This is to certify that the
dissertation entitled

THE ROLE OF THE HIF1 SIGNALING PATHWAY
IN TUMORIGENESIS

presented by

KangAe Lee

has been accepted towards fulfillment
of the requirements for the

Ph.D degree in Program in Cell and Molecular Biology

Major Professor's Signature

July 9, 2007

Date

THE ROLE OF THE HIF1 SIGNALING PATHWAY IN TUMORIGENESIS

By

KangAe Lee

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Program in Cell and Molecular Biology

2007

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ABSTRACT

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The hypoxia-inducible factor 1 (HIF1) is the key transcription factor involved in the cellular responses to hypoxia and it also plays an essential role in adapting a cell to the microenvironment of a tumor through regulation of genes involved in angiogenesis, glycolysis, and many other processes. HIF1 activity is regulated in an oxygen dependent manner by a family of prolyl hydroxylases (PHDs), whose activity is required for HIF1α binding to the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is a component of an E3 ubiquitin ligase complex responsible for instigating proteosomal degradation of HIF1α. This role of regulating HIF1 activity is one reason for pVHL's tumor suppressor activity. Interestingly, this process is completely dependant upon PHD-mediated posttranslational hydroxylation of HIF1α and this requirement raises the possibility of the involvement of PHD in tumor development.

In this study, we characterize the relationship between PHD and HIF1 activity and cellular transformation using a lineage of the MSU1 cell strains of varying tumorigenic potential. We have shown that PHD2 represents the primary HIF prolyl hydroxylase in regulating HIF1 signaling within this lineage of cells and that PHD2 levels decrease as the cell exhibits more transformed characteristics. When PHD2 levels were altered with RNAi in non-tumorigenic fibroblasts we found that moderate decreases in PHD2

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activity can lead to malignant transformation, whereas cells with a more severe loss of PHD2 were unable to form tumors. Consistent with these results, direct chemical inhibition of PHD2 activity in transformed cells reverses a cell's transformed characteristics. Moreover, we found that overexpression of PHD2 in malignant fibroblasts leads to loss of their tumor-forming ability. These changes correlated with HIF1-activated glycolytic rates, vascularization, and the ability to grow under hypoxic stress. These findings suggest a biphasic model for the relationship between PHD2 activity and malignant transformation: With a slight decrease in PHD2 activity the cells gain a growth advantage, such as enhanced glycolysis and angiogenesis and become malignantly transformed. As PHD2 activity is further decreased, the pro-death response might become the dominant signal and the increased adaptation would be overwhelmed. The dual nature of this response is presumably due to PHD2's ability to alter the cellular balance between hypoxic-induced adaptation and pro-death responses.

The hypoxia signaling pathway has many input signals, including low oxygen, reactive oxygen species, TCA metabolites, and various growth factors. It is currently not known how these disparate signals influence HIF1 activity. To address this knowledge gap, we attempted to characterize the PHD2-protein interaction network (PHD2-PIN) using tandem affinity purification and liquid chromatography coupled tandem mass spectrometric analysis (LC-MS/MS). Our result suggested the possibility that PHD2 might have no specific interaction with other proteins under normoxia and that PHD2 might act to coordinate the various input signals without the aid of accessory factors.

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ACKNOWLEDGEMENTS

I would first like to recognize with high regard the guidance and mentorship of Dr. John J. LaPres. His encouragement and advice was vital for my educational and career. Studying under his direction has provided me with the chance to learn the attitude of a scientist which I will take with me through whole my career.

I greatly appreciate the advise and instruction of my guidance committee members, Dr. Susan E. Conrad, Dr. R. William Henry, Dr. Laura R. McCabe, and Dr. Jetze J. Tepe for their support over the years.

I would also like to thank Dr. J. Justin McCormick and Dr. Sandra O'Reilly for their collaborative efforts.

It was very enjoyable to study with former and current members of Dr. LaPres laboratory: Babara G. Wood, Ajith Vengellur, Dorothy Tappenden, Yogesh Saini, Dr. Scott Lynn, Steve Proper, Jeremy Lynd, Amy Bays, and Julie Adkins.

I thank with all my heart my family: parents, Ki-Jun Lee and Young-Ja Pyo, sister, Kang-Hee Lee and brother, Kang-Chual Lee for their love and support. Finally, my special thanks to my fiancée Yong-Bum Kim.

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TABLE OF CONTENTS

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LIST OF FIGURES

Table 3

Table 3-

Table 3-

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Table A-

Table A-

Table B-

LIST OF TABLES

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CHAPTER 1

INTRODUCTION

The reaction of Complex IV of the electron transport chain (ETC) utilizes greater than 95 % of the oxygen we breathe. This reaction, catalyzed by cytochrome oxidase, is required to maintain proper ATP production within the cell. This coupling of energy production to the consumption of oxygen has made the ability to sense and cope with low oxygen tension essential for survival. Decreases in oxygen reaching the tissues of the body, a condition known as hypoxia, is detrimental to cells and tissues because it decreases their ability to produce energy, and thereby disrupts their ability to maintain proper function. Hypoxia and hypoxia-related signaling has been linked to the pathology of all the major causes of death including cardiovascular disease, stroke and cancer. The fact that hypoxia signaling is directly linked to a tumor's ability to thrive has made it and several hypoxia-activated genes the focus of intense drug discovery research (12, 44, 104). To target hypoxia signaling successfully for therapeutics or appreciate its role in xenobiotic toxicity, it is necessary to understand the cellular events following loss of normal oxygen tension and characterize the role of hypoxia in cellular transformation.

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Oxygen is transported from the air that we breathe throughout the body via a respiratory system. The primary function of this system is to deliver inspired oxygen to peripheral tissues and remove carbon dioxide that cells produce as a byproduct of normal cellular function. When a breath is taken, air passes from the airways into microscopic air sacs called alveoli. The exchange of oxygen and carbon dioxide between the alveoli and blood is called external respiration. Although the oxygen carrying capacity of the blood is increased markedly by the presence of hemoglobin, it is the gradient of oxygen partial pressure (pO_2) between the blood plasma and the locus of the oxygen consumption in the mitochondrion that drives O_2 into the tissues by passive diffusion. The pO_2 in the inspired air is 150 mm Hg, whereas the pO_2 in the alveoli and arterial blood is around 100 mm Hg. In the tissues, oxygen dissociates from hemoglobin and diffuses through capillary endothelium into parenchymal cells, so that the pO_2 of the blood draining tissues (i.e. venous pO_2) is far less than arterial pO_2, averaging about 40 mm Hg. Thus, the pO_2 in the cells of the tissues is much lower than that of the arterial blood.

The normal partial pressure of oxygen (pO_2) varies in cells within and among different tissues. For example, the normal pO_2 of skeletal muscle has been reported to be 20 - 30 mm Hg and that of brain is 45 – 65 mm Hg (28, 60). Even within a single organ, cells are exposed to a wide range of oxygen tension in different locations. For example, the pO_2 of the blood in liver varies by site, so that pO_2 in the hepatic artery,

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portal vein, sinusoids and central vein are estimated to be 95 – 105 mm Hg, 50 – 65 mm Hg, 35 – 45 mm Hg, respectively (17, 58, 90). Although estimates of values for "cellular pO_2" have been made in numerous tissues, uncertainty exists due to limitation of methodologies used for the measurements. Moreover, cellular pO_2 must vary with the location of parenchymal cells along the length of the capillary (or sinusoid), and gradients must also exist intracellulary due to the continuous consumption of O_2 in the mitochondria. Thus, cells in the portal triad, where arterial and portal venous blood mixes upon entering the liver, are exposed to a more oxygen-rich environment than cells close to the central vein, since the blood has lost O_2 by diffusion into upstream cells. Nevertheless, each of the cells along the sinusoid considers the degree of oxygen to which they are usually exposed to be "normal". How cells establish or perceive this level of oxygen as "normoxic" is not entirely clear; however, when the pO_2 in the tissues and individual cells drops below this normal level, a state of hypoxia is said to exist.

1.2. Occurrence of hypoxia

Hypoxia arises in biological systems for a wide array of reasons, including normal physiological variation and pathological conditions. During embryonic and fetal development, hypoxia occurs as naturally as the process proceeds and the cellular requirements throughout the developing organism display varying demands for oxygen.

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Studies in vitro have demonstrated that the optimum oxygen tension in a mammalian embryo is approximately 23 – 38 mm Hg, and this oxygen environment is an important physiological factor for vasculogenesis, angiogenesis, and tubulogenesis (68, 126). In addition, normal organogenesis in the developing fetus takes place in a relatively oxygen-poor environment (relative to adult tissues), and increasing oxygen tension during this time is deleterious for heart, kidney, and lung development (70, 115, 126). Hypoxic condition can also occur in the adult under various conditions. For example, during exercise muscle demand for energy can outpace the supply of oxygen, causing localized hypoxia. This type of stress leads to a buildup of lactic acid as the body turns to anaerobic metabolism to address the energy debt. These normal biological processes highlight the balance that is established between oxygen levels and energy productions and the importance of a programmed response to disruption in this balance. Imbalance can occur either through reduced oxygen availability, as in the developing embryo, or increased energy demand, as in the exercising muscle. More importantly, this balance is also central to hypoxia's role in pathological conditions.

Recent interest in hypoxia and its effects in biological systems has stemmed from its role in a wide variety of pathological conditions, most notably cancer. Hypoxia is an important feature of most solid tumors, arising from the process of tumor formation and progression that is characterized by rapid cell proliferation and drastic changes in the local oxygen supply. Rapid cellular expansion quickly outpaces

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the ability of a tumor to create new blood vessels for oxygen delivery, leading to a hypoxic or even anoxic microenvironment within certain portion of the tumor. The hypoxic microenvironment occurs very early during tumor development, beginning as the tumor reaches approximately 2 – 3 mm in a diameter. To this the tumor must respond, primarily by increasing its glycolytic rate and angiogenic potential (44, 47). The resultant newly formed vasculature differs from that in normal tissues, displaying abnormal structure and function. For example, the new tumor vessels are highly disorganized and usually leaky, with incomplete endothelial lining, qualities that lead to irregular blood flow and diminished nutrient and oxygen supply (18, 65). Newly developed vasculature, therefore, does not always address the oxygen debt of the tumor since many portions remain further than 150 μm from the nearest blood vessels, which due to tissue O_2 consumption, is the approximate diffusion limit of oxygen (18). Hypoxia, therefore, remains a constant feature of tumors even after neovascularization. For many years, tumor hypoxia has been considered a challenge for cancer therapy because of its adverse impact on the effectiveness of radiation and chemotherapy. Moreover, low oxygen availability has recently emerged as a major factor that enhances malignant progression, because tumor cells that have adapted to hypoxia gain various advantages in growth. In most cases, this is a sign of poor prognosis (6).

Disruption in oxygen homeostasis also occurs during other pathophysiological conditions such as cardiovascular disease, stroke, and chronic pulmonary disease (100).

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Atherosclerosis often leads to arterial stenosis which ultimately impairs perfusion of the vascular bed and further disruption oxygen and nutrient flow to regions of the heart muscle (98). When this ischemia persists, it can irreversibly reduce myocardial viability (95, 100). Stroke is a well-known cause of cerebral hypoxia because it disrupts the normal flow of blood in the brain and leads to localized loss of oxygen and nutrient supply. In brain tissues, even if this disruption only lasts for a short period of time, it can lead to neuronal cell death and disability (55, 100). Chronic obstructive pulmonary disease (COPD) is the most common form of pulmonary dysfunction and can be subcategorized as asthma, chronic bronchitis, and emphysema. Alveolar hypoxia is common in COPD because of the difficulties in expelling air from the lungs, and it can lead to pulmonary hypertension, pulmonary arteriolar remodeling, and ultimately heart failure (123, 124). The hypoxia-induced injury and tissue remodeling seen in these pathophysiological conditions and others, including diabetes and wounding, can be considered a response to the inability to maintain the balance between oxygen delivery and requirement. The injurious process takes place because the tissue can not adapt to the change in oxygen tension. Toxicant-induced cellular damage that involves the hypoxia signaling system can also be considered in a similar way.

2. Hypoxia signaling; the hypoxia-inducible factor (HIF) system

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Aerobic metabolism provides a significant advantage to multicellular organisms, especially in the area of energy production. When oxygen supply cannot meet the energy demand of the cells, however, an adaptive response must compensate for the energy imbalance to maintain tissue function. A primary mechanism for this adaptive response is the transcriptional regulation of a battery of hypoxia-responsive genes. In fact, genomic screens have shown that thousands of genes are influenced by exposure to hypoxia (108, 116). The most well studied mechanism identified in this process is an interaction of a family of transcription factors, called the hypoxia-inducible factors (HIFs) with a *cis*-acting element, called a hypoxia-responsive element (HRE), located in regulatory region of target genes. This mechanism was first demonstrated for the erythropoietin gene, the expression of which was upregulated more than 100-fold by hypoxia via HIF-induced transcription (2, 92). Since then more than 70 target genes regulated directly by HIF have been identified, and expression of over several hundreds genes are known to be directly or indirectly influenced by HIF (104).

The family of HIFs belongs to the Per-ARNT-SIM (PAS) superfamily of transcription factors and is characterized by the presence of the PAS domain that controls dimerization (48, 50). HIFs are heterodimeric proteins comprising α and β subunits. The alpha class is composed of HIF1α, HIF2α, and HIF3α. The beta subunit includes the aryl hydrocarbon nuclear translocator (ARNT, also known as HIF1β) and ARNT2. HIF1 is the most widely studied HIF heterodimer and is the combination of

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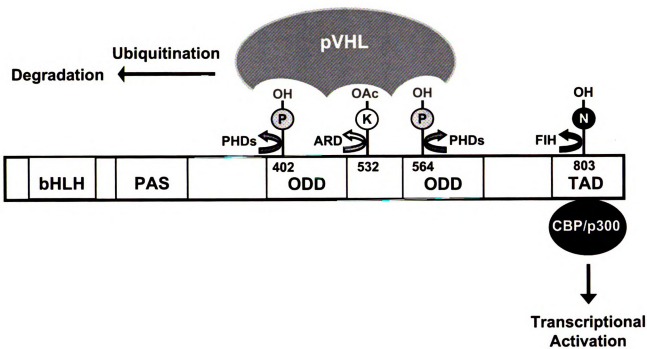
HIF1α and HIF1β (118). HIF1 is the principal regulator of the hypoxic response in most mammalian cells (103). HIF1β is a constitutively expressed, nuclear protein, whereas the expression and activity of HIF1α is tightly regulated by oxygen concentration. It rapidly accumulates upon exposure to hypoxia and on reoxygenation is quickly degraded with a half-life of less than 5 min (123). Given how detrimental hypoxia is to a cell, this rapid response time highlights the speed at which a cell must elicit a reaction to loss of oxygen tension. Equally important is the short half-life of the HIF protein following reoxygenation, and this time frame suggests that prolonged HIF activation might not be beneficial to the cell. Our understanding of the mechanism a cell uses to sense hypoxia and convey this signal to the α subunit has greatly increased over the last several years. This is due to the recent identification of a family of hydroxylases that modify the α subunit in an oxygen dependent manner.

