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The impact of O_2 availability on human cancer

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Abstract

During the last century, it has been established that regions within solid tumors experience mild to severe oxygen deprivation, due to aberrant vascular function. These hypoxic regions are associated with altered cellular metabolism, as well as increased resistance to radiation and chemotherapy. As discussed in this Timeline, over the past decade, work from many laboratories has elucidated the mechanisms by which hypoxia-inducible factors (HIFs) modulate tumor cell metabolism, angiogenesis, growth, and metastasis. The central role played by intra-tumoral hypoxia and HTF in these processes has made them attractive therapeutic targets in the treatment of multiple human malignancies.

Oxygen (O_2) is required for aerobic metabolism to maintain intracellular bioenergetics and serve as an electron acceptor in many organic and inorganic reactions. Hypoxia, defined as reduced O_2 levels, occurs in a variety of pathological conditions, including stroke, tissue ischemia, inflammation, and the growth of solid tumors. The beginnings of hypoxia research in tumor biology can be traced back to observations made in the early 20th century by Otto Warburg who demonstrated that, unlike normal cells, tumor cells favor glycolysis, independent of cellular oxygenation levels. He postulated that tumor growth is caused by mitochondrial dysfunction in neoplastic cells, forcing them to generate energy through glycolysis (reviewed in [1]). This hypothesis appears to be incorrect, but a number of other molecular mechanisms promoting “aerobic glycolysis” have been proposed including mutations and epigenetic changes in genes encoding tumor suppressors (e.g. *p53*), oncogene activation (e.g. *c-Myc*), and hypoxic adaptations {Denko, 2008 #6606; Gatenby, 2004 #6608; Deberardinis, 2008 #6609.

Ambient air is 21% O_2 (150 mm Hg); however, most mammalian tissues exist at 2%-9% O_2 (on average 40 mm Hg). “Hypoxia” is usually defined as ≤ 2% O_2, while severe hypoxia or “anoxia” is defined as ≤ 0.02% O_2. In the decades following Warburg’s observation, scientists sought to determine whether hypoxic or anoxic cells could be found in mammalian tumors and how these cells affected radiation therapy. Of great interest to radiation biologists and oncologists, the radioprotective effect of anoxia in normal tissues was demonstrated in the 1940s by Lacassagne and Evans et al., using whole body anoxia in newborn rodents (reviewed in {Gray, 1953 #6395}). If a subset of tumor cells did in fact

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exist in an environment deprived of O_2, then they might be responsible for tumor recurrence after radiation. This realization is still fueling research today, over 60 years later.

Early demonstrations of tumor hypoxia

Before hypoxic cells could be visualized in tumors, their presence was inferred by some astute observations. In 1955, Thomlinson and Gray studied histology sections of human lung tumors and observed cells growing in "cords" running parallel to vascularized stroma [2]. In large cords, they noted a necrotic core surrounded by a region of viable cells neighboring a capillary vessel. They proposed that necrosis was due to insufficient O_2 and nutrient supply to the rapidly expanding tissue. Regardless of cord size, detectable bands of live cancer cells lying between the necrotic core and the surrounding stroma were consistently 170 microns in width, approximately the calculated distance of O_2 diffusion (145 microns). They proposed that the edges of such necrotic cores harbor viable hypoxic tumor cells [2]. Other human tumors, such as cervical and renal carcinomas exhibited similar histological characteristics, suggesting that this was not unique to the lung (reviewed in [3]). The histological specimens also suggested that tumor cells are exposed to an O_2 gradient ranging from efficient oxygenation near the stroma decreasing gradually to near anoxia bordering the necrotic regions. These varying O_2 concentrations have since been shown to have significantly different effects on cellular processes [4].

Further evidence for the existence of viable hypoxic tumor cells that may influence tumor responses to radiation was offered by Powers and Tholmach. They irradiated lymphosarcomas in mice breathing either ambient air or hyperbaric O_2 (three atmospheres of pressure) before transplanting them to a new mouse host. They observed decreased tumor cell survival if transplanted from mice breathing hyperbaric O_2 as compared to 21%, suggesting that certain tumor cells were protected by decreased O_2 conditions, but sensitized by a surplus of O_2 delivered to the host. Moreover, tumors irradiated in dead mice (likely more hypoxic than viable animals breathing room air), were much less sensitive to radiation [5]. Around the same time, Churchill-Davidson made similarly encouraging observations in cancer patients treated with radiation in combination with hyperbaric O_2 [6]. While these results were promising and suggestive of the existence of hypoxic cells within tumors, they did not provide direct evidence and it was not until the latter decades of the 20^{th} century that precise techniques for measuring O_2 levels were developed.

