THE CELLULAR RESPONSE TO HYPOXIA IN CANCER

WHITE PAPER

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ABSTRACT

Tissue hypoxia exerts significant influence on molecular programs, cellular properties and in tumors often leads to aggressive phenotypes. HIF-1α and HIF-2α belong to the family of hypoxia inducible transcription factors and mediate cellular reprograming under low oxygen conditions. HIFs are stabilized under hypoxia and regulate the expression of genes involved in key cellular adaptations including angiogenesis, metabolic switch and stemness. A standing question in the cancer research field is how variability in the duration of hypoxia affects cellular adaptations and tumorigenesis. Acute and chronic hypoxia co-exist in tumors and differentially shape the phenotype of cancer cells. Differential responses to acute vs chronic hypoxia are partly underscored by differences in the recruitment of HIF-1α vs HIF-2α. Here, we provide an overview of key cellular adaptations triggered by hypoxia and emphasize recent findings highlighting the role of hypoxia-duration in shaping cancer cell phenotypes.

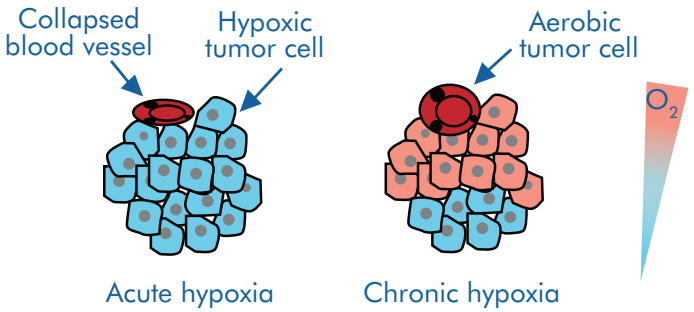


Figure 1. Mechanisms underscoring hypoxia in tumors. Based on duration, hypoxia is classified as acute or chronic. Acute hypoxia is commonly associated with a temporary obstruction of blood flow. Chronic hypoxia, as it may occur in tumor microenvironments distant from vasculature, is underscored by limitations in oxygen diffusion.

INTRODUCTION

Hypoxia refers to a physiological state where the levels of oxygen in organs and tissues fall within a defined sub-optimal range.[1] Low oxygenation or hypoxia is commonly associated with various pathophysiological conditions. For example, in cancer, low oxygen levels occur at different stages during tumor progression, and trigger genetic programs broadly known as "cellular adaptations to hypoxia", which are often linked to negative prognoses.

Tumor-hypoxia results from the combined effects of increased cellular proliferation and decreased or sub-optimal blood supply. Episodic or acute hypoxic events are common in tumors, due to temporary obstruction of blood flow which may limit oxygen supply for short periods of time (minutes to hours).[1,2] In contrast as tumors grow, cells are exposed to extended or chronic hypoxia (hours to days), due to abnormal and insufficient vascularization that limits the diffusion of oxygen to some tumor-regions (**Figure 1**).[1-4] Dynamic changes in oxygen availability within the tumor microenvironment, leading to acute and chronic hypoxia, differentiallymodulate cellular processes including angiogenesis, metabolism, proliferation and metastasis.[1]

Hypoxia inducible factors, HIF-1α and HIF-2α, play central roles in directing the cellular response to low oxygenation. These transcription factors, often referred to as "master regulators of the hypoxia response", are stabilized and activated under low oxygen conditions. Upon activation, HIF-1α and HIF-2α translocate to the nucleus and regulate the expression of a wide range of genes implicated in tumorigenesis.

The genetic programs regulated by HIFs and their outcome for tumorigenesis continue to be the focus of intense study. For example, HIFs regulate or intersect molecular programs that support the maintenance and renewal of stem cells.[5] By promoting tumor stem cells, HIFs positively contribute to tumorigenesis and chemoresistance. Furthermore, HIFs mediate metabolic reprogramming and the epithelial to mesenchymal transition (EMT) process which are quintessential to tumor growth and survival (**Figure 2**). However, cellular adaptive responses to hypoxia driven by HIFs are not always pro-tumorigenic. HIFs may also direct anti-tumorigenic programs, limiting tumor cell proliferation and growth via activation of apoptotic mechanisms or induction of sustained autophagy.[5]

