progenitor cells [79]. Under hypoxic conditions, tumor cells show increased clonogenic potential and Semenza's group in 2016 [77], demonstrated that the exposure of breast cancer cells to hypoxia increases the percentage of breast cancer stem cells, which are required for tumor initiation and metastasis. Importantly, they showed that this response is dependent on the activity of hypoxia-inducible factors (HIFs) which induce the increase in cancer stem cell markers expression. They found that NANOG, SOX2, and OCT4 expression increased in all human breast cancer cells they analyzed in response to chemotherapy or hypoxia [77].

Interestingly, it has been proposed that the switch from HIF1α to HIF2α -dependent signaling plays an important role in the promotion of stemness, aggressive tumor growth, and tumor progression [80,81]. Tumor cells are subjected to a range of oxygen tensions and experience periods of acute/intermittent hypoxia or chronic/prolonged hypoxia. The variability in hypoxic intensity and duration needs distinct sets of cellular responses appropriate for each condition. In this respect, HIF1α seems to have the dominant role in controlling responses to acute hypoxia, whereas HIF2α drives the response to chronic hypoxia [80,82]. Consistent with this, it has been reported that HIF2α drives tumor progression in renal carcinoma cells in which there is a gradual shift from HIF1α to HIF2α expression with increasing tumor grade [83]. HIF2α (but not HIF1α) has been shown to cooperate with a number of oncoproteins frequently deregulated in cancer such as c-Myc, epidermal growth factor receptor (EGFR), and K-Ras [84-86] and has been linked to increased tumor aggressiveness through the promotion of self-renewal and epithelial to mesenchymal transition (EMT). In addition, Koh et al. [80] identified the hypoxia-associated factor (HAF) as an E3 ubiquitin ligase that binds to and ubiquitinates HIF1α by an oxygen- and pVHL-independent mechanism, targeting HIF1α for proteasomal degradation. But interestingly, they found that HAF binding to HIF2α does not lead to its degradation but instead, increases HIF2α transactivating activity. Thus, HAF expression switches the hypoxia response of the cancer cell from HIF1α – to the HIF2α –dependent transcription of genes such as MMP9 and OCT-3/4.

They also showed that this switch by HAF promotes the cancer stem cell phenotype and invasion, resulting in highly aggressive tumors *in vivo*. As mention before, there is experimental evidence that whereas the transcription of HIF2α and HIF3α is consistently increased by hypoxia, transcription of HIF1α is not and can show variable levels of repression. Altogether, these data suggest an important role of HIF2α and HIF3α in the regulation of tumor progression under chronic hypoxia.

These interesting observations have lead to a new paradigm that tumor hypoxia may facilitate the emergence of malignant clones by maintaining cancer stem cells in their undifferentiated stem cell state. Thus, the complete eradication of the total mass of tumor cells, including hypoxic and normoxic cancer stem/progenitor cells and their differentiated progenies, by targeting the HIF signalling network might be crucial to improve current cancer therapies and prevent disease relapse.

Hypoxia Induces Epigenetic Changes to Regulate Target Gene Expression

There is experimental evidence that hypoxia induces epigenetic regulation of transcription of many HIF target genes, by different mechanisms that results in enhancement of expression of some target genes, or in the transcriptional repression of other target genes [87]. With respect to the mechanisms involved, it has been reported that both histone methylation and acetylation status change in the promoters of HIF target genes. Epigenetic changes that promote hypoxia-induced negative regulation of transcription can be produced by the interaction between Reptin and HIF-1α leading to recruitment of histone deacetylase 1 (HDAC1) to some HIF target genes [88].

Epigenetic changes that promote positive regulation of transcription can be mediated by HIF-dependent recruitment of co-activators such as p300/CBP histone acetyltransferases to interact with HIF at HIF target promoters, increasing transcription [87]. Enhancement of transcription can also occur by hypoxia-induced changes in the histone methylation status at promoters of hypoxia-inducible genes: oxygen deprivation activates JMJD1A and inhibits JARID1A histone demethylases, promoting respectively a decrease in H3K9me2, and an increase in HK4me2 levels. Hypoxia also provokes an increase in H3K9me2 levels as a result of G9a up-regulation [89]. Mechanistically, JMJD1A is not essential for stem cell self-renewal but is crucial for tumor suppression, whereas H3K9 methyltransferase G9a is linked with tumor growth and poor diagnosis. In addition, JMJD1A and G9a differentially drive the expression of antiangiogenic factors. As apart from depositing H3K9me2, G9a and its partner protein GLP also form a complex and play a role in the maintenance of DNA methylation at specific loci [90], it is possible that the aberrant G9a-linked DNA methylation downregulates tumor suppressors and leads to malignancy, although its associated H3K9me2 could be erased by JMJD1A. Therefore, investigation of the levels of G9a and the patterns of its associated DNA methylation might create a new direction for the prognosis and treatment of carcinomas in humans [91-95].

Concluding Remarks

Diminished oxygen availability (hypoxia) is a hallmark of the tumor microenvironment. It is well known that cancer cells exhibit several biological properties called as "hallmarks of cancer" that they acquire during the multistep development of cancer. Tumor hypoxia is involved in each hallmark and enabling characteristic displayed by cancer cells.

Cellular adaptation to hypoxia is mediated by the HIF family of transcription factors: three oxygen-dependent different alpha subunits HIF1, HIF2 and HIF3 and two oxygen-independent HIFβ subunits [96-105]. In contrast to the well-established importance of HIF1α and HIF2α in cancer biology, the functional significance of HIF3α is remarkably understudied. Models should be developed to accurately study the temporal regulation of HIF1α, HIF2α and HIF3α.

Enhanced expression and activation of hypoxia-inducible factors (HIFs) frequently occur in cancer cells during cancer progression and is associated with their acquisition of a more malignant behaviour, treatment resistance and poor outcome of cancer patients. Thus, targeting hypoxia is an exciting prospect to improve current anti-cancer therapy and prevent disease relapse [106-112].

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