Even today, scientists lack an optimal method of direct tissue O_2 measurement that is non-invasive, precise, and quantitative; but this goal is clearly within reach (reviewed in [7]). In the 1980's, in vivo polarographic measurements with electrodes and ex vivo cryospectrophotometric measurements of oxyhemoglobin provided some insight into the regional oxygenation status of tumors (reviewed in [8]). Nuclear magnetic resonance spectroscopy of ^{31}P signals detecting tumor cell metabolism and sensitizer-adducts such as misonidazole [9,10] selectively binding hypoxic cells were also used in an attempt to identify hypoxic regions (reviewed in [11]). However, none of these methods were ideal. While electrode measurements were certainly the most direct form of O_2 measurement available, some human tumors were not accessible to electrodes and the electrode diameters were too large to allow precise measurements of cellular oxygenation status. Instead, they likely measured average O_2 tensions in tumor sections, which would not accurately reflect dramatic variations that occur over very small distances [12]. Cryospectrophotometric measurements readily determine the oxyhemoglobin saturation of individual erythrocytes in tumor vessels ex vivo, an indirect assessment of tumor oxygenation [8]. ^{31}P NMR spectroscopy was found to lack sensitivity and technical difficulties still limited the use of sensitizer adducts [11]. Using several of these methods, studies in both the 1980s and early 1990s found decreased average O_2 tensions in human tumor tissue as compared to normal

tissue, as predicted by previous work. Substantial inter-tumor variability was observed however, indicating that predictions of tumor oxygenation would be difficult and underscoring the urgent need for more precise and direct O_2 measurement techniques easily used in clinical settings [8]. In the late 1980s, an additional complication was revealed by Chaplin et al., using Hoechst 33432. This DNA binding stain has a short distribution half life but remains bound to tumor cell DNA, thus labeling cells based on their proximity to the blood supply. Using this technique coupled with cell sorting and responses to treatment, Chaplin differentiated between diffusion limited, chronic hypoxia and acute hypoxia resulting from transient changes in blood flow through tumor vasculature of larger tumors. Cells adjacent to blood vessels may suddenly and intermittently become hypoxic as the vessel becomes momentarily occluded during treatment [13]. These two types of hypoxia may have different effects on treatment responses, a possibility that must be considered in the selection of cancer therapy protocols [14].

The effects of hypoxia on cancer

As described by Otto Warburg in the 1920s, rapidly dividing tumor cells display increased glycolysis, even in the presence of oxygen. As a consequence, lactic acid concentrations are elevated, acidifying the environment. Initially, scientists realized that exposure to hypoxia slowed tumor growth in mice, although early experiments were confounded by hypoxia-induced generalized weight loss in experimental animals. In 1954, Barach and Bickerman repeated these experiments in hypothyroid and acclimatized mice and found similar results without generalized weight loss, confirming that O_2 deprivation impaired tumor growth [15]. In extreme cases of prolonged anoxia, cells would undergo necrosis, as noted in Thomlinson and Gray's tumor sections (reviewed in [16]).

The major focus of hypoxia research for most of the 20th century was its role in cancer treatment responses. As early as 1909, Schwarz and colleagues noted that normal mammalian cells irradiated under conditions of hypoxia or anoxia were less sensitive to radiation than those irradiated in the presence of O_2 [17]. At that time, the changes were attributed to blood flow differences, and not to oxygen availability. In 1923, Petry's studies on the effects of oxygen on vegetable seeds were the first to note a correlation between oxygen and radiosensitivity (reviewed in [18]). In the 1950s, pioneering work by Gray and his colleagues (as well as Churchill-Davidson) established the existence of a hypoxic radioresistance effect in mammalian tumors and sought to quantify it. Gray used Ehrlich mouse ascites tumor cells irradiated in vitro and in mice under various O_2 conditions. Similar to what had been shown in plants and insect tissues, Gray found that the ascites tumor cells showed a dependence of radiation sensitivity on O_2 tension both *in vitro* and *in vivo*. Sensitivity was about three times greater under well-oxygenated conditions as compared to anoxic conditions [19,20]. Subsequently, Churchill-Davidson published a cytological evaluation of damage in patient tumors after radiation. One half of the tumor was irradiated while patients breathed pure O_2 at three atmospheres pressure, the other half while patients were breathing room air. Significantly more damage was observed in the half irradiated under high O_2 conditions [21]. In 1960, Dewey used human embryo liver cells to show that cells grown *in vitro* under anoxic conditions exhibited approximately two and a half fold greater colony forming ability after irradiation than cells grown in air [22]. Hewitt and Wilson irradiated mouse leukemia cells in live mice or two minutes after death and observed an identical sensitivity ratio as observed by Dewey [23]. Several years later, the experiments by Powers and Tholmach described above, further confirmed these findings [5]. Numerous other studies followed evaluating hypoxic effects on transplanted or spontaneous tumor regression after irradiation in mice, confirming earlier experiments (reviewed in [24]). Similarly, hypoxic cells were later found to be more resistant to many commonly used chemotherapeutic agents [25,26].

However, the 1970s and 1980s witnessed a shift in opinions concerning the importance of hypoxia in clinical responses to cancer treatment. Firstly, clinical responses to hyperbaric O_2 treatment[27] and then later to hypoxic cell radiosensitizers [28] were disappointing. Furthermore, hypoxic tumor cells appeared to reoxygenate in response to fractionated radiation treatment and therefore would have substantially less impact on tumor cell responses after fractionated therapy than after a single radiation dose[29,30]. Finally, scientists found that intrinsic cellular radiosensitivity alone at low doses could explain the differences in clinical responses observed [31]. Many became skeptical that the radiosensitization of hypoxic tumor cells was going to be clinically beneficial.