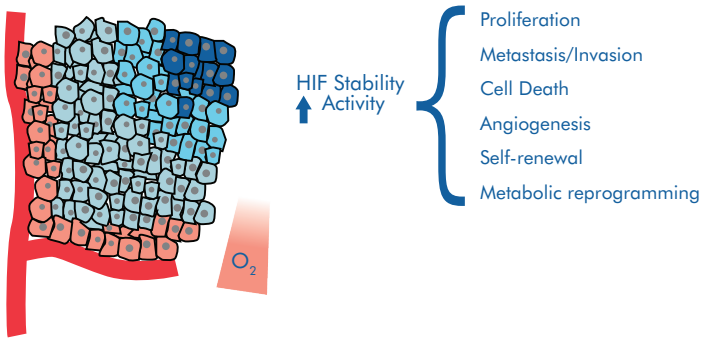
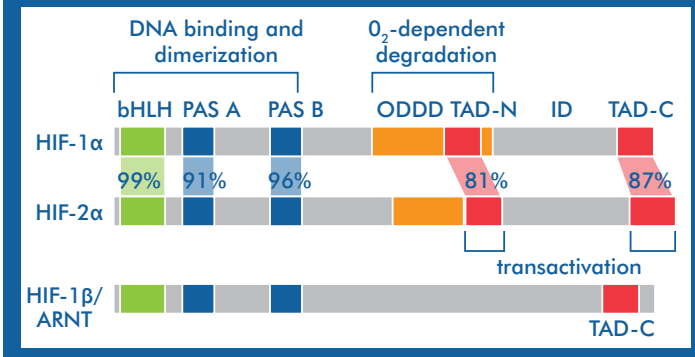


Figure 2. HIF-dependent adaptive responses to hypoxia in cancer. A gradient of oxygenation is present in tumors, with the lowest oxygen tension occurring at distant sites from the vasculature. HIF protein expression becomes stabilized with reduced oxygenation, leading to the induction of various signaling pathways responsible for cellular adaptations to hypoxia.

It is currently recognized that responses to acute hypoxia are predominantly mediated by HIF-1α.[3,5] In contrast, cellular adaptations to chronic hypoxia may predominantly involve HIF-2α regulation.[3,4,6] Acute and chronic episodes of hypoxia may co-exist at different stages of tumor development and have been linked to distinct changes in tumor cell properties. For example, acute hypoxia is thought to result in cellular adaptations leading to increased tumor growth and metastasis; in contrast chronic hypoxia activates programs that inhibit proliferation and promote cell-death.[3] Nevertheless, tumor responses to chronic hypoxia are not fully understood and often lead to more aggressive tumor phenotypes.[7] It has been proposed that the ultimate outcome of hypoxia on tumorigenesis depends on various factors including severity of hypoxia (pO_2), duration of hypoxia (minutes to days), and HIF isoform-expression and -activity.[5]

In this review, we will discuss molecular mechanisms involved in the cellular adaptations to acute versus chronic hypoxia which play critical roles in the modulation of tumorigenesis. Special attention will be given to recent discoveries shedding new light on HIF-dependent cellular adaptations, as well as newly identified pathways that intersect HIF signaling and significantly influence tumorigenesis.

HIF TRANSCRIPTION FACTORS: REGULATORS OF THE ADAPTIVE RESPONSE TO HYPOXIA AND THEIR ROLE IN CANCER



In humans, the gene encoding HIF-1α is located on chromosome 14, and has 16 exons, whereas the gene for HIF-2α resides on chromosome 2 and contains 17 exons.[8,9] The HIF-1α protein is 826 amino acids (aa) long, while HIF-2α protein has 869 or 870 aa.[10, 11] Major domains of HIFs include: basic Helix-Loop-Helix (bHLH); Per-AHR-ARNT-Sim (PAS) homology domains (PAS-A and PAS-B); PAS-associated C-terminal domain (PAC); the oxygen-dependent degradation domain (ODDD); and C- and N-terminal transactivation domains (TAD-C and TAD-N).[12] The homology between HIF-1α and HIF-2α for bHLH, PAS-A, and PAS-B is 99%, 91%, and 96%, respectively.[11] HIF-α isoforms form heterodimers with HIF-1β (aryl hydrocarbon receptor nuclear translocators- ARNT) to regulate gene expression. HIF-1β is constitutively expressed, independently of oxygen levels, and present in the nucleus. HIF domains take part in DNA binding (bHLH), protein-protein interaction and dimerization (PAS-A, PAS-B, PAC), oxygen-dependent degradation (ODDD), and transcriptional activation (TAD-C and TAD-N).[12]