HIF1α contain basic helix-loop-helix (bHLH) and PAS domains that mediate dimerization and DNA binding (Fig. 1-1). HIF1α also contains transcriptional activation domain (TAD) that regulated its transcriptional activity and oxygen dependent degradation (ODD) domain that control the half-life of α subunit in an O_2-dependent manner (Fig. 1-1). The ODD domain includes conserved proline residues, Pro 402 and Pro 564, which are post-tranaslationally hydroxylated under normoxia. Three hydroxylases, known as prolyl hydroxylase domain containing proteins ((PHDs), also known as egg laying abnormal 9 homologues (EGLNs) and hypoxia prolyl

Figure 1-1. Structure of the HIF1α protein and oxygen-dependent modification of HIF1α.

The HIF1α subunit (826 amino acid) is divided into several functional domains: Dimerization and DNA binding domain (**bHLH and PAS domain**), oxygen dependent degradation domain (**ODD**), and transactivation (**TAD**). The expression of HIF1α is regulated by oxygen-dependent post-translational modification. Under normoxia, conserved proline residues (**P**) 402 and 564 within the ODD domain of HIF1α are hydroxylated by the PHD enzyme. This process is required for the binding of the von Hippel-Lindau tumor suppressor protein (**pVHL**), the recognition component of an E3 ubiquitin-protein ligase. Ubiquitination of HIF1α targets the protein for degradation by the 26S proteosome. pVHL binding might also be promoted by acetylation of lysine (**K**) residue 532 by ARD1 acetyltransferase. Oxygen also regulates the interaction of HIF1α with transcriptional co-activators. Oxygen dependent hydroxylation of asparagine (**N**) residue 803 within the TAD of HIF1α by FIH, blocks the binding of CBP and p300 to HIF1α and consequently inhibits HIF1-mediated transcription. Under hypoxic conditions, hydroxylation is inhibited and pVHL cannot bind to the HIF1α leading to HIF1α accumulation and interact with CBP and p300 allowing transcriptional activation of HIF1.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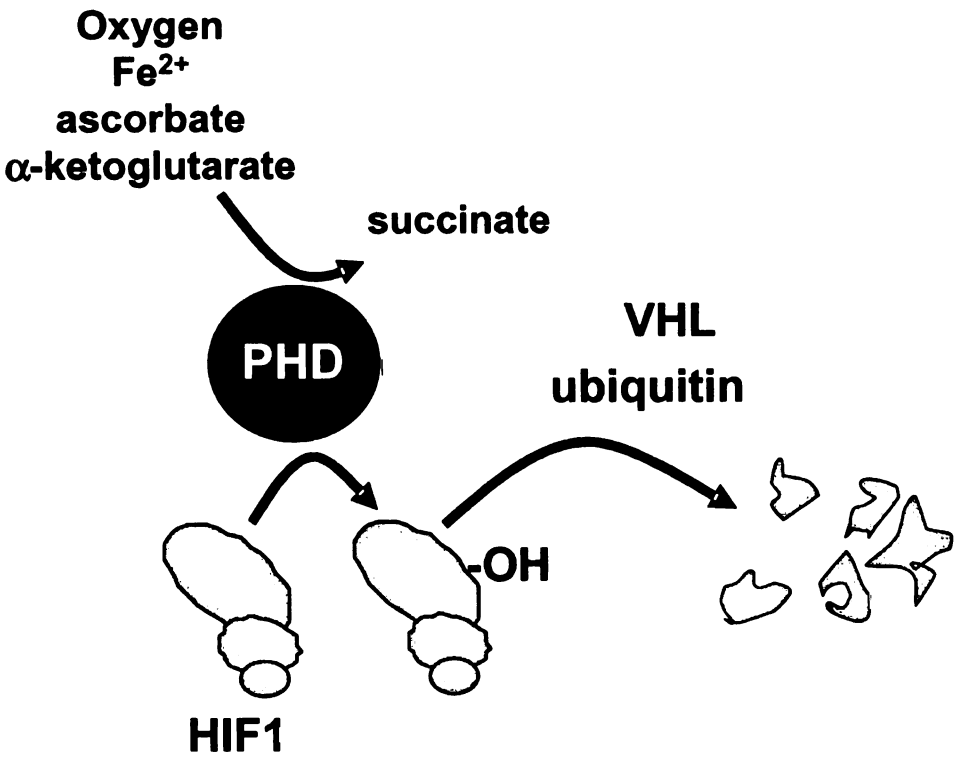
hydroxylases (HPHs)), control HIF1α stability by modifying conserved proline residues within the ODD of HIF1α under normoxia (Fig. 1-1) (7, 30). The proline hydroxylations are required for the interaction of HIF1α with the von-Hippel Lindau tumor suppressor gene product (pVHL). pVHL serves as the recognition component of E3 ubiquitin-ligase that leads to HIF1α ubiquitination and proteosomal degradation (7, 30). PHDs contain a double stranded β-helix (jelly roll) core and iron-binding residues common to members of the dioxygenase family, which includes the collagen prolyl 4-hydroxylase (C-P4H) (85, 97). The catalytic properties of PHDs, including kinetics and affinity constants for the co-substrates, have been clarified (45). These hydroxylases require four factors for activity: oxygen, iron, α-ketoglutarate (αKG) and ascorbate (Fig. 1-2). The enzyme process involves decarboxylation of the αKG to yield succinate and concomitant hydroxylation of the targeted residue. The oxygen, iron, and enzymetic reaction products play a critical role in regulation of the hydroxylase. The proline residues hydroxylated in HIF1α are all present in the context of a Pro-Xxx-Xxx-Leu-Ala-Pro consensus sequence (54). Human type I and type II C-P4H cannot hydroxylate this evolutionary conserved proline (46, 54). All the PHDs hydroxylate the C-terminal hydroxylation site, Pro 564, and Km values of the three PHDs for the peptide are similar. PHD1 and PHD2 showed higher Km values for the peptide containing the N-terminal hydroxylation site Pro 402 and this peptide was not hydroxylated by PHD3 (45, 46). Differences also exist between PHDs in their

Figure 1-2. PHD-mediated hydroxylation of HIF1α.

Prolyl hydroxylases (PHDs) require oxygen, iron, ascorbate and α-ketoglutarate for activity. During the enzymatic process, α-ketoglutarate is decarboxylated, yielding succinate, and the HIF1α ODD is hydroxylated on conserved proline residues. Once hydroxylated, HIF1α is quickly degraded in a pVHL-ubiquitin-26S proteosome-dependent manner.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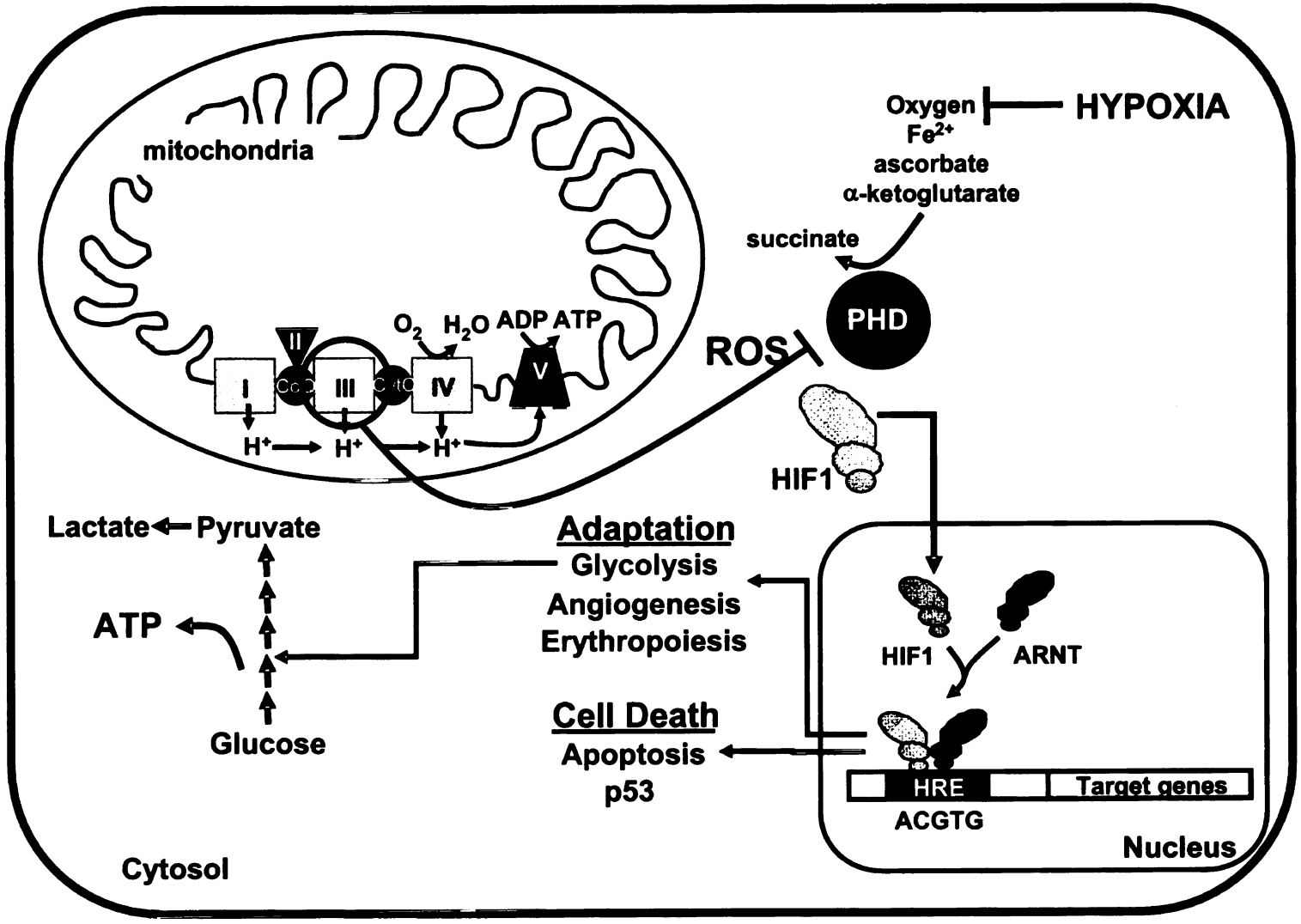
intracellular localization. Human PHD1 is predominantly expressed to the nucleus, whereas PHD2 is present in the cytoplasm (45,82). PHD3 distributes evenly in both compartment in human. The expression of PHDs are induced by hypoxia in cell type specific manner, suggesting a role for these enzymes in a negative feedback pathway responsible for enhanced degradation of HIF1α after reoxygenation (3, 16, 24). These differences in substrate specificity, intracellular distribution and tissue specific inducibility under hypoxia imply distinct function for the three PHDs, however the physiological impact of this diversity is not clear. The PHD-mediated hydroxylation of HIF1α is inhibited under low oxygen conditions, leading to HIF1 α stabilization, accumulation, and consequent induction of HIF1 activity (7, 30, 69). Currently, there are two theories as to how the PHD senses the decrease in cellular oxygen tension (Fig. 1-3). First, since these enzymes require oxygen for activity, hypoxia acts as a loss of substrate, and this might be all that is required for PHD inhibition. The loss of available oxygen would inhibit the enzyme's ability to modify HIF1α and thereby lead to increase transcription factor stability. The disparity between the oxygen requirement for these enzymes and the level of hypoxia needed to induce HIF1α stability in cells has led others to consider alternative mechanisms. In enzyme preparations, a progressive decrease in PHD activity from just below 20% O_2 induces an increasing stability of HIF1α. In contrast, HIF1α stability is not increased in most cell types until the oxygen levels are decreased to less than 7% O_2 (30, 114). In the stabilization of

Figure 1-3. The hypoxia signaling system.

The hypoxia signaling cascade begins in the cytosol where the PHDs are continuously responding to local oxygen concentrations. Once the available oxygen concentration reaches a certain critical point, the PHD becomes inhibited. This can involve direct inhibition of the PHD due to loss of its molecular oxygen substrate or indirect inhibition due to changes in reactive oxygen species (ROS) produced at complex III of the ETC. This change in ROS is caused by the inhibition of the ETC due to decreased oxygen, the terminal electron acceptor at complex IV, and subsequent accumulation of ubisemiquinone at complex III. Once the PHD is inhibited, HIF1α translocates to the nucleus where it interacts with ARNT and becomes transcriptionally active. HIF1 target genes include adaptive and cell death genes. The cellular increase in anaerobic metabolism leads to the overproduction of lactate and a decrease in cellular and local tissue pH.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HIF1α, several laboratories have reported a role of reactive oxygen species (ROS) generated in the mitochondria. These experiments have documented an inability of mitochondria-deficient cells to induce HIF1α stabilization during hypoxic stress, suggesting a role for this organelle in the response (13). Recent reports have elaborated on this, establishing complex III as the likely site of action (8, 13, 41, 77). The proposed mechanism accounts for evidence implicating the mitochondrial respiratory chain in regulation of HIF1 activity (Fig. 1-3). In respiration, electrons donated by NADH and $FADH_2$ flux through complex I-IV of the ETC and are finally transferred to molecular oxygen at complex IV. Hypoxia causes ETC inhibition and the generation of ROS from a number of potential sites, such as complex I and the ubisemiquinone site of complex III. It has been proposed that this oxidative stress within the cell disrupts the catalytic activity of the PHD, possibly through inhibiting the ability of iron to cycle between oxidation states. In recent years, it has become evident that changes in ROS levels within the cytosol result in PHD inhibition, ultimately leading to the induction of HIF1 transcriptional activity (14, 38, 41). Regardless of which mechanism is involved in the inhibition of the PHD, hypoxia leads to HIF1α stabilization, and this begins the cell's attempt to adapt to the decrease in available oxygen.