The next decade witnessed a second shift in conventional wisdom. With the demonstration in the 1980s and 1990s that, like other mammalian tumors, human tumors also contained radioresistant hypoxic cells came the realization that hypoxic cells could be targeted directly with hypoxia-specific cytotoxins. The combination of radiation with a hypoxia-specific cytotoxin would thus have the potential to destroy the entire tumor cell population. Such cytotoxins thus became the focus of intense investigation in the early 1990s [32].

The question of the role of hypoxia in cancer treatment instigated many studies, some of which revealed a number of other critical effects of hypoxia on tumor progression as discussed in detail later, including angiogenesis [33], metastatic potential [34,35], DNA replication [35,36] and reduced protein synthesis [37]. Finally, in the decade immediately preceding the discovery of the transcription factor Hypoxia Inducible Factor (HIF, see below), the expression of a number of genes was determined to be stimulated by O_2 deprivation. The earliest to be identified were Glucose Regulated Proteins (GRPs) and O_2 Regulated Proteins (ORPs)[37,38]. One ORP was later identified as heme oxygenase [39], while two others were revealed to be the same as two GRPs[38,40]. Other genes induced by hypoxia include those regulating hematopoesis and the vasculature (*VEGF*[41], *IL-1α*[42], *endothelin-1* [43], *PDGF*[44], and erythropoietin [*EPO*] [45]), glycolysis [46], metastasis (Cathepsin L [47]), and DNA damage responses (Gadd45, Gadd153 [48]). How the cell sensed O_2 deprivation and how gene transcription was regulated by hypoxia were completely open questions until the discovery of HIFs in the 1990s and further elucidation of HIF-α independent effects of hypoxia on cellular processes over the next decades (see Figure 3).

Identification of Hypoxia Inducible Factors (HIFs)

HIFs are heterodimeric transcription factors consisting of an α and a β subunit [49]. Systematic characterization of the *EPO* gene by multiple groups led to the identification of a cis-acting “hypoxia response element” (HRE; 5′-TACGTGCT-3′) in the 3′-flanking region of this locus that confers O_2 regulation of *EPO* expression [50-53]. “HIF-1” was subsequently described by Gregg Semenza and Guang Wang in 1992 as a nuclear factor that is induced by hypoxia, binds to the *EPO* HRE, and promotes transcriptional activation of *EPO* in O_2-starved cells [54,55]. The HIF-1 binding site within the *EPO* HRE was used for its purification via DNA affinity chromatography and cloning of cDNAs encoding the HIF-1α and HIF-1β subunits in 1995 [49]. This demonstrated that both subunits are members of the basic-helix-loop-helix polypeptide family that contain a “PAS” domain, initially characterized in the *Drosophila melanogaster* Per and Sim proteins and mammalian aryl hydrocarbon nuclear translocator (ARNT). While HIF-1α was a novel protein, HIF-1β was in fact identical to ARNT. HIF-1α protein and HIF-1 DNA-binding activity were rapidly induced by hypoxia (1% O_2), and quickly decayed upon reoxygenation of cells to ambient O_2 levels (21%). Finally, HIF-1α protein levels were shown to increase exponentially as HeLa cells were exposed to decreasing O_2 concentrations (6-0.5%) with a half maximal response between 1.5 and 2% O_2 [56].

The HIF-α/β dimer binds to HREs (5′-G/ACGTG-3′) associated with numerous transcriptional target genes critical to systemic hypoxia responses, such as angiogenesis and erythropoiesis, and cellular hypoxia responses involving metabolism, proliferation, motility, and autophagy (see Figure 1). In fact, hypoxia may be one of the most potent inducers of autophagy, where cellular components are degraded and recycled to restore ATP levels (see [57]). HIF target genes influence development, physiology, and numerous diseases. In mammals, three genes have been shown to encode HIF-α subunits that appear to be similarly regulated by O_2 availability. Of the three HIF-α proteins, HIF-1α and HIF-2α have been the most extensively characterized (see [58]). In addition to being identified first, HIF-1α is expressed ubiquitously, whereas HIF-2α and HIF-3α are found in a subset of tissues. HIF-2α, initially identified as endothelial PAS domain protein 1 (*EPAS1*), also binds to HREs and upregulates gene expression but is restricted to vascular endothelium, liver parenchyma, lung type II pneumocytes, and kidney epithelial cells [59-61]. In contrast, HIF-3α or inhibitory PAS domain protein (IPAS) acts as a dominant negative regulator of HIF-1α and HIF-2α mediated transcription and is found at high levels in the thymus, cerebellar Purkinje cells, and corneal epithelium of the eye [62,63]. Unlike the HIF-α proteins, HIF-1β/ARNT is constitutively expressed and insensitive to changes in O_2 levels. While, HIF-α mRNA levels change in response to hypoxia in select cell types, HIF regulation is primarily based on posttranslational modification and protein stability (See Figure 2).

It is important to note that while the HIFs are critical mediators of the transcriptional response to hypoxia, a number of HIF-independent pathways also respond to changes in O_2 tension (Figure 3). These responses allow cells to acutely adapt to the energetic demands of decreased O_2 availability by limiting energy consuming processes such as protein synthesis. Our understanding of the importance and regulation of these pathways is only beginning to emerge.