Regulation of HIF Expression: Normoxia versus Hypoxia

Four oxygen-sensing enzymes are responsible for regulating HIF expression in an oxygen-dependent manner: prolyl hydroxylases (PHD1, PHD2 and PHD3) and factor inhibiting HIF (FIH).[5] Under normoxia, HIF-1α subunits are short-lived, with a half-life of less than 5 minutes, due to ubiquitin-dependent proteasomal degradation (**Figure 3**).[10] This process is initiated by PHDs, especially PHD2, which catalyzes the post-translational hydroxylation of proline residues Pro-402 and Pro-564 within the oxygen-dependent degradation domain (ODDD) of HIF-1α (**Inset**). Hydroxylated HIF-1α binds von Hippel-Lindau protein (pVHL), which recruits the E3 ubiquitin ligase system and leads to the degradation of HIF-1α by the 26S proteasome (**Figure 4**).[10,12] Knock-down or knock-out of VHL prevents the proteasomal targeting and degradation of HIFs. Additionally, FIH-1 hydroxylates HIF-1α at asparagine residue 803 (Asn-803) in the TAD-C domain, blocking the association of HIF-1α with its coactivators (**Inset**).[13] HIF-2α is also targeted for degradation under normoxia, but the specific residues targeted for modification differ from those in HIF-1α. For example, in the TAD-C domain of HIF-2α, Asn-851 is hydroxylated by FIH-1, and in the ODDD domain, Pro-405 and Pro-531 are hydroxylated by PHDs.[10]

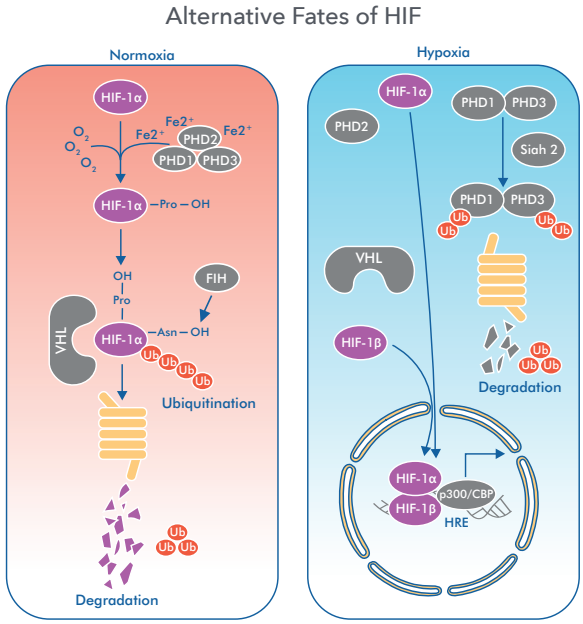


Figure 3. Key steps in the regulation of HIF-1α. Under normoxic conditions, HIF-1α is targeted for hydroxylation by a group of PHD enzymes, ensuring together with pVHL the proteasomal targeting of HIF-1α. Under hypoxia HIF-1α is stabilized and freely moves to the nucleus where it forms a heterodimer with HIF-1β and by interacting with co-activators p300/CBP regulates the expression of HRE positive target genes.

Under hypoxia, PHDs are targeted for degradation by E3 ubiquitin ligases Siah1a and Siah2, which prevents the association of HIFs with pVHL (**Figure 3**).10 Siah2 is phosphorylated and regulates PHDs, primarily PHD1 and PHD3, downstream of p38 and Akt signaling.10, 14 Overall, stabilization of HIFs occurs under hypoxic conditions because PHDs are inactivated, and HIFs escape ubiquitination and degradation. However, several enzymes control HIF protein function. **Table 1** highlights major enzymes regulating the transcriptional activity of HIF-1α.