Hypoxia is not the only signal that can lead to HIF1α stabilization. It has long been known that a number of chemicals, such as cobalt, deferoxamine and αKG analog, dimethyloxaloglutarate (DMOG), can induce the stabilization of HIF1α experimentally

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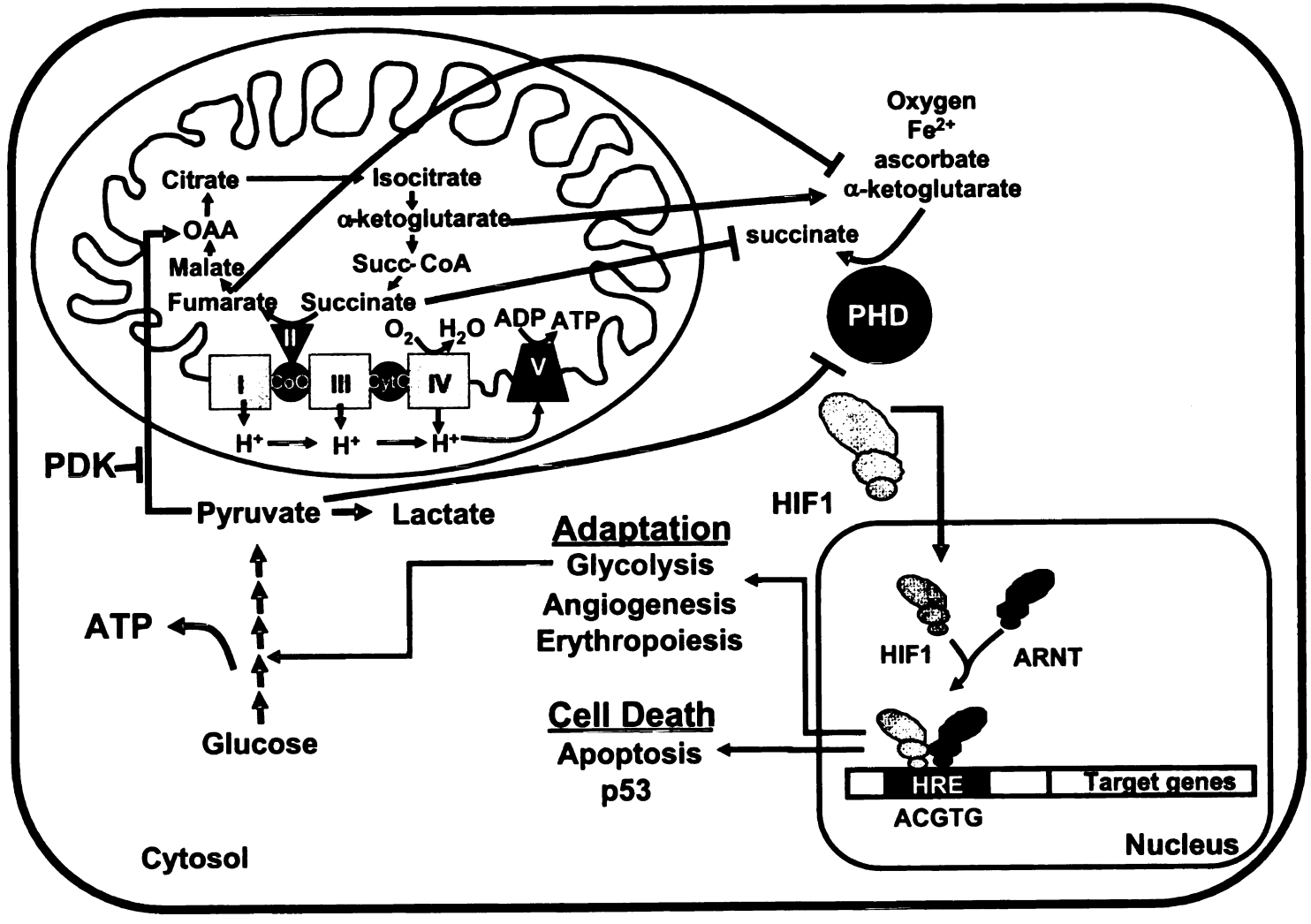
(30, 78). They act by directly inhibiting PHD activity by either removing or competing with iron or αKG. More recently, a link has been established between key metabolites and HIF1α stabilization. As discussed above, PHD requires αKG for activity and, during the catalytic process, generates succinate. Recently, families with mutations in the gene encoding succinate dehydrogenase (SDH, complex II of the ETC) have been demonstrated to display characteristics of a pseudo hypoxic response (5, 22, 99). These people have a gene expression profile similar to that induced by hypoxia in normal humans and are prone to pheochromocytomas. In addition, people with mutations in the fumarate hydroxylase (FH) gene, another citric acid cycle enzyme, also display signs of aberrant hypoxia signaling (53). People with FH mutations are also prone to certain types of cancer, presumably this involves direct inhibition of PHD through modulation of the decarboxylation step within the enzyme. The mutation in SDH would tend to increase intracellular concentrations of succinate, leading to PHD dysfunction via product inhibition (Fig. 1-4). The FH mutation would be expected to lead to increased fumarate levels within the cell, and fumarate can act as a competitive inhibitor for the αKG binding site in PHD (Fig. 1-4). Finally, the glycolytic end product, pyruvate, regulates HIF1α stability, and this activation might be responsible for the Warburg effect (75).

In the early part of the last century, Otto Warburg described a tumor's increased dependence upon fermentation or anaerobic glycolysis (119). He suggested that the

Figure 1-4. Link between intermediary metabolism and HIF1 signaling.

Recent reports have established a direct link between key metabolic intermediates and HIF1 signaling. These metabolites include α-ketoglutarate (α-KG), which is necessary for PHD activity. Recently, it was demonstrated that patients with mutations in their succinate dehydrogenase gene (Complex II of the ETC) display characteristics of a pseudo-hypoxic state. Another link was established between fumarate disregulation and HIF1 signaling, and it was suggested that fumarate competes for α-KG binding within the PHD enzyme. Finally, pyruvate acts in a feed-forward mechanism to stabilize HIF1α in the absence of hypoxia. The recent characterization of pyruvate dehydrogenase kinase (PDK) as a HIF1 target gene might amplify this linkage between pyruvate and hypoxia signaling, since PDK over-regulation would serve to trap pyruvate in the cytosol.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increased glycolytic activity and metabolic adaptation might be the reason for the cellular transformation. The demonstration that metabolic intermediates of glycolysis, pyruvate and oxaloacetate, can activate HIF1α through inhibition of PHD raises the possibility that it can participate in feed-forward activation of HIF1α leading to a prolonged hypoxic signal (74). In addition, recent reports have shown that the pyruvate dehydrogenase kinase (PDK) gene is a HIF1 target gene (61, 116). PDK is responsible for inactivating the pyruvate dehydrogenase complex, thus trapping pyuvate in the cytosol. This would further serve to prolong the hypoxic signal by maintaining the cytosolic concentration of pyruvate. The resultant extended signal might explain the Warburg effect and directly links HIF1 activation to increased glycolytic activity. These combined results suggest that a better characterization of the metabolic state of the cell, as well as oxygen and PHD levels, is required if the HIF signaling cascade is to be understood and targeted successfully by therapeutic agents.

Other modifications, in addition to PHD-mediated hydroxylation, have been linked to hypoxia signaling. The transcriptional activity of HIF1α is also reduced under normoxia by oxygen-dependent hydroxylation of asparagine (Asn) 803 within the C-terminal transactivation domain (CTAD) of this transcription factor (Fig. 1-1) (76). Factor inhibiting HIF (FIH) mediates this modification of the conserved Asn residue, and the hydroxyl-asparagine prevents the binding of co-activator p300/CBP to HIF1α (69). This effectively removes the ability of HIF1α to direct p300-dependent transcription; however, it remains possible for the transcription factor to modulate

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transcription in a p300-independent manner. It has also been reported that acetylation of HIF1α at lysine 532 by arrest defective-1 (ARD1) acetyltransferase might regulate HIF1α stability by enhancing the interaction between hydroxylated HIF1α and pVHL (Fig. 1-1) (35, 56). Phosphorylation is also involved in regulating HIF1 activity. During hypoxia, p42/p44 mitogen activated protein kinase (MAPK) phosphorylates HIF1α and enhances the transcriptional activity of HIF1 (93, 96).

HIF1 activity is also regulated in an oxygen-independent manner at the HIF1α expression level by certain growth factors that ensure the maintenance of oxygen homeostasis in normal growing tissues. For example, signaling via HER2 and IGF-1 receptor tyrosine kinases increase HIF1 expression. These responses result from the activation of signaling pathways involving phosph-inositide 3-kinase (PI3K)/AKT/ mTOR that lead to the increases in the translation factor elF-4E, which in turn enhances HIF1α mRNA translation (96, 104). HIF1 is also regulated by additional oxygen-independent molecular processes. Recently, several studies have demonstrated that exposure of cells to certain nitric oxide (NO) donors or gaseous NO molecules modulated HIF1 activity. For example, treatment of cells with S-nitrosoglutathione (GSNO), DETA-NO, or NOC18 induces HIF1 activity under non-hypoxic conditions (59, 88). In contrast, sodium nitroprusside (SNP) inhibits hypoxia-induced HIF1 activation (42, 111). The molecular mechanisms regulating HIF1 signaling in response to NO molecules are under investigation but appear to involve effects on both HIF1α

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synthesis and stability. In summary, there are many ways in which HIF activity can be regulated, some of which are oxygen-dependent and others of which are not. The relative importance of these various conditions on normal and pathophysiology remains to be determined.

3. The integrative HIF1-mediated responses to hypoxia

Multicellular organisms have developed sophisticated physiological; infrastructure to maintain oxygen homeostasis. Hemoglobin, a major constituent of red blood cells, is responsible for carrying oxygen to peripheral tissues, and the number of circulating erythrocytes is a major determinant of tissue oxygenation (9, 102). Decreased oxygen availability results in compensatory stimulation of erythropoiesis by upregulating the production of erythropoietin (EPO) (102). EPO enhances the production of red blood cells, and its hypoxia-induced transcription is regulated by HIF1 through an HRE site in the 3' flanking region in the EPO gene (2, 92). The hypoxia-induced expression of the EPO gene is thought to be part of a systemic response that an organism initiates to cope with the decrease in available oxygen. Angiogenesis is a tissue-oriented response to hypoxia and is critical to restoring oxygen transport to ischemic areas by improving blood supply to the affected tissues. Vascular endothelial growth factor (VEGF) is a central factor controlling physiological and pathological angiogenesis, and, in most cases, it is induced in response to hypoxia (113). The expression of VEGF is regulated by the binding of HIF1 to HREs located in

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the 5'-flanking region of VEGF (107). In addition, VEGF receptors and the pro-angiogenic cytokine interleukin-8 are upregulated in response to hypoxia (33, 83).

The cellular response to hypoxia is focused on addressing the energy deficit created by the decrease in available oxygen. When aerobic metabolism is diminished due to the lack of molecular oxygen, the cell utilizes the HIF1 signaling cascade to increase the expression of the complete battery of glycolytic enzymes, since it is the one place the cell can turn to address its energy debt. The glycolytic process is not as efficient as the ETC and ATP synthase in producing ATP, generating only 2 moles of ATP per mole of glucose, and this inefficiency can lead to impairment of physiological function (101). The increased dependence upon anaerobic glycolysis is coupled to the upregulation of various glucose transporters and other growth factors in an attempt to supply the cell with the necessary catabolic reagents to carry out this adaptative response (15). The importance of HIF1 in this switch to anaerobic metabolism has been well demonstrated with *in vivo* and *in vitro* models. Cells with a loss of HIF activity display reduced hypoxia-induced expression of 13 different glucose transporters and glycolytic enzymes, compare with their wild type counterpart (15, 55, 116). Finally, this adaptive response also involves HIF1-mediated transcription of the PHD themselves (3, 16, 31). This ability is PHD isoform- and cell type-specific. PHD2 and PHD3 are upregulated in response to hypoxia in a wide variety of cells, whereas PHD1 is only marginally regulated by loss of oxygen tension in most cells. There has been speculation that this HIF1-mediated upregulation of PHD functions as

a shut-off mechanism for the hypoxic response once normal oxygen tension is restored. This might be one role for such feedback inhibition; however, there may be a more subtle role for this regulatory loop, that is, that this feedback loop actually establishes the set-point for "normoxia" within the various cell types of body. The cells in various tissues and even within a single tissue are exposed to a wide range of oxygen tensions. For example, liver cells close to portal vein and hepatic artery are exposed to a greater oxygen tension than those near the central vein, and yet each cell type perceives the oxygen concentration to which it is exposed as normal. It is possible that the decreasing oxygen concentration along the sinusoid leads to a partial activation of HIF1-mediated transcription of the appropriate PHD. The increased PHD levels along the sinusoidal oxygen gradient could compensate for reduced delivery of oxygen, similar to the way an increased receptor concentration can compensate for lack of available ligand in signal transduction. In support of this notion, recent studies have suggested that HIF1-dependent regulation of PHD levels might provide a self regulatory loop which defines a tissue specific threshold for HIF1 activation as a function of pO_2 rather than simply accelerating HIF1α degradation following reoxgenation (110). In this manner, the HIF1:PHD-linked transcription can establish the required levels of HIF1 signaling necessary for the cell's microenvironment and normal cellular function.

The adaptive response, including altered PHD level and upregulation of EPO, VEGF and anaerobic glycolysis, is not always capable of addressing the hypoxia-

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induced disruption of cellular homeostasis. In these cases, the cell begins modulating various cell death pathway presumably as a mechanism to eliminate irrecoverable stressed cells (18, 106). This pathway involves HIF1-dependent transcription of various pro-apoptotic B-cell chronic lymphocytic leukemia (CLL)/ lymphoma (BCL) family members, such as BCL2/adenovirus E1B19 kDa interacting protein 3 (BNIP3) and NIX (26, 89). The activation of HIF is able to contribute to p53-mediated cell cycle arrest and cell death by stabilizing p53 through cross talk (1, 11). This increased "suicide" response seems in opposition to the adaptive response described above. It is currently thought that this pathway to cytotoxicity is an attempt by the cells to maintain the tissue as a whole. Removal of cells under severe hypoxic stress by programmed death could increase the chance of survival for neighboring cells by increasing nutrient and oxygen supply and maintaining appropriate tissue architecture.

