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Hypoxia Inducible Factors: Central Regulators of the Tumor Phenotype

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Abstract

Low oxygen levels are a defining characteristic of solid tumors, and responses to hypoxia contribute substantially to the malignant phenotype. Hypoxia-induced gene transcription promotes characteristic tumor behaviors including angiogenesis, invasion, metastasis, de-differentiation and enhanced glycolytic metabolism. These effects are mediated, at least in part, by targets of the Hypoxia Inducible Factors (HIFs). The HIFs function as heterodimers, made up of an oxygen-labile α-subunit and a stable (β-subunit, also referred to as ARNT. HIF-1α and HIF-2α stimulate the expression of overlapping as well as unique transcriptional targets, and their induction can have distinct biological effects. New targets and novel mechanisms of dysregulation place the HIFs in an ever more central role in tumor biology, and have led to development of pharmacological inhibitors of their activity.

Introduction

Hypoxia occurs when available oxygen falls below 5%, triggering a complex cellular and systemic adaptation mediated primarily through HIF transcription. HIF-1α was first identified as a critical regulator of erythropoietin expression in response to low oxygen [1]. HIF-2α and HIF-3α have also been described, with HIF-3α (also known as IPAS) functioning as an inhibitor of transcription [2,3]. More than 100 HIF targets have been identified in a variety of systems. These include promoters of angiogenesis, such as Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor, glycolytic enzymes such as Aldolase A and Phosphoglycerate Kinase, and cell cycle regulators such as p21 and p27, as well as genes involved in extracellular matrix remodeling, differentiation and apoptosis [4–7]. HIF-1α and HIF-2α bind hypoxia response elements (HREs) in a complex with the (β-subunits, ARNT and (more rarely) ARNT2 [8,9]. The biological significance and transcriptional effects of HIF-3α remain somewhat obscure, and only HIF-1α and HIF-2α will be discussed further in this review.

HIF-α subunits are continuously transcribed and translated, and their stability is regulated by oxygen availability. Under normoxic conditions, two prolines (402 and 564 in human HIF-1α and 405 and 531 in human HIF-2α) in the HIF-α oxygen-dependant degradation

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domain (ODD) are hydroxylated by a family of oxygen dependant proline hydroxylases (PHD1–3) [10–13], allowing binding and ubiquitylation by the von Hippel-Lindau (VHL) tumor suppressor, a component of an E3 ubiquitin ligase complex [14]. HIF-α interaction with the transcriptional co-activator p300 is also regulated by oxygen levels, with binding inhibited by oxygen-dependent asparaginyl hydroxylation (asparagines 803 in human HIF-1α and 851 in human HIF-2α) of the HIF transactivation domain by Factor Inhibiting HIF (FIH) [15,16].

VHL disease is a hereditary cancer syndrome marked by clear cell renal carcinoma (RCC), pheochromocytoma, and hemangioblastoma. The VHL tumor suppressor protein (pVHL) is required for normoxic degradation of the HIF-α subunits and can also target atypical protein kinase C λ and some subunits of RNA polymerase for degradation [17]. Of the *VHL*-associated malignancies, RCC and hemangioblastoma result from normoxic HIF-2α stabilization [18–21], whereas pheochromocytoma results from a HIF-independent effect of pVHL on JunB [22]. The HIFs also play an important role in non-inherited malignancies. There is substantial clinical data associating HIF-α protein expression with poor outcomes in patients with a broad range of sporadic cancers. These include adenocarcinoma of the breast, lung, and GI tract, as well as CNS malignancies and squamous cell tumors of the cervix and head and neck [5]. Data from mouse allograft studies have been less consistent. In some cases, disruption of *Hif-1α* inhibited allograft growth [23,24], but in others it promoted it [25,26]. Consistent inhibition of tumor growth has been observed following normoxic stabilization of HIF-1α due to *Vhl* loss [20,27–29] and the overexpression of HIF-1α or HIF-2α in glioma [25,30].