HIFs and cellular O_2 sensing

How the HIF pathway “senses” changes in O_2 concentration has been intensively studied since the cloning of the HIF-1α and HIF-1β subunits in 1995. While much has been elucidated concerning O_2 sensation by metazoans, key questions remain such as how HIF-independent pathways (Figure 3) perceive changes in O_2 availability. Huang et al. determined that HIF-α subunit stability is largely regulated via a region containing 200 amino acids referred to as the oxygen-dependent degradation domain (ODD) [64]. Direct analysis of the HIF-1α protein revealed that it is ubiquitinated and degraded by the 26S proteasome in O_2 replete cells [64-66]. Peter Ratcliffe, Patrick Maxwell, and colleagues demonstrated in 1999 that renal carcinoma cells lacking the von Hippel-Lindau tumor suppressor protein (pVHL) constitutively express both HIF-1α and HTF-2α as well as multiple HIF target genes [67]. Hypoxic regulation of HIF could be restored upon reintroduction of plasmids encoding the pVHL polypeptide. pVHL is the substrate recognition component of a ubiquitin-protein ligase complex that includes ElonginB, ElonginC, Cul2, and Rbx1 [67-71] . William Kaelin, Nikola Pavletich, and coworkers determined that this complex is related to the Skp1-Cul1-F-box (SCF) family of ubiquitin-protein ligases that catalyze the transfer of ubiquitin from ubiquitin conjugating enzymes to specific lysine residues of their substrates [72].

Biochemical studies performed by the research groups of Ratcliffe, Kaelin, and Frank Lee indicated that HIF-α interaction with pVHL requires the O_2-, 2-oxoglutarate-, and iron-dependent hydroxylation of proline residue 564 (within the human HIF-1α ODD) by an enzymatic activity reminiscent of procollagen prolyl hydroxylases [73-75]. A family of three HIF-specific prolyl hydroxylases was subsequently identified based on characterizing candidate genes that encode 2-oxoglutarate-dependent dioxygenases as well as biochemical

purification [76-78]. The mammalian enzymes, referred to as prolyl hydroxylase domain (PHD) proteins, are orthologs of the Cainorhabditis elegans *egl-9* gene [76]. Like *vhl-1* mutants, *egl-9* deficient worms constitutively express HIF-1α. Hydroxylation and pVHL-mediated polyubiquitination of HIF-α is inhibited in O_2-deprived cells, resulting in HIF-α accumulation, translocation to the nucleus, ARNT dimerization, and target gene activation (see Figure 2). The ability of HIF-α to interact with coactivators such as p300/CBP exclusively under hypoxic conditions is also regulated by hydroxylation, in this case that of a C-terminal asparagine residue by Factor inhibiting HIF-1 (FIH-1) [79-82]. FIH-1 is also an iron- and 2-oxoglutarate-dependent dioxygenase that likely fine tunes the regulation of HIF by O_2 availability.

The role of HIFs in cancer

Based on the analysis of human cancer biopsies and experimental animal models, it has become increasingly clear that HIFs play a critical role in cancer progression (see [83-85]). Immunohistochemical techniques have demonstrated that HIF-1α is overexpressed in a broad spectrum of human malignancies [84]. Furthermore, HIF-1α accumulation has been associated with poor patient survival in early stage cervical cancer [86], breast cancer [87,88], oligodendroglioma [89], ovarian cancer [90], endometrial cancer [91], and oropharyngeal squamous cell carcinoma [92]. Significant associations between HIF-2α overexpression and increased patient mortality have been reported for other diseases, including non-small-cell lung cancer (NSCLC) [93], neuroblastoma [94], astrocytoma [95], and head and neck squamous cell carcinoma [96]. Interestingly, HIF-1α overexpression was associated with decreased patient mortality for head and neck cancer [97] and NSCLC [98], suggesting that the two HIF-α subunits can have opposing effects on disease progression depending on tumor type. Along these lines, HIF-1α expression gradually decreases, whereas HIF-2α expression increases as renal carcinomas develop in patients with VHL disease, the cancer susceptibility syndrome associated with germline *VHL* mutations [99,100]. An association between HIF-3α expression and patient prognosis has not been examined.

Numerous experiments involving subcutaneous injection of murine and human cancer cell lines into immunodeficient mice have supported the notion that HIFs figure prominently in tumor growth. The first experiment was performed by the Ratcliffe group using ARNT-deficient mouse hepatoma cells [101]. Compared with tumors generated from wildtype cells, mutant tumors grew more slowly and exhibited decreased angiogenesis. Randall Johnson and coworkers demonstrated that immortalized and transformed *Hif-1α*$^{-/-}$ mouse embryo fibroblasts also produced smaller tumors in subcutaneous models [102]. David Livingston, Andrew Kung, and colleagues disrupted HIF transcriptional activity by introducing C-terminal HIF-1α peptides into human breast and colon cancer cells, resulting in decreased tumor growth in nude mice [103]. This peptide functions by inhibiting the ability of HIF-1α to interact with the CBP and p300 cofactors. Consistent with VHL patient sample analysis (see above), inhibiting HIF-1α in renal carcinoma cells results in increased xenograft tumor mass, while HIF-2α inhibition results in decreased tumor growth in nude recipients [99]. Ectopic expression of HIF-3α in hepatoma cells decreases tumor growth and angiogenesis in a subcutaneous tumor model, consistent with the dominant negative function of HIF-3α [63]. Finally, downregulating HIF-2α via RNA interference resulted in decreased subcutaneous neuroblastoma growth [94]. The studies in renal carcinoma, which were the first to directly demonstrate that HIF-1α and HIF-2α could have opposing effects on tumor growth, spurred a new interest into understanding the molecular differences between the α subunits. These observations also demonstrated that the relative contributions of the α subunits to tumor growth versus suppression are likely to be tissue specific.