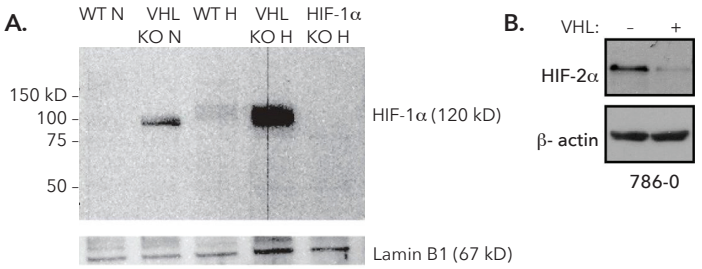


Figure 4 . Knockout of pVHL leads to increased stabilization of HIF proteins. A. Naive CD4 T cells from WT, VHL-deficient (VHL KO), or HIF-1α–deficient (HIF-1α KO) mice were differentiated under IL-22–skewing conditions for a total of 60 hours. Some cells remained at normoxia for the duration of the culture (N); others were at normoxia for 35 hours and then hypoxia (1% O_2) for 24 hours (H). At 60 hours, nuclear extracts were harvested, and HIF-1α; Antibody (H1alpha67) [NB100-105] and Lamin B1 levels were analyzed by Western blot. **B.** Western blot with HIF-2α/EPAS1 Antibody [NB100-122] of 786-O cells without or with VHL overexpression.

Table. 1 Major enzymes regulating HIF-1α[12]

	Regulators of HIF-1α1[2]	Regulatory Mechanisms
Negative	Casein kinase 1 (CKI)	Phosphorylation within PAS-B (Ser-247) destabilizes the HIF-1α–ARNT complex and reduces transactivation
	PHDs	Hydroxylation within ODDD (Pro-402) and TAD-N (Pro-564) lead to reduced stability of HIF-1α
	FIH	Hydroxylation within TAD-C (Asn-803) causes faulty transcriptional activation by HIF-1α
Positive	Glycogen synthase kinase 3β (GSK3β)	Phosphorylation within ODDD (Ser-551, -555, -588) results in diminished stability of HIF-1α
	ERK1	Phosphorylation within C-terminal domain (Ser-641, -643) masks a nuclear export signal and results in nuclear accumulation and enhanced activity of HIF-1α
	Protein kinase A (PKA)	During intermittent hypoxia, PKA functions like ERK1[15]
	p300/CBP-associated factor (PCAF)	Acetylation within C-terminal domain (Lys-674) leads to increased HIF-1α levels and binding of p300
	p300	Acetyl-transferase activity of p300 within C-terminal domain (Lys-709) blocks ubiquitination-induced degradation of HIF-1α
	Casein kinase II (CKII)	Hypoxia-induced phosphorylation within TAD-C (Thr-796) decreases affinity of HIF-1α for FIH

HIF GENE TARGETS AND THEIR ROLE IN TUMORIGENESIS

Discoveries in the 1990s revealed that HIF-1α translocates to the nucleus under hypoxic conditions, and binds to the consensus sequence, 5′-CGTG-3′, of hypoxic response element (HRE) containing genes. To transcribe genes involved in the hypoxic response, HIF-1α recruits and associates with co-activators CBP/p300.[16-18] Later, HIF-2α was found to regulate HRE containing genes in a similar manner.[19, 20] Together, HIFs activate the expression of over 100 genes involved in a broad range of tumorigenic functions, including angiogenesis, a process regulated by multiple hypoxia-induced genes.[21, 22]

Under hypoxia, HIFs may activate pathways that promote cancer cell growth and survival or activate key pathways that inhibit tumor growth.[23-25] The ultimate outcome for tumorigenesis is thought to be significantly dependent on the combined activities of HIF-1α and HIF-2α, and dependent on tissue, cell, and cancer type. Ahead, we discuss some adaptations to hypoxia in cancer cells regulated by HIFs paying special attention to novel findings.

I. ANGIOGENESIS

Angiogenesis is a process by which new vasculature is formed from pre-existing blood vessels, allowing delivery of oxygen and nutrients to tumor cells. This process plays a critical role in facilitating dissemination of tumor cells, or metastasis.[5] The importance of HIFs in angiogenesis is highlighted by the observation that reduced HIF-2α expression in endothelial cells disrupts tumor vascularization.[26] VEGF is perhaps the most important proangiogenic factor that is transcriptionally activated by HIFs.[5, 27] Other HIF target genes that promote angiogenesis, maintain vascular tone, and facilitate adaptation to hypoxic conditions in cancer cells include:

HIF Regulated Angiogenic Targets[11, 22, 28]
Angiopoietin-2 (Ang-2)
Adrenomedullin (ADM)
Cyclin G2 (CCNG2)
Endothelial TEK tyrosine kinase (Tie-2, TEK)
Endothelin-1 (ET-1)
Inducible nitric oxide synthase 2 (iNOS2)
Platelet-derived growth factor-β (PDGF-β)
Plasminogen activator inhibitor-1 (PAI-1)
VEGF-R1
VEGF-R2

HIF-dependent induction of VEGF and the aforementioned genes promote adequate oxygen delivery to hypoxic tissues.[22, 28] Significantly, even with the activation of angiogenesis signaling, hypoxia tends to persist in tumors, due to the poor-quality and leakiness of newly formed vessels which often fail to match robust tumor growth.