4. Hypoxia (or HIF1) signaling and cancer

Hypoxia is frequently exhibited in human cancers especially in solid tumors when its mass reaches a certain size and oxygen delivery becomes limited. Interest in the role of hypoxia in cancer biology has grown exponentially in the two decade since its identification (105). Hypoxia was initially studied because of its effects on response to radiotherapy. Radiation treatment requires free radicals from oxygen to destroy target cells, and cells in hypoxic area were found to be resistant to radiation-induced cell death. Tumor cells within the hypoxic areas were observed to survive and continue

proliferating, in contrast to those in perfusion-limited areas (120). The association between tumor hypoxia and malignant transformation has been extensively documented for a variety of tumors (4, 47, 109). For example, cellular adaptation to hypoxia and altered glucose metabolism are well known fundamental characteristics of cancer biology. It is also known that tumors can not grow beyond several mm^3 because of the restricted capacity of oxygen, glucoses, and other nutrients from blood vessel without proper adaptation. Once a tumor reaches this size, its volume is subject to minor changes as the rate of cell death equals that of cell division. Several factors can assist carcinomas in conquering it limits in tumor growth, for example new vessel formation. As a matter of fact, angiogenesis is considered as an essential process for tumor growth and metastasis. However, as mentioned above, rapid cancer cell proliferation easily outpaces the rate of angiogenesis and newly formed tumor vessels are much less functional. New vessel formation, therefore, does not address the oxygen debt; consequently hypoxia remains a constant feature of tumor, forcing the tumor to adapt to these environmental conditions.

4.1. HIF1 activity as a unique characteristic of tumor

The ability of cells to adapt to hypoxia is important for cancer cell survival. As mentioned above, cells possess mechanisms to response to low oxygen condition and one of the major regulators in this response is a transcription factor HIF1. HIF1-mediated signaling would appear to be critical to promote tumor cell survival and

even tumor growth. In fact, significant HIF1α levels are one of the unique characteristic of various human cancers including lung, prostate, breast, colon carcinomas, which are the leading causes of U.S. cancer mortality (73, 127). Moreover, overexpresison of HIF1α occurs very early in carcinogenesis before histological evidence of angiogenesis or invasion. For example, HIF1α protein is detectable in preneoplastic and premalignant lesion such as breast ductal carcinoma, colonic adenoma, and prostate intraepithelial neoplasia (127). HIF1α, indeed, has been assessed for its use as a novel biomarker for precancerous lesions that warrant clinical surveillance or therapeutic intervention.

Significant basal HIF1α and its activity are seen not only in poorly oxygenated solid tumors but also in well oxygenated tumor area and metastatic nodules. Moreover, many cancer cell lines cultured in normal growing condition (i.e. 21% oxygen) display significant basal levels of HIF1α protein as well as HIF1 mediated gene expression. Tumor cells possess an increased basal glycolytic activity (Warburg effect) to supply required energy under the lack of oxygen availability. In addition, cancer cells use glycolysis for energy production preferentially even in the presence of oxygen (75, 119). The aerobic glycolysis of cancer cells, in fact, contributes directly to tumor progression and malignant transformation by promoting HIF1 activation. As mentioned, glycolysis may represent a feed forward mechanism to the expression of genes turned on by HIF1, since glycolytic metabolites can promote HIF1 activation and many genes encoding glycolytic enzymes, glucose transporters

and glucose regulatory hormones are induced by HIF1 (73, 75, 23, 102). Glycolysis may also contribute to the hypoxia-independent induction of HIF1 by several endocrine agents and environmental toxins. Elevated hexokinase and phosphofructokinase activities are hallmarks of cancer cells and phosphofructokinase activity is stimulated by insulin, insulin-like growth factor, and epidermal growth factor (10), all of which can induce HIF1 under normoxia through a mechanism involving the phosphatidylinositol 3-kinase (PI3K) signaling pathway (10, 32). Cancer cells, consequently, display significant HIF1 activity throughout their progression, suggesting that hypoxia adapted cells with HIF1-induced glycolytic activity as well as others signaling pathway may maintain HIF1 activity even after they escape the hypoxic environment to take an advantage of HIF1-mediated processes.

4.2. Positive role of HIF1 signaling in tumor progresses

HIF1 induces expression of various growth factors that are known to promote cell proliferation. This is normally involved in initiating cell migration and regeneration after hypoxia damage. Growth factors such as transformation growth factor-α and -β (TGF-α and -β), insulin-like growth factor 2 (IGF2), and platelet derived growth factor (PDGF) are known HIF1 target genes and whose role in cellular transformation have been extensively studied (27, 62, 67). The most well studied HIF1 activated growth factors regulate endothelial-cell proliferation and blood vessel

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formation. HIF1 induces expression of a key angiogenic factor, VEGF, and one of its receptors, VEGF receptor 1 (VEGFR1/FLT1). HIF1 signaling also leads to reduced expression of anti-angiogenic proteins such as Thrombospondin-1 and -2 (25, 64). As mentioned, tumors cannot grow beyond a certain size without angiogenesis because of the limited diffusion of O_2, glucose, and other nutrients. In many cancers, the degree of vascularization is strongly correlated with malignancy and patient death (23, 43). Hypoxia-induced angiogenesis is blocked by inhibitors of oncogene signaling pathways, such as agents that inhibit RAS, epidermal growth factor receptor (EGFR), and the receptor tyrosine kinase ERBB2 (HER2/NEU), which indicates that there is crosstalk between oncogenic and hypoxia response pathways.

Hypoxic cancer cells use glycolysis as a primary energy source and cellular transformation and clonal expansion of cancer cells depends on enhanced glucose transport and glycolysis (23, 119). In addition, recent studies indicated that cells also use this pathway as an energy source during metastasis (81). As mentioned, HIF1 regulates the expression of all the enzymes in the glycolytic pathway, as well as expression of the glucose transporter GLUT1 and GLUT3, which mediate glucose uptake (15). The intermediates of glycolytic pathway provide the precursor for synthesis glycine, serine, purines, pyrimidines and phospholipids, all of which are required for tumor cell growth and maintenance (44). Tumor cells that are deficient in the HIF1 signaling have lower ATP and glycine concentration *in vivo* (44).

The glycolytic activities of cancer cells also affect the overall pH of tumor. Tumors have been shown to adapt to pH changes and grow at lower pH than are found in normal tissues, giving the tumor growth advantage. Glycolysis is thought to be the main mechanism by which tumors lower their pH, through generation of lactic acid. Carbonic anhydrase, which reversely converts carbon dioxide and water to carbonic acid, might also be involved. The activities of carbonic anhydrase 9 and 12, were reported to be regulated by HIF1 and strongly induced by hypoxia in a range of tumor cells (121).

HIF1 signaling also contributes invasion and metastasis in tumor cells. Previous studies showed direct link between HIF1 and a cells' invasiveness. Cells transfected with HIF1α expression vector resulted in significant increase in invasiveness of cells under both normoxic and hypoxic conditions (63). Consistence with their results, the complementary experiment using siRNA targeting HIF1α decreased the colon carcinoma invasion (63). Cancer cells produce proteases including the urokinase-type plasminogen-activator receptor (uPAR), cathepsin D, and matrix metalloprotease-2 (MMP2), which enhance the cells' motility by digesting basement membrane/ extracellular matrix (ECM) (29, 72). The analysis of mouse ES cells showed that the expressions of these genes were regulated by HIF1. HIF1 also increases the expression of autocrine motility factor (AMF), transforming growth factor-α (TGF-α), and surface receptor such as c-MET tyrosine kinase, fibronectin 1, keratins, and vimentin, which promote the fluid structure that required for pathophysiology of invasion (63).

In addition, hypoxia signaling is involved in many other aspects of tumor progression, further decreasing a patient's chance of survival. It can select cells within the tumor that harbor mutations via cross-talk between HIF1 and other oncogenes including H-Ras and v-Sac (57). For example, HIF1 overexpression is associated with aberrant tumor suppressor accumulation in human cancer (80, 127). It also provides cancer cells resistance to apoptosis by expression of adrenomedulin (ADM), EPO, endothelin 1 (ET 1), nitric oxide synthase 2 (NOS 2). HIF1 signaling, in addition, indirectly affects cellular immortalization and genomic instability by enhancing telomerase activity, inducing DNA breaks at fragile site and disrupting repair of DNA damage (19, 125). Finally, HIF1-mediated cell cycle arrest through cyclin kinase such as WAF1 (p21) and KIP (p27) can also act as a selective force by inducing cell differentiation (36).

4.3. Hypoxia signaling in cancer therapy

It has long been known that the hypoxic nature of solid tumors limits the efficacy of chemo- and radio-therapies. The decreased level of molecular oxygen within tumor has repeatedly thwarted the therapeutic effect of ionizing radiation to create oxygen free radicals, which is the basis of the pharmacological efficacy (86, 112). It has been estimated that hypoxic cells are approximately three times less susceptible to radiation-induced damage (112). The limitations of chemotherapy are influenced both by the decrease in available oxygen at the level of the organism as well

as site-specific loss, such as in the central portion of tumors. A decrease in tissue oxygen tension can influence therapies in several ways. First, changing expression of the P450s within hypoxic tissues can serve to increase or decrease a drug's effectiveness. In addition, low oxygen concentration can influence the catalytic activity of the P450s by removing required cosubstrates. Second, distribution of the drug can be decreased within the affected tissue due to inadequate vascularization and perfusion (91). For example, within a tumor, the main cause of the localized hypoxia is an inability of the angiogenic process to keep up with the fast-growing tumor cells. Finally, the changes in intermediate metabolism that occur as a result of hypoxia can also influence drug metabolism and effectiveness. For example, as discussed above, metabolic switch to glycolysis lead to tissue lactic acidosis, especially within a tumor and changes in local pH can influence drug delivery and metabolism. Each of these factors will influence a drug's effectiveness in the hypoxic microenvironment and, in case of a tumor, that might ultimately determine prognosis (4).

The hypoxic microenvironment within a tumor might also select for a more aggressive phenotype. It has been shown that hypoxia increases the rate of mutation within cell (109, 125). This increased mutation rate might promote an already partially compromised cell to full transformation, or it might start a normal cell down the path to full tumorigenic potential. It has also been suggested that hypoxia can select for mutated forms of p53 (39). This selective pressure within the tumor could lead to tumor cells that have removed themselves from one principal regulator of cellular

homeostasis. In addition, hypoxia can select for cells that are resistant to apoptotic signals (122). Finally, the inability of therapy to kill all of the cells within a tumor due partly to a combination of the systemic and localized effects of hypoxia could produce cells refractory to later treatment. Indeed, each of these factors might play a role in the ability of hypoxia to promote more aggressive tumors and ultimately increase the chance of a poor prognosis.

Recently many investigations have focused on targeting hypoxia and the HIF1 signaling cascade for cancer therapeutics. The goal of these new therapies is to manipulate the HIF signaling pathway to inhibit tumor growth or to exploit the hypoxic microenvironment of a tumor to make the therapies more specific and efficacious. There are 4 major areas of research in this field: (1) designing drugs that directly inhibit HIF1 signaling, (37, 52), (2) influencing other signaling cascades that indirectly alter HIF1 signaling (66), (3) exploiting the hypoxic microenvironment to increase specificity and decreasing toxicity (20, 34), (4) altering regulation of HIF target genes that are critical for tumor growth or for the function of their protein products (20, 117).

Taking advantage of the hypoxic environment as a drug target has its limitations. The efficacy of these therapeutics depends upon the level of hypoxia, the cells exposed, the tissue's normal pO_2, and levels of P450 and P450 reductase and the activation range of the drug itself. To begin to cope with these limitations, these bioreactive compounds are now being used in conjunction with gene therapy vector (21, 71).

These therapies have also exploited HRE-driven gene constructs to insure that the enzyme is only expressed in hypoxic tissue, where the drug should be activated (40). Development of each of these compounds as drugs has utilized our understanding of the hypoxic environment and the HIF1 signaling system to target hypoxic tissues. These therapies, however, are still restricted by our limited knowledge of the HIF1 signaling to regulate a wide range of cellular responses essential to the adaptation but also cell death signaling and how the resulting changes in cells can affect to tumor formation and progression. Characterizing the role of HIF1 signaling in tumorigenesis, therefore, is important for understanding of the basic cause of cellular transformation and tumor growth as well as targeting this signaling for cancer therapy.

Hypothesis and Specific Aims

Research projects were designed and developed to investigate the role of the HIF1 signaling pathway in tumor development. The central hypothesis tested was: **The activity of PHD is related to the process of cellular transformation via its ability to modulate HIF1 signaling.** This hypothesis includes the possibility of altering PHDs activity can modulate the HIF1 regulated balance between adaptation and cell death and direct manipulation of the PHD activity within a cell will alter the cell's tumor forming potential. Previous studies strongly support this hypothesis: (1) Hypoxia and significant HIF1 activity are common characteristic of fast growing tumors and HIF1 signaling has a positive impact in tumor formation and progression. (2) PHD activity has the ability to control the HIF1 transcriptional responses. (3) There is a link between cancer and perturbation of PHD activity and subsequent HIF1 mediated signaling. (4) Finally, VHL functions as a tumor suppressor, primarily because it can regulate HIF1 activity, and the latter activity is dependant upon PHD-mediated posttranslational hydroxylation of HIF1α.

The MSU1 lineage of cell strains was used to test this hypothesis. These cell strains were established in Dr. McCormick's laboratory at MSU. Initially, normal human fibroblast cells were derived form the foreskin of a neonate and designated LG1 (84). LG1 cells were transfected with v-myc and selected using neomycin to generate the cell strain with an infinite life span, MSU-1.0 cells (84). A fast growing and spontaneous variant strain emerged from the MSU-1.0 cells, resulting in the MSU-1.1 cells (51). The MSU1.1 cell strain acquired partial growth factor independence; however, they are negative in anchorage independent growth and tumor formation in athymic mice, indicating their non-tumorigenic nature. To generate malignantly transformed cells, MSU-1.1 cells were transfected with the V12-H-RAS oncogene or exposed to γ-radiation (51, 87). Transformed foci derived from these cell strains formed solid tumors in athymic mice and these tumor cells were cultured resulting in cell strains designated PH3MT and γ2-3a, respectively.

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Each of the chapters in this dissertation is focused on investigating the hypothesis by addressing the following specific aims.

Aim 1: Characterization of HIF1 signaling in a lineage of cell strains of varying tumorigenic potential.

The correlation between HIF1 activity and tumorigenic potential in the MSU1 lineage of cells was assessed by determining the expression levels of HIF1α and HIF1 target genes. PHDs' expression levels were also characterized to investigate the relationship between PHDs levels and tumorigenicity as well as HIF1 activity in MSU1 cell strains. Finally, individual PHD isoforms were evaluated for their ability to regulate HIF1 activity in these cells.

Aim 2: Characterize the effects of reduced levels of PHD2 on the tumorigenicity of non-transformed cells.