While the injection of human cancer cells into immunocompromised mice has aided our understanding of the role of HIFs in cancer, this approach exclusively evaluates the terminal stages of malignancy. It fails to investigate the processes of tumor initiation, progression, and metastasis over a prolonged period of disease. The use of conditional alleles of murine *Hif-1α* and *Hif-2α* in spontaneous mouse models of cancer should greatly advance the field. Recently, Liao et al. have demonstrated a pivotal role for HIF-1α in pulmonary metastasis using a transgenic model of breast cancer [104]. Here, HIF-1α was conditionally ablated in the mammary epithelium of mice expressing the polyoma middle T oncoprotein in mammary glands. HIF-1α deficiency resulted in delayed tumor onset, reduced tumor growth and somewhat fewer tumor blood vessels. However, the number of metastases to the lung was significantly decreased. These results indicate that HIF-1α is unnecessary for breast cancer initiation or blood vessel recruitment, but plays an unexpected role in promoting metastatic potential. A role for HIF-1α in promoting breast cancer metastasis was also described by Hiraga et al. [105]. Validating observations made in mouse models through expression profiling and immunohistochemistry of patient samples will also be an important tool to extend experimental findings to the clinic [106].

A variety of genetic alterations that activate oncogenes and inactivate tumor suppressor genes result in increased HIF-1α expression, such as *VHL, PTEN,* and *ARF* loss of function and *ERBB2* and *SRC* gain of function (reviewed in [84]). However, one of the first links between tumor hypoxia and a specific cancer genetic program was that of *p53*. Regions of hypoxia and necrosis are common features of solid tumors. In 1996 Amato Giaccia, Thomas Graeber, and coworkers demonstrated that loss of the p53 tumor suppressor protein reduced hypoxia induced cell death in these lesions [107]. The authors proposed that O_2 deprivation provides a selective pressure within tumors for the clonal expansion of rare cells acquiring p53 mutations. The molecular mechanism(s) carrying the hypoxic signal to p53 was unclear until An et al showed that p53 was actually stabilized as a result of physical association with HIF-1α [108]. More recent studies have supported these initial observations, although some controversy concerning interactions between the HIF-1α and p53 pathways remains to this day.

HIFs also impact the c-Myc pathway: whereas HIF-1α opposes c-Myc [109], a potent regulator of proliferation and anabolic metabolism, HIF-2α promotes c-Myc activity [110]. These observations describe crosstalk between responses to changes in O_2 availability and a key transcription factor regulating cell growth [111]. Because c-Myc is commonly dysregulated in cancer, this is also an important interaction to consider when developing anticancer therapies.

Therapeutic implications of tumor hypoxia

As described previously, early work on the role of O_2 levels in cancer centered on the observation that poorly perfused tissues are more resistant to radiation [112]. It was later that Gray made the direct connection between tissue oxygenation and tumor cell radioresistance, a principle known as the "O_2 enhancement effect" [19]. Oxygen was thought to act as a direct radiosensitizer, increasing damage to DNA through the formation of free radicals. Indeed, experiments conducted by Richard Hodgkiss and colleagues in the late 1980s demonstrated that the oxygen effect required O_2 to be present at the exact time of radiation, leading to the "oxygen fixation" hypothesis (see textbox 2) [113]. The relative contribution of cellular responses to hypoxia versus the direct effect of free radicals to hypoxic radioresistance is still being deciphered.

Early attempts to exploit the oxygen effect for therapeutic benefit focused on interventions to increase tumor oxygenation during radiation. Hyperbaric O_2, red blood cell transfusions

and erythropoietin administration were all attempted as methods to increase tumor oxygenation. However, these approaches did not gain widespread use due to conflicting reports of their efficacy in clinical trials [114]. In the 1970s, nitroimidazole derivatives, which act as molecular mimics for O_2, were tested in conjunction with radiation. Despite initial promise, clinical trials with nitroimidazoles demonstrated limited benefit in part due to dose-limiting toxicities [115]. As an alternative to increasing tumor oxygenation, more recent strategies have attempted to take advantage of hypoxia to selectively kill tumor cells. Tirapazamine, described over 20 years ago, is a prototypic example of a hypoxia-activated prodrug [116]. Tirapazamine is specifically reduced in hypoxic cells, forming radical species that poison topoisomerase II leading to DNA double-strand breaks [117]. Clinical trials with this agent have demonstrated benefit in patients with lung as well as head and neck cancer, when used in combination with radiation or chemotherapy [118,119].