Regulation of HIF Stability and Expression

The normoxic degradation of the HIF-α subunits is well characterized, but its inhibition under hypoxia is an area of active investigation, and remains controversial. As oxygen is required for hydroxylation, it is a limiting substrate under anoxic (0% O_2) conditions. However, HIF-α's are stabilized in a reactive oxygen species (ROS)-dependent fashion well above this threshold. Early evidence showed that inhibitors of mitochondrial ROS generation were able to block hypoxic HIF-α stabilization [31]. However, such drugs may have toxic or off target effects on HIF-α regulation. It has also been suggested that these drugs may cause redistribution of oxygen away from the mitochondrion, leaving more available for PHD activity and thus maintaining it under moderate hypoxia [32,33]. However, genetic studies have shown that disruption of electron transport chain (ETC) Complex III, cytochrome c and Rieske iron-sulfur protein also block hypoxic HIF stabilization [34,35], while disruption of ETC Complex IV did not [36]. These data suggest that respiration is not required for HIF-α stabilization but the delivery of electrons to cytochrome c is, supporting a requirement for ROS (but not oxygen consumption) in hypoxic HIF-α stabilization. Further evidence comes from the analysis of $junD^{-/-}$ mice, which show enhanced ROS production and normoxic HIF-α expression. In this case, enhanced intracellular H_2O_2 levels were shown to inhibit PHD activity by altering the reduction of Fe^{3+} to Fe^{2+}, which is required for PHD activity [37]. This is also a plausible mechanism for PHD regulation in hypoxic cells.

Normoxic HIF-α stabilization is both necessary and sufficient for RCC development following *VHL* inactivation. pVHL can also be inhibited by the E2-EPF ubiquitin carrier protein, which targets pVHL for proteasome mediated degradation [38]. Overexpression of this protein occurs in breast, lung, ovarian and CNS cancers, and correlates strongly with tumor grade and poor patient outcomes [39]. The alteration of metabolic pathways impinging on PHD activity can also promote normoxic HIF-α stabilization and tumor formation. In addition to oxygen and Fe^{2+}, the PHDs require 2-oxoglutarate as a substrate

and ascorbic acid as a co-factor to catalyze HIF-α hydroxylation, and produce succinate and carbon dioxide in addition to hydroxylated proline residues. Inactivation of fumarate hydratase, a rare cause of inherited RCC, promotes HIF-α stabilization due to inhibition of the PHDs by fumarate, which competes with 2-oxoglutarate for active site binding [40]. Similarly, inactivation of succinate dehydrogenase, which occurs in some renal, thyroid and colon cancers, leads to succinate accumulation and product inhibition of the PHDs [41].

Control of HIF-α translation

The mTOR kinase responds to nutrient and growth factor availability to regulate translation. Normoxic HIF-α expression is promoted by disruption of mTOR regulation, resulting from increased HIF-α translation rates despite unaltered levels of degradation. This is likely to occur in many tumors which show hyperactivation of receptor tyrosine kinases, and thus translation [42], but is also seen in several inherited tumor syndromes. Loss of the TSC2 tumor suppressor gene, an inhibitor of mTOR activity, causes normoxic stabilization of the HIF-α subunits by enhancing their translation rate, leading to the formation of highly vascular tumors [43]. Enhancement of HIF-α translation under hypoxia by disruption of the promyelocytic leukemia tumor suppressor (PML) can also promote tumor growth. Originally identified as part of a leukemogenic fusion protein, PML has since been appreciated to have a tumor suppressive effect, and is lost in multiple sporadic tumors [44]. Genetic disruption of *Pml* correlates with increased VEGF and HIF-α expression through attenuation of the hypoxic inhibition of mTOR, normally effected by the sequestration of mTOR in PML containing nuclear subdomains [45]. Thus, the regulation of HIF-α translation is likely to have a contributing role in a broad range of tumor types.

HIF-1α vs. HIF-2α

Discovered first and expressed ubiquitously, HIF-1α is by far the best characterized α-subunit. HIF-2α expression is limited to endothelium, kidney, heart, lung and gastrointestinal epithelium, and some cells of the CNS [3,46,47]. Differences exist in their targets, with HIF-1α uniquely activating glycolytic enzyme genes and HIF-2α preferentially activating VEGF, transforming growth factor-α (TGFα), lysyl oxidase, Oct4 and Cyclin D1 [7,48–52]. Similarly, the effects of *Hif-1α* and *Hif-2α* gene disruption are substantially different, with *Hif-1α* knockout leading to impaired cardiac and vascular development and E10.5 lethality [23,26,53] while *Hif-2α* loss leads to a broad range of phenotypes including embryonic lethality due to bradycardia and vascular defects, perinatal lethality due to impaired lung maturation, and embryonic and post-natal lethality caused by multi-organ failure and mitochondrial dysfunction [54–57].