New cell strains with reduced levels of PHD2 were generated using lentiviral infection of shRNA targeting PHD2 in non-tumorigenic MSU-1.1 cells and continuous clonal selection. Newly created shPHD2 cell strains were examined for their HIF1 activity and tested for their tumorigenic potential and tumor forming ability in athymic mice. Finally, the expression of HIF1 target genes and their functional consequence were determined in shPHD2 cell strains. A PHD inhibitor was also applied to test the reproducibility of the results.

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Aim 3: Characterize the effect of overexpression of PHD2 on the tumorigenicity of malignantly transformed cells.

New cell strains with that overexpressed PHD2 were created using retroviral gene transfer of a PHD2 cDNA in malignantly transformed PH3MT cells and clonal selection. Newly created PHD2 cell strains were characterized for their HIF1 activity, tumorigenecity and the functional expression of HIF1 regulated genes.

Aim 4: Identify the protein components associated with PHD2.

The protein network associated with PHD2 was characterized using Tandem-Affinity Purification (TAP)-tagging method. Cells expressing a TAP-tag version of PHD2 were created and verified for their functional activity. Purified proteins were identified using mass-spectrometry and interactions were verified using co-immunoprecipitation and Western blot analysis.

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CHAPTER 2

THE BIPHASIC ROLE OF THE HIF PROLYL-4-HYDROXYLASE, PHD2, IN MODULATING TUMOR-FORMING POTENTIAL

This chapter represents a manuscript that was submitted to Mol. Cell. Biol. in May, 2007. Authors included: KangAe Lee, Sandra O'Reilly, Matti Kiupel, J. Justin McCormick, and John J. LaPres.

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ABSTRACT

Hypoxia is a common feature of solid tumors. The cellular response to hypoxic stress is controlled by a family of prolyl hydroxylases (PHDs) and the transcription factor HIF1. To investigate the relationship between PHD and HIF1 activity and cellular transformation we characterized the expression levels of PHD isoforms across a lineage of cell strains with varying transformed characteristics. We found that PHD2 is the primary functional isoform in these cells and its levels are inversely correlated to tumor-forming potential. When PHD2 levels were altered with RNAi in non-tumorigenic fibroblasts, we found that small decreases can lead to malignant transformation, whereas severe decreases do not. Consistent with these results, direct inhibition of PHD2 was also shown to influence tumor-forming potential. What is more, we found that overexpression of PHD2 in malignant fibroblasts leads to loss of the tumorigenic phenotype. These changes correlated with HIF1α activity, glycolytic rates, VEGF expression, and the ability to grow under hypoxic stress. These findings support a biphasic model for the relationship between PHD2 activity and malignant transformation.

INTRODUCTION

Many solid tumors frequently exhibit areas of hypoxia because they have a high rate of cellular proliferation and form aberrant blood vessels (8, 32). Since tumor hypoxia was first identified, it has been well documented that the decreased oxygen tension has a strong impact on tumor progression in a variety of ways. Most prominently, hypoxia induces glycolysis and angiogenesis which are important changes for tumor growth and clonal expansion (9, 10, 18). In addition, hypoxia promotes the stepwise progression along a benign to malignant pathway by selecting cells that have acquired transformed characteristics and have lost tumor suppressor function (4, 19, 38). Finally, the transcription factors that regulate the cellular response to hypoxia are important for tumor growth and progression (31). Characterizing the role of hypoxia signaling in tumor development, therefore, is important for understanding the basic causes of cellular transformation and tumor growth.

The cellular responses to hypoxia are primarily regulated by the transcription factor, hypoxia inducible factor 1 (HIF1) (24, 36). HIF1 is a heterodimer of HIF1α and HIF1β (also referred to as aryl hydrocarbon receptor nuclear translocator (ARNT)) and both belong to a superfamily of basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) proteins. HIF1β is a constitutive nuclear protein and interacts with other transcription factors, such as the aryl hydrocarbon receptor. HIF1α is specific to the response of hypoxia and is constantly synthesized and under normoxia, it is rapidly degraded by the

ubiquitin-proteosomal pathway (25). The degradation of HIF1α is mediated by the product of the von Hippel-Lindau (VHL) tumor suppressor gene, which specifically interacts with the oxygen-dependent degradation domain (ODD) of HIF1α. The oxygen-dependence of this process is regulated by a family of prolyl hydroxylase domain-containing enzymes (PHDs, also designated EGL 9 homologues (EGLNs) and HIF1 prolyl hydroxylases (HPHs)) (7, 14).

Three mammalian PHDs (PHD1-3) regulate HIF1 signaling and each has a distinct tissue distribution, pattern of subcellular localization, and substrate specificity (23, 28). For proper activity, PHDs require oxygen, iron, α-ketoglutarate, and ascorbate. The oxygen requirement suggests that PHDs are "sensors" for hypoxia (16, 25, 33). In the presence of adequate oxygen, PHDs hydroxylate HIF1α at conserved proline residues within the ODD domain. Once hydroxylated, HIF1α becomes a substrate for VHL-mediated ubiquitination and degradation (7, 14). Under hypoxic conditions, PHDs are inactive and HIF1α is stabilized and translocates to the nucleus, where by dimerizing with HIF1β, it forms the functional transcription factor HIF1. HIF1-mediated transcription regulates many processes involved in cellular homeostasis and transformation, including anaerobic metabolism, O_2-carrying capacity, and angiogenesis (9, 35). Alternatively, under severe hypoxic stress, HIF1 can induce a pro-death response through transcriptional activation of pro-apoptotic factors such as BCL family members and modulation of the p53 signaling pathway (1, 8, 29, 40). HIF1,

therefore, regulates a balance between cellular adaptation through upregulation of survival genes, such as glycolytic enzymes and cell death through modulation of various pathways.

Several studies show that modulation of PHD activity has the capability to control the HIF1 transcriptional response (3, 15, 21, 22). Recent studies indicated a link between cancer and perturbation of PHD activity and subsequent HIF1-mediated signaling (16, 33). What is more, it has been suggested that VHL is a tumor suppressor, at least under some circumstances, because it can modulate HIF1 activity, and the latter activity is dependent upon a functional PHD. In view of these reports, we hypothesize that PHD activity is linked to a cell's tumor-forming potential, and that direct manipulation of the PHD activity within a cell will alter the cell's tumor-forming capacity.

To test this hypothesis, we made use of three human fibroblast cell strains from the MSU1 lineage of cells. These cells, derived one from the other, each one having acquired a characteristic related to malignant transformation, until the last change gave rise to a cell strain that by acquiring one more change, e.g., a RAS oncogene, becomes capable of forming malignant tumors in athymic mice with a short latency (26). The three cell strains used for the present study are: MSU-1.0, MSU-1.1, and PH3MT. MSU-1.0 is the infinite life span precursor to MSU-1.1. The MSU-1.1 acquired partial growth factor independence. The PH3MT cell strain was derived from a malignant

tumor formed in an athymic mouse that was injected with MSU-1.1 cells transfected with an overexpressed hRAS oncogene and selected for focus formation.

Using these cells we have shown that PHD2 represents the primary functional HIF prolyl hydroxylase within the MSU1 lineage of cells and that PHD2 activity decreases as the cell exhibits more transformed characteristics. Moderate decreases in PHD2 activity resulting from the use of RNAi in the non-tumorigenic cell strain, MSU-1.1, resulted in malignant transformation. Interestingly, MSU-1.1 cells with a more severe loss of PHD2 activity were unable to form tumors. Consistent with these results, chemical inhibition of PHD2 activity in transformed cells decreases the cell's tumor-forming potential and the overexpression of PHD2 in malignant fibroblasts lead to inhibition of tumor growth. These results suggest a biphasic relationship between a cell's tumor-forming potential and its PHD activity and highlights potential difficulties when targeting this signaling cascade for therapeutics.

MATERIAL AND METHODS

Cell culture, DMOG treatment, and Western blot analysis

MSU1 cell strains were maintained in αMEM (Mediathech Inc, Herndon, VA), and human embryonic kidney (HEK) 293 cells and phoenix-ampho cells were cultured in DMEM (Mediathech Inc) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 100 unit/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA). Cells were grown in a 37 °C incubator with 5% CO_2 (Precision, Winchester, VA). Dimethyloxallyl glycine (DMOG, Sigma, St. Louis, MO) was dissolved in 1x PBS prior to use in these studies.

Preparation of total and nuclear proteins and Western blot analysis were performed as described previously (40, 43). The following antibodies were used; rabbit polyclonal anti -PHD1, -PHD2, and -PHD3, mouse monoclonal anti-HIF1α (Novus, Littleton, CO), rabbit polyclonal anti-β-Actin (a generous gift from Dr. John Wang, MSU), and goat anti-rabbit and mouse (Sigma).

Quantitative Real Time-PCR Analysis

For gene expression analysis, cells were exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 16 hr. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined by UV spectrometry and analyzed for integrity using spectrometry and denaturing gel electrophoresis. 1 μg total RNA was reverse-transcribed using

SuperScript First-Stranded Synthesis System (Invitrogen) according to the manufacturer's protocol and primed with oligo$(dT)_{18}$ primers. Gene expression was measured by quantitative real time-PCR (qRT-PCR), based on Sybr-Green methodology (Applied Biosystems, Foster City, CA) as previously described (42). Analysis was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers used for qRT-PCR were (5' to 3'): VEGF: tcctcacaccattgaaacca and gatcctgccctgtctctctg; PHD1: caggatgggagtggagagtt and agtggtagaggtggctgtgg; PHD2: gagctgtgcgggaagatg and gcacacgagcttgtgcttct; PHD3: agcttcctcctgtccctcat and acgtggcgaacataacctgt; HIF1α: acaagtcaccacagggacqag and agggagaaaatcaagtcg; GAPDH: cagcctcaagatcatcagca and gtcttctgggtggcagtgat; LDH: aggcccgtttgaagaagagt and tgcacaacctccacctagaa; BNIP3: gctg gaacacgtaccatcct and atctgcccatcttcttgtgg. The level of each gene was normalized to the expression level of the hypoxanthine phosphoribosyltransferase (HPRT) gene and each primer set was determined to be specific by BLAST and dissociation curve analysis.

Design of shRNA and shRNA-lentiviral constructs

The sequences of siRNA targeting PHD1, PHD2 or PHD3 (three independent sequences per hydroxylase) were designed using the Ambion web-based design tool (http://www.ambion.com/techlib/misc/siRNAfinder.); PHD1-a: tcagaactgggacgttaag; PHD1-b: gactatatcgtgccctgcatg; PHD1-c: cgcaggaaggccatggtggcg; PHD2-a: taaagactgggatgccaag; PHD2-b: gacgaaagccatggttgcttg; PHD2-c: cttcagattcggtcggtaaag;

PHD3-a: tcggccctcactgaagact; PHD3-b: gtctaaggcaatggtggcttg; PHD3-c: caggttatgttcgccacgtgg. Control scrambled shRNA, tgcgtcttgttcatctcct, was also designed using a web-based tool (https://www.genscript.com/ssl-bin). The sequence of each shRNA was analyzed by BLAST to ensure specificity for each target. Small hairpin RNA (shRNA) constructs were generated using a two-step PCR approach. Briefly, the first round PCR generated an amplicon of the U6 promoter with the sense strand of the shRNA cassette and the loop. The second round of PCR added the antisense strand of shRNA cassette. The shRNA cassettes that had been generated were then cloned into the pGEM-T-Easy vector (Promega, Madison, WI). Once shRNA cassettes were verified as functional, they were sub-cloned into the lentiviral vector, pVCwPBam vector (a generous gift from Dr. David Looney, UCSD). pVCwPBam is a lentiviral that was designed for the expression of shRNA cassette and it contains a puromycin resistant sequence for the creation of stable cells.

Luciferase Assay

Each cell strain was transiently transfected utilizing Lipofectamine^{2000} via manufacturer's instructions (Invitrogen). DNA used in the transfections was diluted in OptiMEM media (Invitrogen) and consisted of an HRE-driven luciferase reporter (17) and shRNA-pGEMT-easy plasmids described above. A β-galactosidase expression vector was also employed to control for transfection efficiency. Separate transfections were performed utilizing a similar protocol with expression plasmid for PHD1, PHD2,

or PHD3 (a generous gift of Dr. Steven McKnight, Univ. of Texas, Southwestern (7)). Each transfection was adjusted to contain the same total concentration of transfected DNA using an empty expression vector. Following transfection, the cells were incubated for 16 hr and then exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 18 hr. Cells were lysed and analyzed for luciferase activity using Luciferase Assay System (Promega) according to manufacture's protocol.

Viral packaging, infection and selection

The three independent shPHDs- and scrambled- shRNA-pVCwPBam vectors were co-transfected into HEK293 cells with the packaging plasmids, pC34N and pVSV-G as described above (a generous gift from Dr. David Looney, UCSD). Following a 24 hour incubation, the media was replaced with fresh growth medium and the cells were incubated for an additional 24 hr. Viral particles were purified by centrifugation (650g for 5mins), followed by filtering with 0.45 μm membrane filter (Millipore, Billerica, MA). MSU-1.1 cells ($1x10^5$) were plated 24 hr prior to infection on 60 mm tissue culture dishes. Purified virus containing polybrene (5 μg/ml, Sigma) was added to the cells and incubated for 24 hrs. Media was replaced and the cells were cultured for 48 hrs. Infected cells were then selected with puromycin (0.4 μg/ml, USBiologicals, Swampscott, MA) and individual clones were isolated.

The cDNAs for PHD2 and GFP were inserted into the retroviral vector pZOME-1N and were packaged using Phoenix-ampho cells (a generous gift from Garry Nolan,

Stanford Univ.) (30). Purified viral particles were used to infect PH3MT cells and infected cells were selected and individual clones were isolated as described above.

Anchorage Independence and Tumor Formation Assays

Assessment of a cells ability to grow in an anchorage-independent manner was performed as previously described (20). Cells were fed weekly for 3 weeks and fixed in gluteraldehyde (2.5%).

Athymic BALB/c mice, 5 weeks of age, were implanted subcutaneously in the rear flank with 1 cm^3 absorbable gelatin sponges (Pharmacia/Upjohn Company, Kalamazoo, MI) to serve as a matrix for cell injection. One week after implantation 10^7 cells suspended in 0.2 ml MEM medium were injected into each sponge. The mice were monitored weekly for tumor growth and the size of tumor was measured. When the tumor reached 1 cm in diameter, the mice were euthanized and the tumors were removed. Tumors were then fixed with neutral buffered formalin for histological examination. All mice were euthanized 5 months after injection, even if tumors were not observed.