In addition to the direct role of molecular O_2 in generating radiation induced DNA damage, the biological effects of hypoxia on tumor cells can also modulate their response to therapy. This was first demonstrated several decades ago in experiments showing that the duration of hypoxia influenced radiation sensitivity [120]. Collectively, hypoxia modulates multiple pathways that contribute to radiation and chemotherapy resistance. Hypoxia exerts a selective pressure on cells for loss of p53, a key mediator of apoptosis, and upregulates the expression of the multidrug resistance gene (MDR1), leading to efflux of chemotherapeutic drugs [121-123]. Hypoxia has also been shown to contribute to increased mutagenesis rates and metastasis [124-127]. *Lysyl oxidase, c-MET,* and *CXCR4* are examples of genes directly regulated by hypoxia that play important roles in metastasis, and could serve as druggable targets.

Given the impact of hypoxia on therapeutic efficacy and patient prognosis, the ability to measure and image hypoxia in tumors has garnered significant attention. The introduction of the polarographic O_2 microelectrode (Eppendorf electrode) in the 1990s allowed the direct and most accurate measurement of O_2 tension in tumors [128]. However, due to the invasive nature of this technique and limitation to superficial tumors it cannot be broadly utilized in the clinic. Thus the emphasis has shifted to imaging modalities to indirectly observe tumor hypoxia. Increased glycolysis by tumors can be imaged using positron emission tomography (PET) with ^{18}fluorodeoxyglucose (FDG) as a tracer. However, since many tumors display increased rates of aerobic glycolysis, this is not a selective marker for tumor hypoxia. Several other compounds have also been developed that selectively accumulate in hypoxic tissues, including fluoromisonidazole (FMISO), etanidazole penta-fluoride (EF-5), and Cu(II)-diacetyl-bis(N4-methylthiosemicarbazone (Cu-ATSM) [129-131]. Nitroimidazoles are reduced under hypoxic conditions, forming stable adducts in the cell that can be detected by the PET scanner. While many of these approaches are currently being investigated for their ability to predict treatment response, they still have not attained routine use in the clinic.

The cloning of HIF, understanding of the pathways that regulate it, and identification of downstream HIF targets has produced numerous molecular targets for therapeutic intervention. Targeting the HIF pathway has generated significant attention because of the potential to selectively target hypoxic tumor cells and the role that hypoxia plays in key tumorigenic processes such as metastasis, angiogenesis and metabolism. Due to the difficulty in directly targeting transcription factors, the most promising interventions include strategies that block HIF accumulation, either by inhibiting translation or promoting degradation. Another approach is to inhibit key HIF target genes, such as VEGF, TGFα, or lysyl oxidase. Mutations in the PI3K/AKT/mTOR and RAS signaling pathways are commonly found in human tumors and both of these pathways have been shown to upregulate HIF-1α protein. Indeed, inhibition of the mTOR kinase with RAD-001 in a mouse model of prostate cancer induced by oncogenic Akt was found to decrease HIF-1α

accumulation and HIF-dependent gene expression [132]. Renal carcinoma cells, which often exhibit mutations in the *VHL* tumor suppressor, have also been shown to downregulate HIF-1α expression in response to mTOR inhibition [133]. In addition to the hypoxia-regulated VHL-dependent HIF degradation pathway, HIF is degraded through interaction with receptor of activated protein kinase C (RACK-1) [134]. RACK-1 competes with Hsp90 for binding to HIF-1α and Hsp90 inhibitors have been shown to promote HIF degradation. Targeting the interaction between HIF and the transcriptional co-activator p300 has also been demonstrated to inhibit HIF target gene expression and impair tumor growth [103,135]. Numerous other small molecules, including topoisomerase inhibitors, microtubule destabilizers, and histone deacetylase inhibitors, inhibit HIF-1α accumulation, although the mechanism for these effects is not yet clear [136].

Finally, drugs against several key HIF transcriptional targets have been developed and approved for clinical use. Chief among these is bevacizumab (Avastin, Genentech), a monoclonal antibody against VEGF, a key endothelial growth factor involved in tumor angiogenesis [137]. Bevacizumab is now commonly used for the treatment of metastatic colorectal cancer [138]. VEGF activity can also be inhibited by VEGF receptor tyrosine kinase inhibitors such as sorafenib, which has shown clinical benefit in renal and hepatocellular carcinoma [139]. The therapeutic effects of anti-VEGF therapy appear to involve several mechanisms, often depending on the tumor type and chemotherapy regimen. These effects include inhibition of angiogenesis, endothelial cell apoptosis, and vessel normalization [140]. Additionally, epidermal growth factor receptor inhibitors such as Iressa have seen some success in the treatment of lung cancer[141,142].