A recent study demonstrated differential regulation of HIF-1α targeted genes involved in angiogenesis in colorectal cancer (CRC). Briefly, the expression of angiogenic genes including VEGF-A, Smad7, Jun, IL-8, CXCR-4, PDGF-A, TGF-A and ANG-PTL-4, was found by microarray analysis to be more elevated under conditions of acute (24 hours) versus chronic (72 hours) hypoxia.[29] *In vitro* studies showed that both hypoxic conditions promoted an invasive phenotype, albeit an induced differential gene expression.[29]

II. METABOLISM

To meet the increasing energy demands of cancer cells, HIFs bring about changes in energy production pathways. For example, intra-tumoral HIF-1α induces transcriptional activation of genes involved in lactate production, pyruvate metabolism, glucose transport, and glycolysis. Together these metabolic changes underscore the Warburg effect, which involves a dramatic increase in both the rate of glucose uptake and the fermentation of glucose to lactate even in the presence of adequate oxygen and fully functioning mitochondria.[5, 30] The expression of glycolytic enzymes is predominantly under the control of HIF-1α. Additionally, HIF-1α affects the metabolism of amino acids, iron, and nucleotides.[31] HIF-2α may contribute to other mechanisms in support of the metabolic changes occurring under hypoxia. For example, HIF-2α regulates the expression of glucose transporter-1 (GLUT1) in VHL-null renal cell carcinoma.[32]

Cancer cell metabolism may be also modulated by c-Myc oncogene dependent programs downstream of HIFs.[25] In CRC cells, c-Myc expression is induced during acute hypoxia. In contrast, prolonged hypoxia suppressed c-Myc expression, a process regulated at the transcriptional level by HIF-2α.[25, 33] Interestingly, the expression of HIF-1α and HIF-2α is differentially regulated by the extent of hypoxia; HIF-1α is induced under acute hypoxia and HIF-2α predominates under chronic hypoxia.[25] These findings highlight the importance of hypoxia-duration as a factor influencing the regulatory interplay between HIF isoforms.

III. CANCER CELL-SURVIVAL AND -DEATH

The first HIF-1α target gene identified was erythropoietin (EPO), which stimulates erythropoiesis, and is upregulated in hypoxia.[22] EPO is also an important target of HIF-2α, demonstrating that HIFs often share similar regulatory mechanisms.[10, 20] By maintaining adequate blood/oxygen supply, EPO and pro-angiogenic genes (VEGF, iNOS2, and ADM) ensure HIF-mediated cancer cell survival.[10, 20, 22, 28] Paradoxically, overactivation of HIF-1α in certain cancers can lead to cell cycle arrest and/or apoptosis by the following three mechanisms:[24]

1. **HIF-1α stabilizes p53, potentially by binding to E3 ubiquitin ligase MDM2.[24]**
2. **HIF-1α induces the expression of proapoptotic genes (NIP3, NIX, and RTP801) under hypoxia.[24]**
3. **HIF-1α transcriptionally activates MIX-1, which represses p21-inhibitor c-Myc, leading to cell cycle arrest.[24]**

Additionally, HIF-1α and HIF-2α may target the same downstream genes with opposing outcomes. For example, in contrast to HIF-1α, HIF-2α inhibits the activation of the tumor suppressor p53, promoting tumorigenesis in lung and clear cell renal cell carcinoma (ccRCC).[23]