GAPDH, LDH and MTT Assays

Cells were lysed with 100 µl of 1% Triton X-100 (Sigma) and cell debris was removed by centrifugation (3500g for 5min). 2.5 µL of supernatants were transferred into 96 well plate and 200 µl of GAPDH reagent (100 mM $C_7H_{16}NOSNa$ (TAPS), pH 8.6, 1 mM NAD^+, 1.5 mM DL-glyceraldehyde 3-phosphate (Sigma), and 20 mM

NaH_2PO_4, 6 mM Cysteine (Fisher Scientific, Fair Lawn, NJ)) or LDH reagent (50 mM K_2HPO_4, 200 μM NADH.Na_2 (Sigma) and 6.5 mM Pyruvate (Invitrogen)) were added to each well for GAPDH or LDH assays, respectively. NADH kinetics was measured by absorbance at 340nm for 5 min at 37 °C with a 12 second reading interval. Values were normalized to protein concentration of sample. MTT assays were performed as previously described (40).

RESULTS

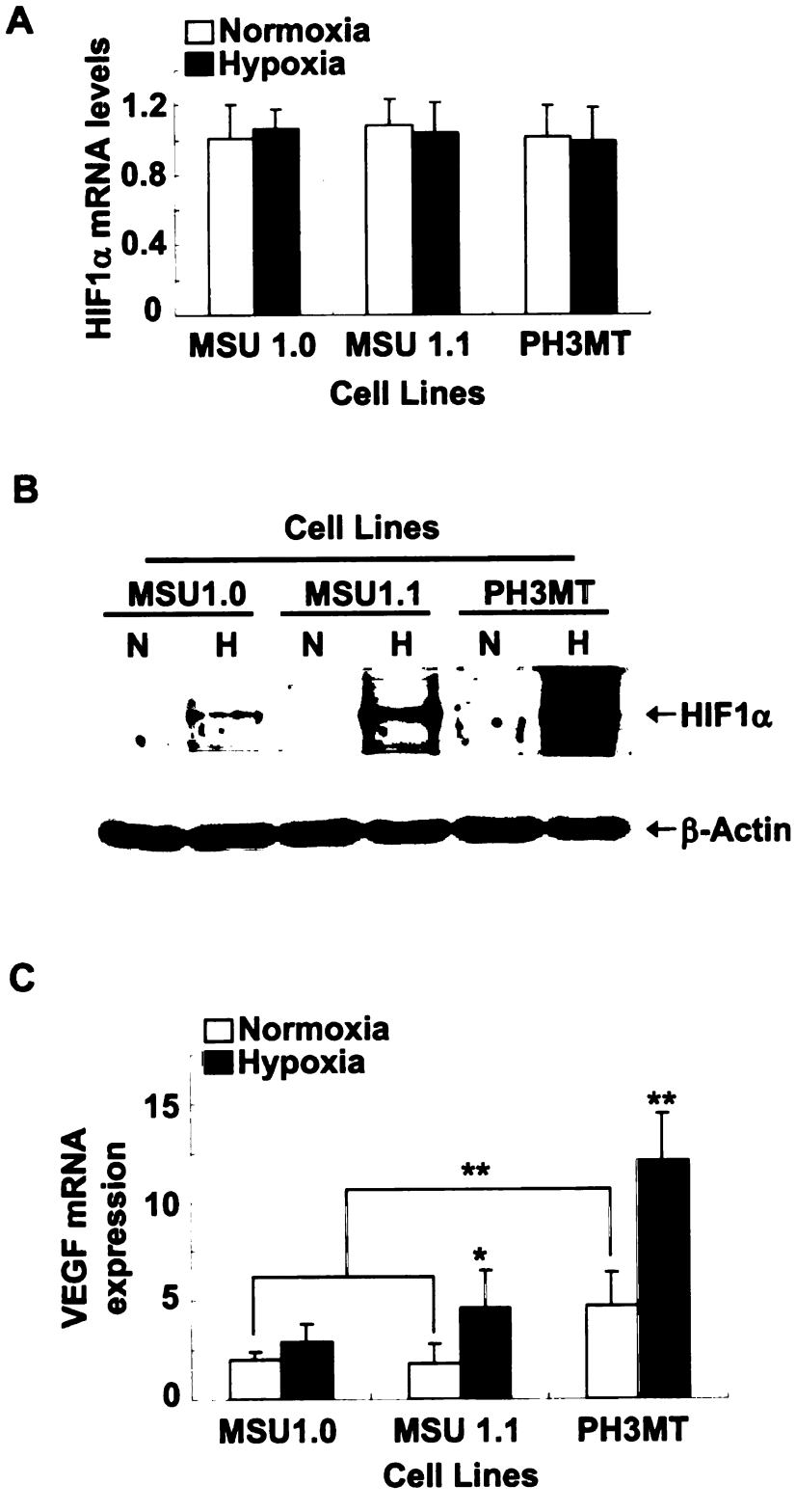
Increasing HIF1 protein levels and activity and decreasing PHD2 levels in the MSU1 cell lineage as cells become more transformed

High levels of HIF1α protein and HIF1 signaling are hallmarks of solid tumors and cells derived from such tumors (4, 32, 45). To determine if this relationship exists in the cell strains from the MSU1 lineage, we compared the level of HIF1α protein in three key cell strains from the lineage using Western blot analysis. We found that the HIF1α mRNA levels were consistent across the cell strains, both in the presence and the absence of oxygen (Fig. 2-1A). HIF1α protein was undetectable under normoxia. Its levels, however, were substantially increased following exposure to hypoxia, and there was a more pronounced increase in the cells that had the acquired characteristics of malignant cells (Fig. 2-1B). In addition, the levels of VEGF mRNA, a classic HIF1 target gene, were significantly increased in a manner that correlated to HIF1α levels under hypoxia (Fig. 2-1C). Although HIF1α protein was undetectable under normoxia, the tumor-derived PH3MT cells displayed higher basal levels of VEGF mRNA, than the two non-tumorigenic cell strains (Fig. 2-1C). These results show that the levels of HIF1α and HIF1 activity are higher in a tumor-derived cell strain than in the non-tumorigenic precursor cells.

Since PHDs are the primary regulators of HIF1α stability we analyzed the levels of these hydroxylases in the MSU1 cell lineage. Comparative analysis of PHD mRNA

Figure 2-1. Expression levels of HIF1α and HIF1 activity in MSU1 lineage of cells.

(A) HIF1α mRNA levels in three MSU1 lineage of cells were determined using qRT-PCR. Cells were exposed to normoxia (20% O_2, white bar,) or hypoxia (1% O_2, black bar) for 16 hr. (n=6). **(B)** HIF1α protein levels were determined in MSU1 lineage of cells by Western blot analysis. Cells were exposed to normoxia (N, 20% O_2) or hypoxia (H, 1% O_2) for 6 hr, and nuclear proteins were prepared and analyzed for HIF1α protein levels. To verify equal loading, the blot was stripped and reprobed with a β-Actin antibody. **(C)** VEGF mRNA levels were determined in each of the MSU1 lineage of cells using qRT-PCR. Cells were exposed to normoxia (20% O_2, white bar) and hypoxia (1% O_2, black bar) for 16 hr (n=10, * $p<0.05$ ** $p<0.01$).


A
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Hypoxia
HIF1α mRNA levels
1.2
0.8
0.4
0
MSU 1.0
MSU 1.1
PH3MT
Cell Lines
B
Cell Lines
MSU1.0
MSU1.1
PH3MT
N
H
N
H
N
H
←HIF1α
←β-Actin
C
Normoxia
Hypoxia
VEGF mRNA expression
15
10
5
0
**
**
*
MSU1.0
MSU 1.1
PH3MT
Cell Lines

levels revealed that PHD2 is the predominant isoform in these cells, comprising greater than 75% of the total PHD mRNA in the cells (Fig. 2-2A). Western blot analysis showed that PHD2 protein levels were inversely correlated with the increasing transformation of the MSU1 cell strains (Fig. 2-2B). These results show that PHD2 is the predominant isoform within the MSU1 cell strains, and its levels are inversely correlated with the transformed characteristics and HIF1 activity of these cells.

HIF1 activity can be regulated by PHD2 in MSU1 cell strains

To test PHD2's role in HIF signaling in these cell strains, a series of transient transfections was performed with an HRE-driven luciferase reporter construct. First, the specific silencing of PHD isofoms was confirmed by Western blot analysis using MSU 1.1 cells transfected with each independent shRNA construct (Fig. 2-2C). In the presence of a PHD2-specific shRNA construct, there was an increase in luciferase activity under normoxic conditions (20% O_2) in the MSU-1.1 cells that was not observed for the PHD1 or PHD3 shRNAs (Fig. 2-2D). There was no significant change in the presence of hypoxia for any of the shRNA constructs, suggesting that the HRE-mediated transcription was maximally stimulated. In a similar set of experiments, PHD1-3 were overexpressed in PH3MT cells to determine if increases in endogenous levels of the various PHDs could inhibit hypoxia-induced transcription (Fig. 2-2E). The results mirrored those of the shRNA data, in that only PHD2 was capable of inhibiting HRE driven luciferase activity under hypoxia. The luciferase activity in the PHD1 and

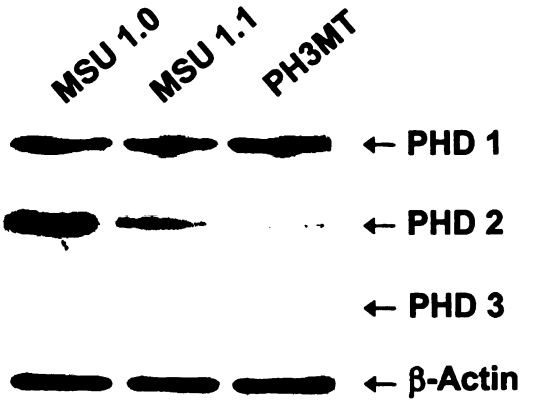
Figure 2-2. Expression levels of PHDs and the effects of modulating PHD levels on HIF1- hypoxia signaling in MSU1 lineage of cells.

(A) The mRNA levels of each PHD isoform were analyzed in the MSU cell lines using qRT-PCR and expressed as a percent of total PHD message. (n=12) **(B)** The protein levels of the PHD isoforms were determined in the MSU cell lines by Western blot analysis with PHD1-3 specific polyclonal antibodies or a β-Actin specific antibody. **(C)** Specific silencing of PHD isoforms using shRNA. MSU-1.1 cells were transiently transfected with no transfectant (MSU-1.1/Ctrl), scrambled shRNA cassette (Scram) or three independent shRNA cassettes targeting each PHD isoform (PHD1-3). Total protein was isolated and analyzed by Western blot with specific antibodies for each PHD or β-Actin. **(D)** MSU-1.1 cells were transiently transfected with no shRNA cassette (Ctrl), a scrambled shRNA (Scram), or shRNA cassettes targeting a specific PHD isoform (PHD1-3), together with an HRE-driven luciferase reporter construct and a β-gal expression vector for normalization. After transfection, cells were exposed to normoxia (20% O_2, white bar) or hypoxia (1% O_2, black bar) for 16 hr and analyzed for luciferase activity. (n=10, ** $p<0.01$) **(E)** PH3MT cells were transiently transfected with nothing (Ctrl), an empty expression vector (Vector) or an expression vector for the PHDs (PHD1-3) as described for MSU-1.1 in B. (n=10, ** $p<0.01$).

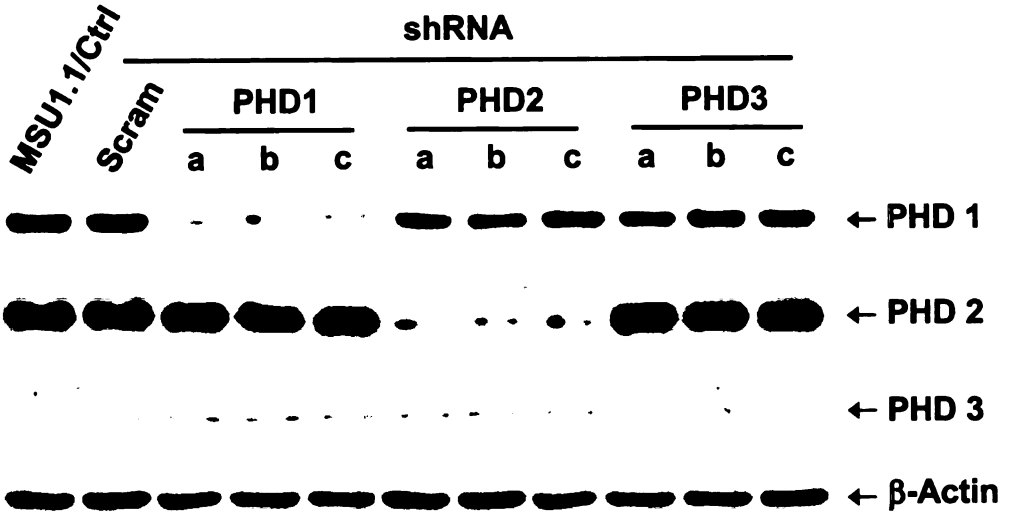
A

Cell Lines	PHD1	PHD2	PHD3
MSU 1.0	25.1 %	74.8 %	0.1 %
MSU 1.1	16.6 %	88.1 %	0.3 %
PH3MT	12 %	87.7 %	0.3 %