Differences in gene targets and knockout phenotypes suggest that HIF-2α may promote a distinct phenotype in tumors expressing it. This has been observed in CNS, colorectal, non-small cell lung and head and neck tumors, where expression of HIF-2α is more strongly associated with poor patient outcomes than expression of HIF-1α [5,58]. Data from genetic models suggest that HIF-2α may preferentially promote tumorigenesis, where ES cell derived teratomas with HIF-2α “knocked in” to the *Hif-1α* locus exhibiting a four-fold increase in mass over HIF-1α expressing controls, largely due to increased proliferation [59]. Enhanced proliferation likely results from increased expression of TGFα and Cyclin Dl. Additional effects on the tumor phenotype may result from HIF-2α mediated induction of the stem cell factor Oct4 and activation of c-Myc transcription, as described below [49,60].

HIF transcriptional targets

A series of microarray studies have defined HIF target gene families [6,7,50,61–64]. Erythropoeisis, angiogenesis, and glycolytic metabolism are controlled through multiple gene targets, with differential activation based on cell type and which HIF-α subunit is expressed. Continued analysis is expanding our understanding of how some of these responses are mediated. HIF-1α induction of glycolytic metabolism has been well appreciated, but the inhibition of aerobic metabolism through the induction of pyruvate dehydrogenase kinase (PDK1) was only recently described. PDK1 phosphorylates pyruvate dehydrogenase, inhibiting the conversion of pyruvate to acetyl-CoA. The inhibition of aerobic metabolism at moderate levels of hypoxia may free limited oxygen supplies for other cellular processes and avoid the accumulation of toxic metabolites [65,66].

Metastasis is a defining characteristic of cancer, and is also promoted by tumor hypoxia. Metastasis is a coordinated process, where chemokines direct cell migration, adhesion molecules mediate attachment in distant organs, and proteases and other secreted enzymes degrade or alter the extracellular matrix. Studies in breast cancer and RCC demonstrated that the chemokine receptor CXCR4, a major metastatic mediator, is upregulated by HIF [67], while analysis of lung epithelium further showed matrix metalloproteinases (MMPs) 2 and 9 are regulated by hypoxia [68]. Another key mediator of metastasis is the HIF target lysyl oxidase, which is strongly associated with hypoxia and poor patient outcome in several tumor types. Lysyl oxidase alters extracellular matrix components such as elastin and collagen and its inhibition blocks *in vitro* migration and *in vivo* metastasis from subcutaneous xenografts or after tail vein injection [52].

HIF targets known to be important in development also have a substantial role in tumor biology. Oct4, a POU-domain transcription factor and HIF-2α target, is a key regulator of stem cell behavior. Well known for a role in embryonic stem cells, Oct4 has more recently been observed in some adult stem cell populations [69]. In studies of a knock-in model where HIF-2α was expressed from the *Hif-1α* promoter, a dramatic disruption of embryonic development was observed, correlating with an enhancement of TGF-α, VEGF and Oct4 expression. *In vitro* models of these developmental phenotypes were mostly reversed by shRNA knockdown of Oct4 [49]. Interestingly, Oct4 knockdown also substantially reversed the growth advantage seen in subcutaneous teratomas derived from the knock-in ES cells compared to teratomas from *Hif-1α* WT ES cells [49]. The mechanism by which Oct4 modulates tumor behavior is not yet clear, but one intriguing possibility is that it promotes the growth of a ‘cancer stem cell’ population, and thus self-renewal and chemotherapy resistance.

In addition to its direct gene targets, HIF can regulate the transcription factors Notch and c-Myc [70,71]. HIF-1α was found to require Notch and its target genes in models of hypoxia-induced muscle and neural cell de-differentiation. In fact, HIF-1α interacts directly with the intracellular domain of Notch1, increasing its half-life and transcriptional activity [72]. The *in vivo* implications of this interaction remain to be understood, but given Notch’s role in development and tumor biology they are likely to be significant [73]. The implications of HIF-1α inhibition of c-Myc are somewhat clearer. Though HIF-1α has long been connected with cell cycle arrest, the mechanism by which this occurs has not been well understood. HIF-1α directly inhibits c-Myc, causing de-repression of its targets p21 and p27 [70]. c-Myc targets involved in mismatch repair are also modulated by HIF-1α, suggested a role for HIF in hypoxia induced genetic instability [71]. In assessing the effects of HIF-2α on c-Myc, we have observed that HIF-2α promotes c-Myc transcriptional activity, which may also contribute to HIF-2α mediated tumor progression [60].