The past century of hypoxia research has provided significant advancement in our understanding of the molecular pathways by which O_2 tension influences the properties of tumors, from proliferation and angiogenesis to radiation and chemotherapy resistance. Current studies are focused on extending observations made *in vitro* to more physiologic contexts. The cloning of the HIFs has provided a genetic tool that when coupled with established mouse models of cancer will help clarify HIF-dependent effects on disease progression. Accordingly, studies in which one or both of the HIF-α subunits is conditionally deleted are now underway and will allow us to determine the tissue and stage specific effects of these factors on tumor progression. Mouse models will also be a useful platform to test newly identified therapies that target the HIF pathway for the treatment of cancer. The recent identification of a number of hypoxia-regulated microRNAs provides another mechanism by which O_2 levels can influence gene expression. A number of these microRNAs are highly expressed in tumors and their predicted targets (eg. BID, VEGF, cyclin D2) have roles in apoptosis, angiogenesis and proliferation. In future studies it will be important to identify the targets of these microRNAs to understand their role in tumorigenesis. [143]. Nearly a century after Warburg's initial observation of aerobic glycolysis in tumors, the unique metabolism of cancer cells will be important to reexamine. Specifically, the crosstalk between the HIF pathway and dysregulated oncogene and tumor suppressor function in driving metabolic changes will be an important area of study. Collectively, these observations will be critical for the successful development of novel imaging and treatment modalities, with the promise to improve targeted therapy of cancer and overcome therapeutic resistance induced by hypoxia.

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Box 1: Timeline

The history of research on tumor hypoxia

1909 Schwartz first observed the effect of changes in vascular function on radiation sensitivity.

1923 First correlation between oxygen and radiosensitivity made by Petry in Germany.

1924 Otto Warburg describes increased tumor cell glycolysis in the presence of high O_2 levels.

1953 Gray and colleagues establish the existence of hypoxic radioresistance.

1955 Thomlinson and Gray describe tumor cell hypoxia in clinical specimens.

1964 Hyperbaric O_2 used to promote the efficacy of radiation treatment of cancer patients.

1984 First “oxygen regulated proteins” (ORPs) described.

1986 Polarographic electrodes and nuclear magnetic resonance spectroscopy used to measure tumor cell oxygenation.

1986 First attempts to exploit tumor cell hypoxia by selectively killing O_2-starved cells with tirapazamine.

1991 Hypoxia regulated DNA elements characterized in human *erythropoietin* (*EPO*) gene.

1995 “Hypoxia Inducible Factor-1” (HIF-1) biochemically purified and cloned by Semenza and colleagues.

1996 Giaccia and coworkers determine that tumor cell hypoxia selects for rare clones exhibiting p53 inactivating mutations.

1997 First demonstration of tumor promoting effects by HIF in subcutaneous mouse models.

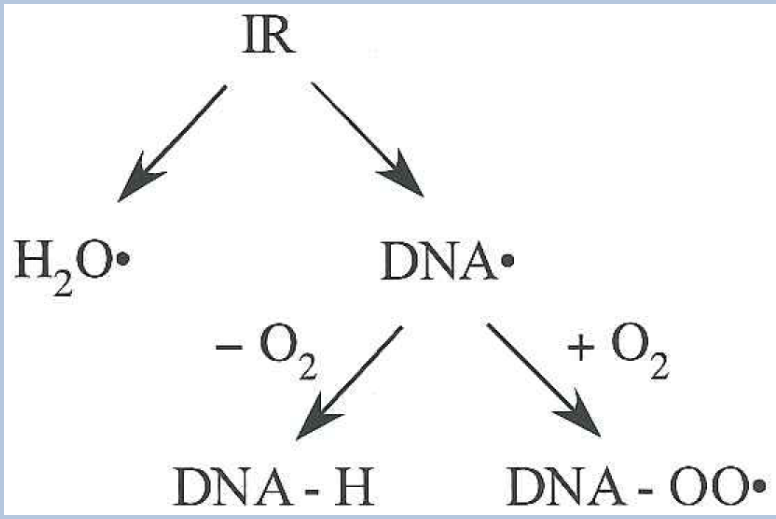
1999 HIF regulation by the von Hippel-Lindau tumor suppressor protein (pVHL) described by Ratcliffe et al.

2001 Ratcliffe, Kaelin, and Lee research groups demonstrate HIF-α subunit prolyl hydroxylation.

2001 HIF-prolyl hydroxylase domain (PHD) enzymes identified.

Box 2: The "oxygen fixation" hypothesis

Irradiation of cells generates free radicals either in DNA or water (H_2O) molecules. Free radicals in DNA (DNA•) can react with available O_2 to generate a peroxy-radical (DNA – OO•), thus chemically modifying the DNA ('oxygen fixation'). In the absence of O_2, the DNA radical will be reduced, restoring the DNA to its original composition (DNA – H). In addition to the direct effects on DNA, free radicals produced from H_2O (H_2O•) can further damage nearby DNA.



Box 3: HIF contributions to other diseases

- **Polycythemia:** Point mutations in HIF-2α have recently been identified in multiple families with erythrocytosis [144,145]. A point mutation in VHL leading to Chuvash polycythemia preferentially stabilizes HIF-2α [146].
- **Cardiovascular disease:** Cardiac ischemia is a significant source of patient mortality. The adaptive responses mediated by the HIFs are important for protecting ischemic tissues from injury and activating the HIF pathway selectively in these tissues could provide an important therapeutic intervention [147-149].
- **Pulmonary hypertension:** Both HIF-1α and HIF-2α regulate vascular remodeling and development of pulmonary hypertension in response to chronic hypoxia [150,151].
- **Inflammation:** Hypoxia is commonly observed in inflammatory states, and HIF-1α can have protective as well as pro-inflammatory effects. Macrophage specific deletion of HIF-1α revealed an important role in mediating innate immune responses [152]. Alternatively, deletion of HIF-1α in colonic epithelium revealed a protective function in drug-induced colitis [153].