B



C



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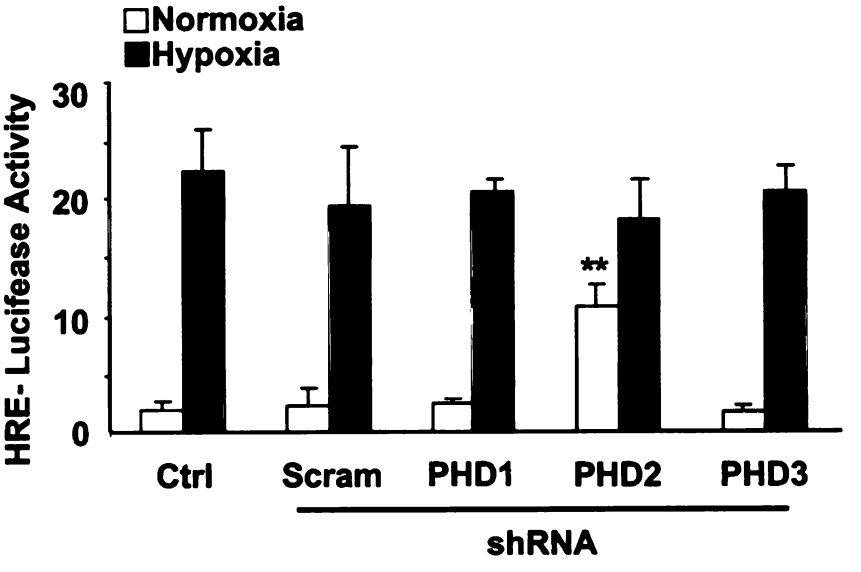
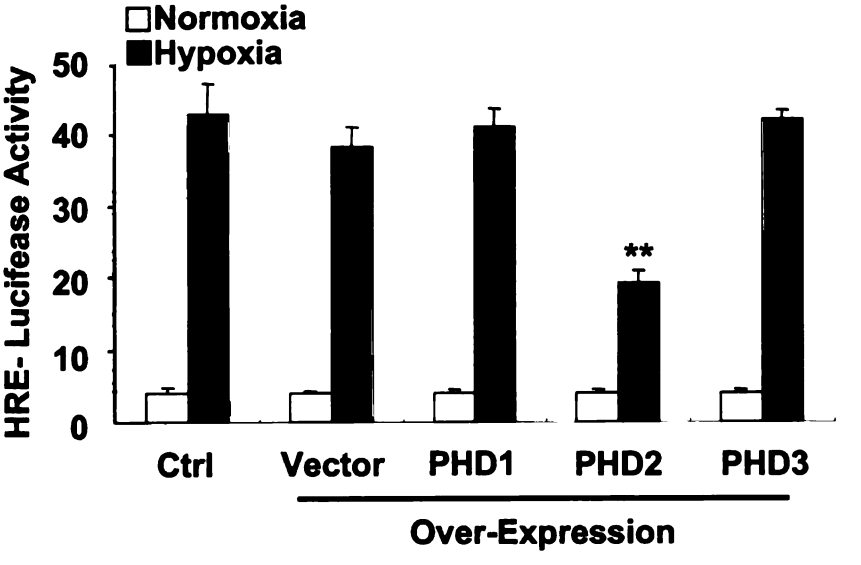

Normoxia
Hypoxia
HRE- Lucifease Activity
30
20
10
0
**
Ctrl
Scram
PHD1
PHD2
PHD3
shRNA

E


Normoxia
Hypoxia
HRE- Lucifease Activity
50
40
30
20
10
0
**
Ctrl
Vector
PHD1
PHD2
PHD3
Over-Expression

PHD3 transfected cells was similar to that found using the empty expression vector or the mock transfected controls. These results are evidence that PHD2 is the major functional isoform in regulating HIF1 activity in the MSU1 cell strains and that direct modulation of PHD2 activity can alter a cell's ability to respond to hypoxic stress.

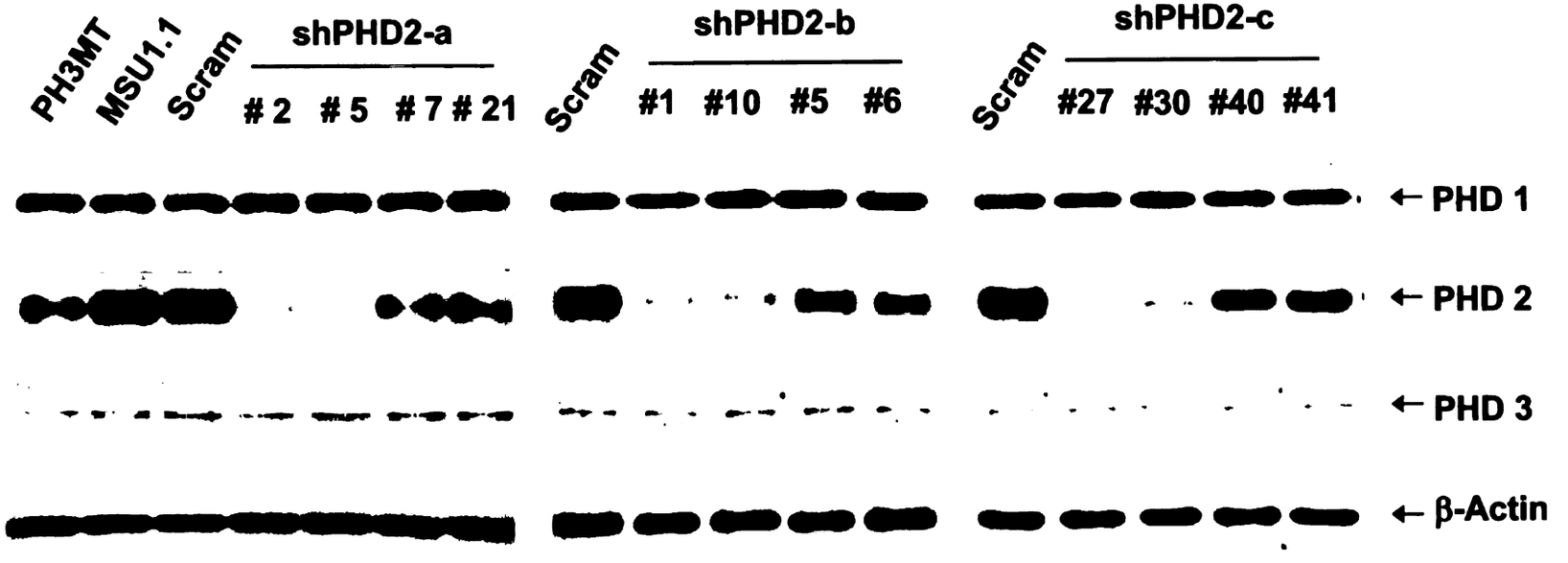
Decreases in PHD2 levels alter a cell's tumor-forming potential

Our data show that the MSU1 cell strains are a suitable system to directly test the link between PHD levels, HIF1 activity, and tumorigenesis and prompted us to examine whether the loss of PHD2 could bestow tumor forming ability upon a non-tumorigenic cell strain. To answer this question, a series of stable cell strains was created that have decreased levels of PHD2. Non-tumorigenic MSU-1.1 cells were infected with the lentiviral vector, pVCwPBam, encoding three distinct shRNA targeting PHD2 or scrambled shRNA. Initially, cell strains (shPHD2-a, -b, and -c) were assessed for PHD2 levels using Western blot analysis. Among those, four clonal cell strains were chosen from each shPHD2 strain that exhibited decreased PHD2 levels compared with the parental MSU-1.1 cells and scrambled shRNA expressing controls (Fig. 2-3A). The strains showed differences in PHD2 levels, with shPD2-a strains #7 and #21, shPHD2-b strains #5 and #6, and shPHD2-c strains #40 and #41 having moderate reduction in PHD2, which is similar to the levels of PHD2 in PH3MT, malignantly transformed MSU-1 lineage of cells and shPHD2-a strains #2 and #5, shPHD2-b strain #1 and #10, and shPHD2 strain #27 and #30 showing an almost complete loss of PHD2 expression.

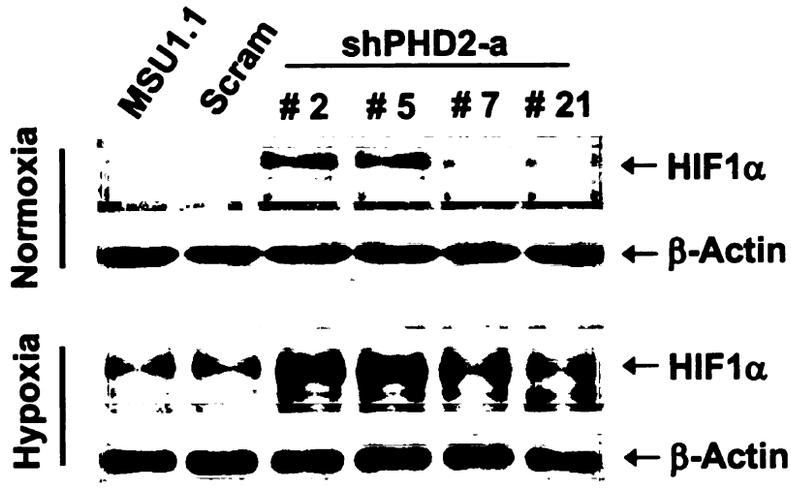
Figure 2-3. Characterization of shPHD2 infected MSU 1.1 strains.

(A) MSU-1.1 cells were infected with three independent lentiviral construct that expresses distinct shRNA cassette targeting PHD2. The levels of PHD were characterized in newly created shPHD2-a strains (#2, #5, #7, and #21), shPHD2-b strains (#1, #10, #5, and #6), and shPHD2-c strains (#27, #30, #40, and #41) by Western blot analysis using isoform-specific PHD antibodies or a β-Actin specific antibody. The parental cell line (MSU-1.1) and a scrambled shRNA cell strain (Scram) were included as controls. **(B)** HIF1α protein levels were analyzed in shPHD2-a strains exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 6 hrs by Western blot analysis. To verify equal loading, the blot was stripped and reprobed with a β-Actin antibody. **(C)** shPHD2 strains (shPHD2-a #2, #5, #7 and #21, shPHD2-b #1, #10, #5 and #6, and shPHD2-c #27, #30, #40 and #41) and scrambled shRNA clone (Scram) were assessed for anchorage independent growth by forming colonies in agarose. Parental MSU-1.1 cells and A210, hRAS-transformed cells, were included as a negative and positive control, respectively. (n=10).

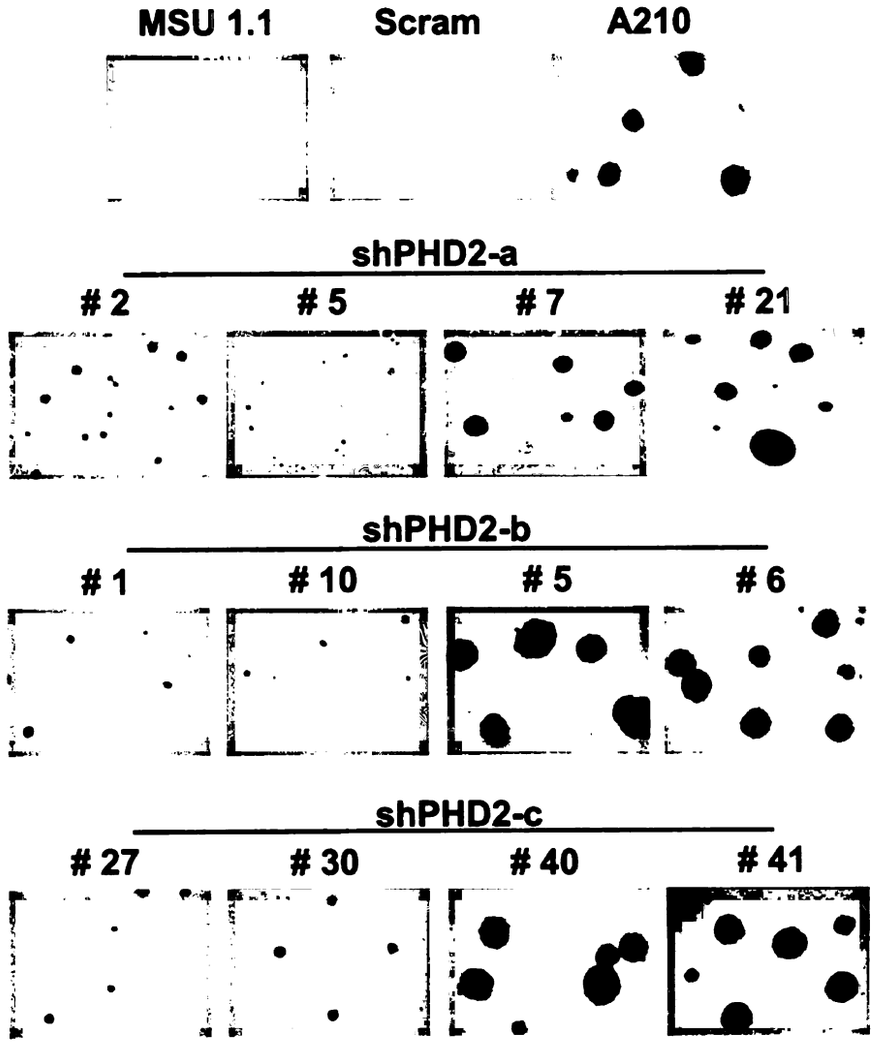
A


PH3MT
MSU1.1
Scram
shPHD2-a
#2
#5
#7
#21
Scram
shPHD2-b
#1
#10
#5
#6
Scram
shPHD2-c
#27
#30
#40
#41
← PHD 1
← PHD 2
← PHD 3
← β-Actin

B


MSU1.1
Scram
shPHD2-a
2
5
7
21
Normoxia
← HIF1α
← β-Actin
Hypoxia
← HIF1α
← β-Actin

C


MSU 1.1
Scram
A210
shPHD2-a
2
5
7
21
shPHD2-b
1
10
5
6
shPHD2-c
27
30
40
41

The levels of PHD1 and PHD3 were unaffected in any of the shPHD2 infected cell strains (Fig. 2-3A). To determine if the decreased PHD2 levels within the four strains had a functional consequence, we characterized HIF1α protein levels (Fig. 2-3B). There was substantial HIF1α protein in strains shPHD2-a #2 and #5 under normoxic conditions, compared to that of the parental or scrambled shRNA cell strains. The level of HIF1α protein was moderately up-regulated in strains shPHD2-a #7 and #21 under normoxia. All of the shPHD2 strains displayed hypoxia-induced HIF1α stabilization.

To examine whether our shPHD2 cell strains had acquired transformed characteristics, we first characterized their ability to form colonies in an anchorage-independent manner (soft-agar assay) (Fig. 2-3C). The strains with the least PHD2 (i.e. shPHD2-a #2 and #5, shPHD2-b #1 and #10, and shPHD2-c #27 and #30) were capable of forming colonies only marginally better than the scrambled shRNA strain and the parental MSU-1.1 cells; however, they did not perform as well as the positive control, the RAS-transformed A210 cells . The strains with a moderate reduction in PHD2 (i.e. shPHD2-a #7 and #21, shPHD2-b #5 and #6, and shPHD2-c #40 and #41) exhibited strong anchorage-independent growth, forming colonies larger than the positive controls (Fig. 2-3C). Additional shPHD2 strains with moderate or severe decreases in PHD2 levels also displayed similar results (data not shown). These results indicate that a small loss in PHD2 expression can aggressively promote a cell's ability to grow in an anchorage-independent manner, however, further loss of PHD2 does not significantly

change the cells' anchorage independent growth phenotype.