HIF and Cancer Therapy

Pharmacologic inhibition of the HIF target VEGF has proven efficacy as a cancer therapeutic [74], and has generated interest in targeting global HIF activity. Direct approaches, such as inhibition of p300-mediated co-activation [75] and DNA binding [76], are being explored as is HIF inhibition through repression of its translation. HIF-α subunits appear to be particularly sensitive to translational regulation as the use of pharmacological mTOR inhibitors can block HIF-α expression even following *VHL* loss [77]. In fact, the mTOR inhibitor CCI-779 resulted in a statistically significant survival advantage in patients with metastatic renal cancer [78].

Mouse models have shown that HIF can have a substantial impact on the response to cytotoxic cancer therapies. Ionizing radiation (IR) treatment in subcutaneous tumor models causes HIF-1α stabilization through ROS induction. HIF-1α induction leads to release of cytokines including VEGF that promote endothelial cell survival, and thus blunt the therapeutic effect of IR [79]. The stabilization of HIF-1α in endothelial cells is also likely to occur following IR, and can substantially promote tumor growth [80]. On the other hand, HIF-1α enhances the effect of IR on tumor cells themselves. In a similar model, induction of HIF-1α promotes p53 phosphorylation and stabilization, as well as cell death following IR. These effects, combined with HIF effects on the endothelium, suggest a particular advantage to combination treatments using IR followed at a later point by HIF inhibition [81]. Thus, the inhibition of the HIFs, either at the level of protein expression or transcriptional activity, should be consider on a case-by-case basis, depending on tumor type and other therapies used concurrently.

Conclusion

In addition to important roles in development, hematopoeisis, and ischemic disease the HIFs also have a broad range of effects on tumor biology. They are directly responsible for tumor angiogenesis and metastasis, and contribute substantially to metabolic changes, the evasion of apoptosis, and genomic instability. Despite the appreciation of their relevance to tumor biology, novel targets and mechanisms are reported frequently. Their pharmacological inhibition represents an opportunity and a challenge, and an important area for future study.

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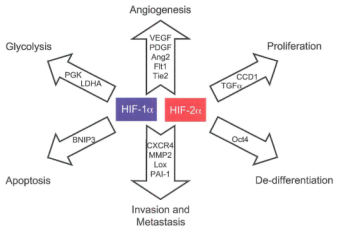

Figure 1. HIF-1α and HIF-2α activate overlapping but distinct genes

HIF-1α and HIF-2α share the regulation of target genes involved in angiogenesis, invasion and metastasis, while HIF-1α alone activates genes involved in glycolysis and apoptosis. HIF-2α uniquely activates the stem cell factor Oct4 and Cyclin D1, while it preferentially regulates the growth factor TGFα. Abbreviations: Platelet derived growth factor (PDGF), fms- related tyrosine kinase 1 (Flt1), Tunica internal endothelial cell kinase 2 (Tie2), Plasminogen activator inhibitor 1 (PAI-1), Lactate dehydrogenase A (LDHA), BCL2/ adenovirus E1B 19kDa interacting protein 3 (BNIP3), Cyclin D1 (CCD1).

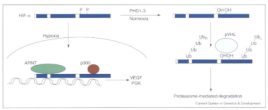

Figure 2.

Regulation of HIF-α stability: continuously transcribed and translated, the HIF-α subunits are degraded under normoxic conditions. Two prolines in the ODD are hydroxylated by PHD1, 2 or 3, allowing recognition by an E3 ubiquitin ligase complex including the VHL tumor suppressor protein. Following pVHL-mediated ubiquitylation, the HIF-α subunits are degraded in a proteasome-dependent fashion. When oxygen levels fall below ~5%, the PHDs are no longer active and the HIF-α subunits can translocate to the nucleus, where they bind co-factors including ARNT and p300 and transactivate hypoxia response genes, such as *VEGF* and *PGK*.