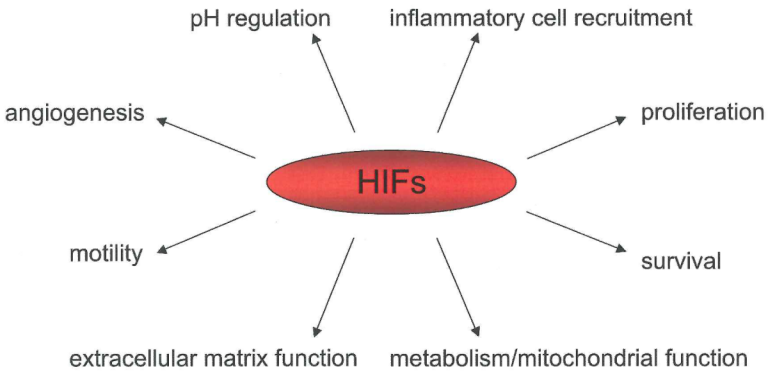


Figure 1.

Genes activated by hypoxia-inducible factors (HIFs) involved in tumor progression. Genes encoding proteins involved in numerous aspects of tumor initiation, growth, and metastasis are transcriptionally activated by either encoding HIF-1α or HIF-2α. Examples include: inflammatory cell recruitment (SDF-1α, CXCR4), proliferation (cyclin-D2, IGF-2), survival (VEGF, erythropoietin), metabolism/mitochondrial function (glycolytic enzymes, PDK-1), extracellular matrix function (fibronectin-1, collagen type-5), motility (c-MET, SPF-1α), angiogenesis (VEGF, PDGF), and pH regulation (carbonic anhydrase-9).

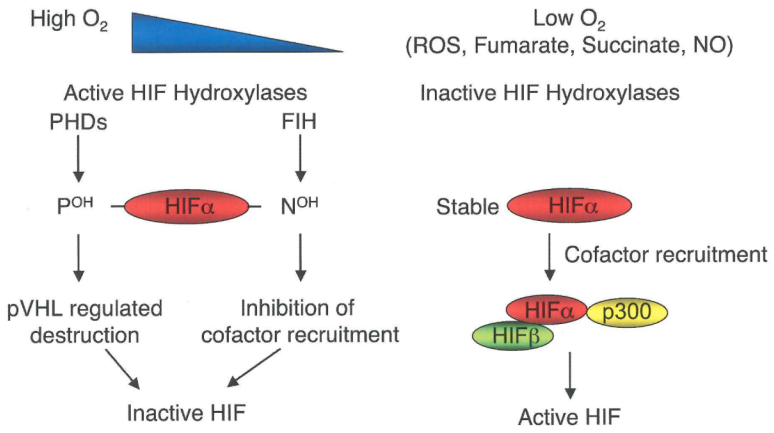


Figure 2.

Regulation of HIF-α subunits by O_2 availability and other intracellular metabolites. In oxygen replete cells, the HIF-prolyl hydroxylases (PHDs) are active, resulting in the hydroxylation of proline residues in HIF-α and their targeted degradation via the pVHL-proteosome pathway. "Factor-inhibiting HIF" (FIH) hydroxylation of an asparagine residue in the C-terminus of the HIF-α subunit, blocks p300 co-factor recruitment. This results in the inactivation of HIF-α subunit transcriptional activity. All of these processes are inhibited when O_2 levels decrease or cells exhibit increased levels of reactive oxygen species (ROS) and other metabolites such as fumerate, succinate, and nitric oxide (NO). Here, the HIF-α subunits are stabilized, they recruit co-activators such as p300, and activate HIF target genes, as described in Figure 1.

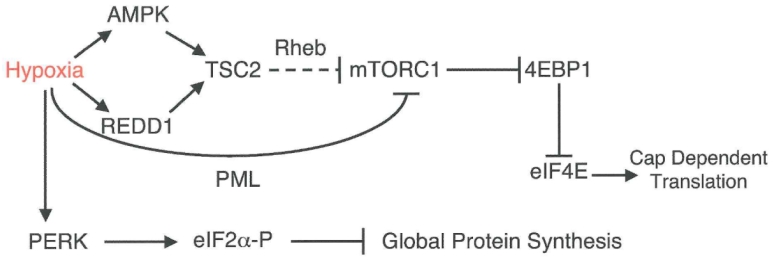


Figure 3.

Hypoxia regulates other critical pathways that impact tumor progression in a HIF-independent fashion. A schematic diagram of signaling pathways which regulate mRNA translation is shown. These include the mTOR pathway and the integrated stress response. Hypoxia influences AMPK and REDD1 activity, which act upstream to inhibit mTOR kinase activity, resulting in a decrease in cap-dependent translation. Hypoxia also inhibits the ER resident kinase PERK, which phosphorylates eIF2α, resulting in global protein synthesis inhibition. While HIFs contribute to mTOR regulation via the induction of REDD1 during chronic hypoxia, all of these pathways can be regulated by O_2 deprivation in a HIF-independent manner. Although the mechanisms for mTOR and PERK regulation by changes in O_2 remain unclear, they represent important additional therapeutic targets.