Recent studies have demonstrated that HIFs regulate expression of non-coding genes including miRNAs. In multiple myeloma (MM), HIF-1α regulates the expression of miR-210 under conditions of chronic hypoxia.[34] miR-210 has both oncogenic and anti-tumorigenic functions. As an oncogene, miR-210 promotes neovascularization, whereas as a tumor suppressor, it prevents cellular proliferation.[35, 36] Under chronic hypoxia in MM, HIF-1α dependent upregulation of miRNA-210 was found to suppress the expression of dimethyladenosine transferase 1 homolog 1 (DIMT1)–interferon regulatory factor 4 (IRF4) axis. Inhibition of the DIMT1-IRF4 axis would be expected to promote apoptosis, as this axis induces cell survival and proliferation.[34] Interestingly, the suppression of the DIMT1-IRF4 axis under chronic hypoxia failed to increase apoptotic cell death in MM cells, suggesting that other HIF-1α anti-apoptotic dependent pathways may determine the overall phenotype (**Figure 5**).[34]

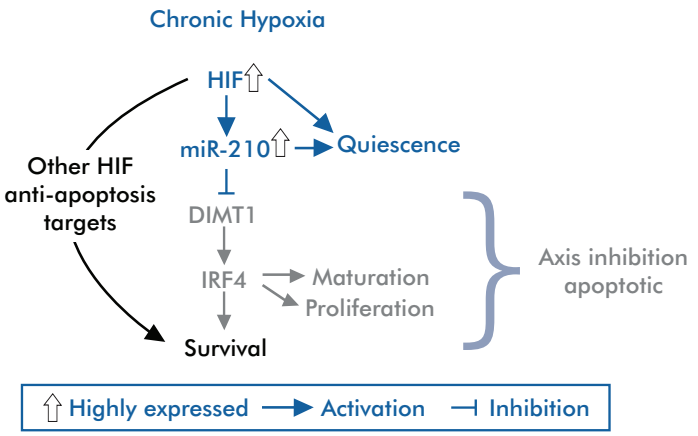


Figure 5. Regulation of DIMT1-IRF4 in chronic hypoxia. Stabilization of HIF-1α, and upregulation of miR-210 lead to inhibition of DIMT1/IRF4 axis, which should have a pro-apoptotic outcome. In myeloma cells, activation of other HIF-1α targets may support survival albeit inhibition of the DIMT1/IRF4 axis.

IV. THE EPITHELIAL TO MESENCHYMAL TRANSITION (EMT) AND STEMNESS

Functional transition of polarized epithelial cells, which interact with basement membrane using their basal surfaces, to a mobile mesenchymal cell phenotype, has been implicated in embryogenesis, organ development, tumorigenesis, and metastasis.[37] Cells with a mesenchymal phenotype have increased migratory capacity, resistance to apoptosis, invasiveness, and an enhanced capacity to produce components of extracellular matrix.[37]

Phenotypic changes associated with the process of EMT are underscored by transcriptional, post-transcriptional, translational and post-translational programs and modifications.[2, 38] Hypoxia and HIF-1α, either directly or indirectly, regulate the expression of many transcription factors that govern the process of EMT, including Notch, PDGF, VEGF, Snail, TCF3, TGF-β, Twist1, ZEB1 and ZEB2.[39, 40] In CRC, HIF-1α directly binds to the proximal promoter of ZEB1 to increase ZEB1 expression, which leads to EMT and cell migration.[41] In gastric stem cells, HIF-1α induces EMT by increasing Snail, an E-cadherin repressor that facilitates metastasis.[40] Reduction of E-cadherin levels is a critical step in the EMT process and may result from various mechanisms including decreased expression, proteolysis and endocytic recycling (**Figure 6**).[38] Once E-cadherin is removed from the cell membrane, catenins translocate to the nucleus to regulate mesenchymal genes.[38]

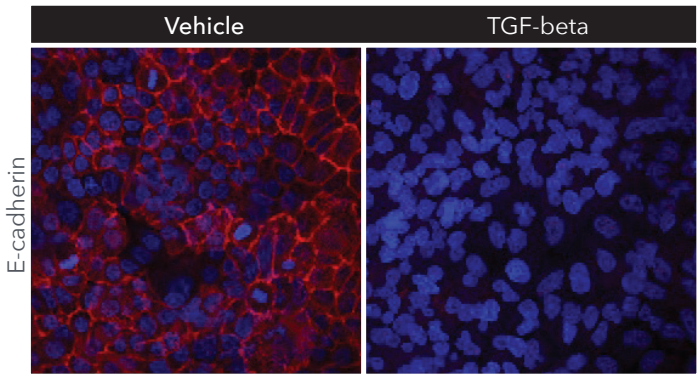


Figure 6. TGF-β regulates expression of E-cadherin. Expression of E-cadherin was detected in immersion fixed A549 human lung carcinoma cell line vehicle treated (left panel) or treated with 10 ng/mL TGF-beta (Catalog # 240-B, right panel) for 48 hours using Goat Anti-Human/Mouse E-Cadherin Antigen Affinity-purified Polyclonal Antibody (Catalog # AF648) at 10 µg/mL for 3 hours at room temperature. Cells were stained using the NorthernLights™ 557-conjugated Anti-Goat IgG Secondary Antibody (red; Catalog # NL001) and counterstained with DAPI (blue).

In carcinomas, hypoxia represents a trigger for the induction of EMT programs which may also lead to the development of stemness, giving rise to cancer stem cells (CSCs) with self-renewal capacity.[2] CSCs are responsible for the aggressive phenotype and metastatic potential of tumors.[2, 42] Hypoxia is a driver for the development and maintenance of CSCs partly through the stabilization of HIF-1α and HIF-2α. Induction of HIF signaling is associated with the expression of pluripotency markers, supporting the initiation and maintenance of stem cells.[43-45] In neuroblastoma and breast cancer, CSCs may arise by a process of de-differentiation regulated by hypoxia.[43] Several transcription factors induced in hypoxia are downstream of HIFs and play key roles in cancer cell-renewal including OCT4, NANOG, and SOX2 (Figure 7).

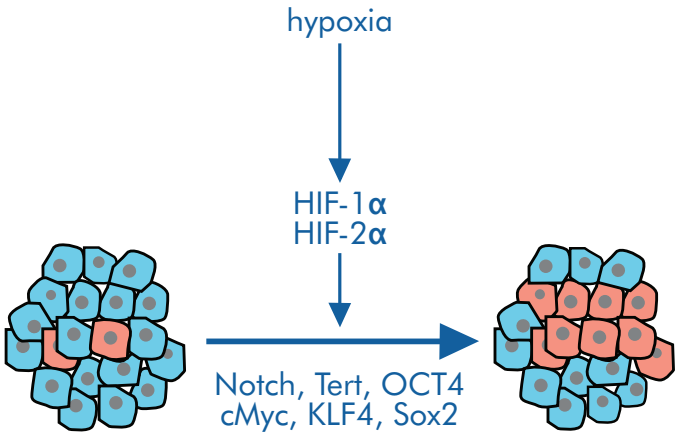


Figure 7. Hypoxia and HIF proteins are critical for the development of CSCs. In the hypoxic tumor microenvironment, HIF proteins regulate the expression of genes involved in the initiation and maintenance of stem cells. Populations of stem cells may differ in their expression of pluripotency markers.

Recently, SOX2 was shown to contribute to tumorigenesis and invasiveness in prostate cancer.[42] Regulation of SOX2 expression by HIFs was isoform specific and dependent on the extent of hypoxia. For example, acute hypoxia leads to SOX2 induction downstream of HIF-1α and to increased invasiveness. In contrast, under chronic hypoxia, HIF-2α induced SOX2 leads to greater capacity for sphere formation and thus increased stemness.[42]

SUMMARY

Hypoxia plays key roles in development and disease. In cancer, hypoxia frequently results from the combined effects of poor blood supply and increased proliferation. Cellular adaptations to hypoxia commonly lead to more aggressive and invasive phenotypes that are resistant to therapy. Cellular responses to h ypoxia determine cancer progression and are predominantly shaped by the regulatory activities of HIF proteins.

Understanding cellular adaptations to low oxygen levels is complicated by heterogeneity within tumors. Different microenvironments within tumors may expose cells to diverse oxygenation levels from normoxic to anoxic. It has become increasingly clear that the duration of hypoxic events significantly influences the genetic programs activated and the ensuing phenotypes. Significantly, recent studies highlight the importance of hypoxia duration for the interplay between HIF-α isoforms and tumorigenesis outcome.

Overall, cellular adaptations to hypoxia are predominantly regulated by HIFs, however signaling pathways including PI3K/AKT/mTOR, MAPK/ERK and the unfolded protein response (UPR) participate in cellular re-programming. Understanding how these different signaling mechanisms interact to shape tumor progression under variable oxygenation conditions is critical for the development of effective cancer therapies.

ACKNOWLEDGEMENT:

Reviewed and Edited by: Bethany Veo, PhD., Research Associate, University of Colorado (AMC)

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