To determine if the shPHD2 strains were capable of forming tumors, five BALB/c athymic mice (5 weeks of age) were injected at two sites per mouse for each shPHD2-a cell strains (shPHD2-a #2, #5, #7, and #21) or the scram cell strain, as a control (Fig. 2-4). As expected, five months after injection, the scram cell strain did not show any tumor growth, like the parental cell strain, MSU-1.1(27). In contrast, two shPHD2-a strains, #21 and #7, yielded high grade fibrosarcomas at all ten injection sites in weeks 3 and 5, respectively (Fig. 2-4). shPHD2-a strains #2 and #5, which exhibited the lowest levels of PHD2, were negative for tumor-forming ability even after five months (Fig. 2-4). These results show that moderate decreases in PHD2 activity lead to malignant transformation, while further loss of PHD2 activity produces cells that do not form tumors.

These results suggest that a biphasic role exists for PHD2 in tumor formation (Fig. 2-5). At normal levels of PHD2 (e.g. MSU-1.1), the hypoxic response is regulated properly and no tumors are formed. With a slight decrease in PHD2 activity (e.g. shPHD2-a strains #7 and #21 and PH3MT cells) the cells gain an advantage and become malignantly transformed. As PHD2 activity is further decreased (e.g. shPHD2-a cell strains #2 and #5), the pro-death response might become the dominant signal and the increased adaptation would be overwhelmed. The dual nature of this response is presumably due to PHD2's ability to alter the cellular balance between

Figure 2-4. Tumor-forming ability of shPHD2-a strains.

shPHD2-a strains (#2, #5, #7 and #21) and scrambled shRNA clone (Scram) were injected into athymic mice and tumor growth was monitored weekly for 5 months. (n=10) (Images in this thesis/dissertation are presented in color)

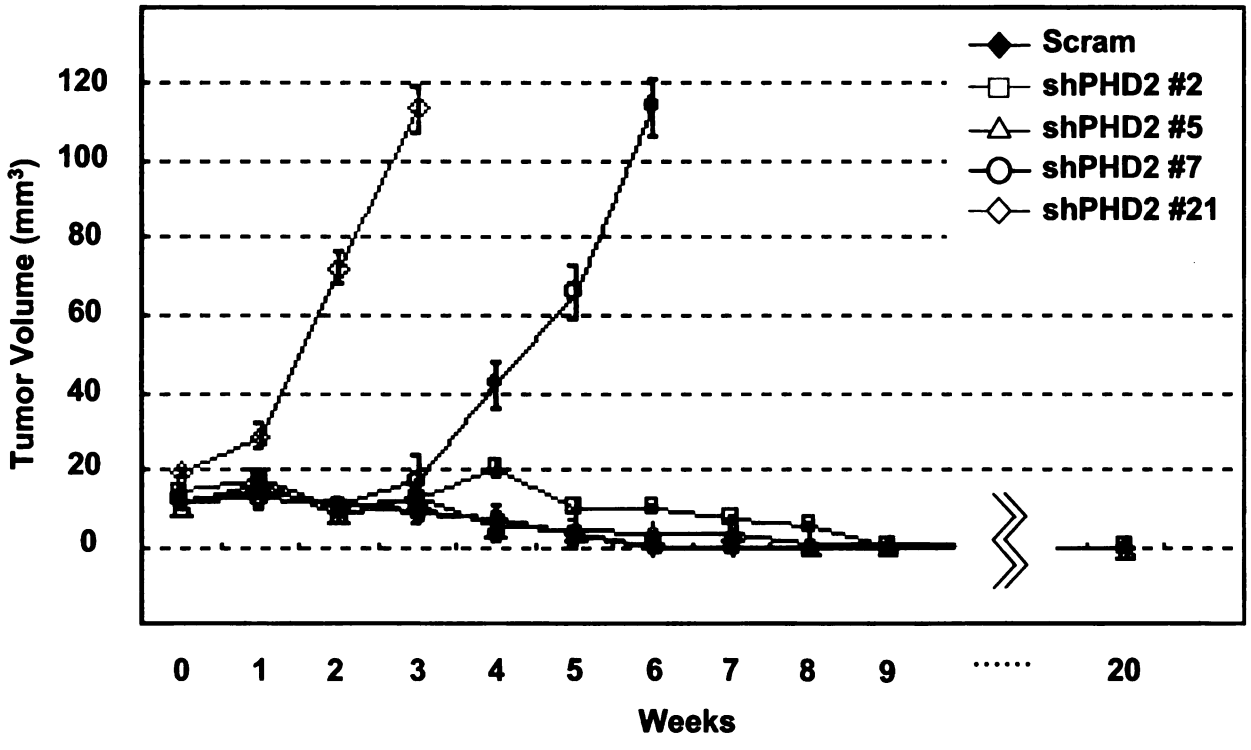
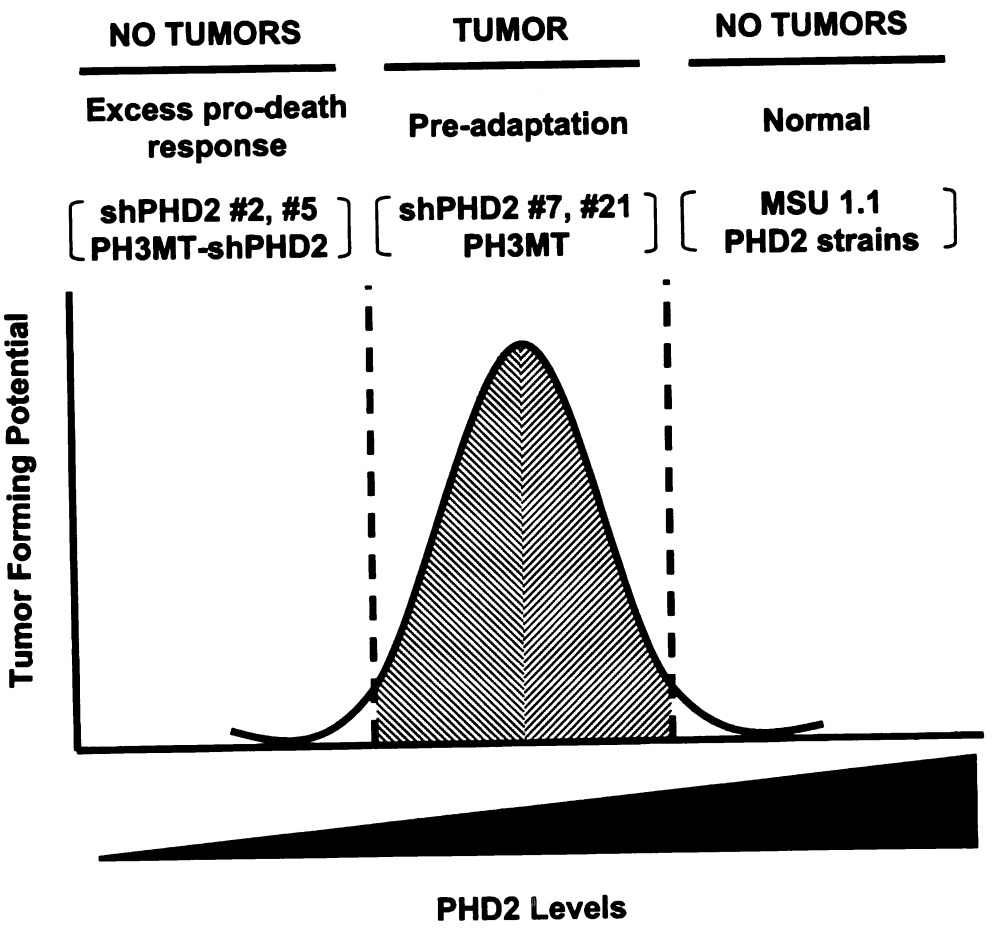

Scram
shPHD2 #2
shPHD2 #5
shPHD2 #7
shPHD2 #21
Tumor Volume (mm³)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 20
Weeks

Figure 2-5. A graphical representation of the relationship between PHD2 levels and tumor forming potential


NO TUMORS
TUMOR
NO TUMORS
Excess pro-death response
Pre-adaptation
Normal
shPHD2 #2, #5
PH3MT-shPHD2
shPHD2 #7, #21
PH3MT
MSU 1.1
PHD2 strains
Tumor Forming Potential
PHD2 Levels

hypoxic-induced adaptation and pro-death responses.

Biphasic role of PHD in balancing HIF1-mediated adaptation and cell death

The proposed model presented in Figure 5 suggests that PH3MT and shPHD2-a strains #7 and #21 (small decrease in PHD2) have a growth advantage, such as a higher rate of glycolysis and angiogenesis via HIF1-mediated pre-adaptation. HIF1 also regulates cell-death through transcriptional activation of pro-apoptotic factors (1, 41). The model also suggests that shPHD2-a strains #2 and #5 (severe loss of PHD2) have higher expression of pro-death genes, such as Bcl-2/adenovirus E1B 19 kd-interacting protein 3 (BNIP3), and a decreased viability under hypoxic stress.

To characterize the level of glycolysis and angiogenesis, the clones were analyzed for the expression of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and vascular endothelial growth factor (VEGF). To determine the potential cell death signals within the cell strains, BNIP3, a known HIF1 regulated BCL2 family member, was also measured. PH3MT and shPHD2-a strains #7 and #21 showed a higher basal expression of GAPDH and LDH when compared to the parental MSU-1.1 cells or scrambled control (Fig. 2-6A). In contrast, shPHD2-a strains #2 and #5 had higher BNIP3 expression levels than the other clones tested (Fig. 2-6A). Interestingly, VEGF basal expression was increased in the four PHD2 shRNA cell strains compared to the parental strain (Fig. 2-6A). To determine if these expression patterns had functional significance, GAPDH and LDH enzyme assays were performed

(Fig. 2-6B). These assays confirmed the mRNA data and showed that shPHD2-a strains #7 and #21 have a higher rate of glycolytic activity. This activity was similar to that of the malignantly-transformed PH3MT cell strain. Cell growth assays were also performed on each of the cell strains, in the presence and absence of hypoxia. The malignant strains, shPHD2-a #7 and #21 and PH3MT, had an increased growth characteristics under hypoxic stress as compared to the parental MSU-1.1 and scrambled control (Fig. 2-6C). shPHD2-a #2 and #5 exhibited increased doubling time and an inability to grow under hypoxic stress (Fig. 2-6C). Finally, the functional consequence of increased VEGF expression in shPHD2-a clone #21 was confirmed by immunostaining of the tumors derived from these clones with the endothelial specific antibodies for CD31 (PECAM) and Factor VIII. The tumors resulting from cells with moderately decreased PHD2, PH3MT and shPHD2 #21, showed a range of vascularization (Fig. 2-6D). These data support the biphasic model and are evidence that small decreases in PHD2 activity can lead to malignant transformation, increased glycolysis and vascularization, and severe loss of PHD2 activity can inhibit cell viability and tumor-forming potential through stimulation of cell-death pathways.

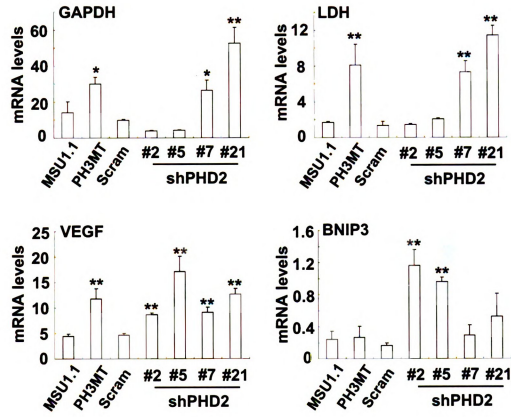
The inhibition of PHD activity reverses a cell's transformed characteristics

To further support the biphasic model and provide evidence that the results described in figures 3-6 were directly related to PHD activity, we inhibited PHD2 in malignantly transformed PH3MT cells and shPHD2-a strains #7 and #21 using the

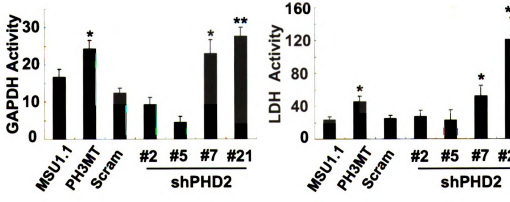
Figure 2-6. HIF1-mediated cellular responses in shPHD2 strains.

(A) mRNA levels of GAPDH, LDH, BNIP3, and VEGF were determined in MSU-1.1, PH3MT, scrambled shRNA infected strain (Scram), and shPHD2-a strains (#2, #5, #7 and #21) using qRT-PCR. (n=6, * $p<0.05$, ** $p<0.01$). **(B)** GAPDH and LDH activity were determined in MSU-1.1, PH3MT, scrambled shRNA infected strain (Scram), and shPHD2-a strains (#2, #5, #7 and #21). Kinetic activity was normalized to protein concentration. (n=8, * $p<0.05$, ** $p<0.01$). **(C)** Each cell strain was analyzed by MTT assay following exposure to normoxia (20% O_2, white bar) or hypoxia (1% O_2, black bar) for 3 days. (n=4). **(D)** CD31 (PECAM) and Factor VIII immunostaining was used to visualize vascularization in formalin fixed tumor derived form shPHD2 strain #21, PH3MT, and a benign growth derived from an early MSU lineage (Ctl).

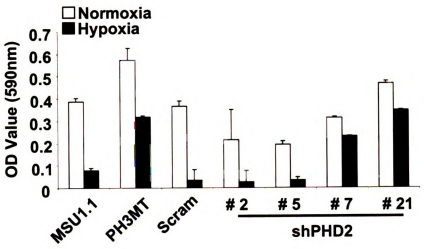
A


GAPDH
mRNA levels
60
40
20
0
MSU1.1
PH3MT
Scram
#2 #5 #7 #21
shPHD2
LDH
12
8
4
VEGF
25
20
15
10
5
BNIP3
1.6
1.2
0.8
0.4

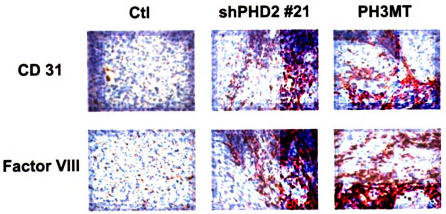
B


GAPDH Activity
30
20
10
0
MSU1.1
PH3MT
Scram
#2 #5 #7 #21
shPHD2
LDH Activity
160
120
80
40

C


Normoxia
Hypoxia
OD Value (590nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MSU1.1
PH3MT
Scram
2
5
7
21
shPHD2

D

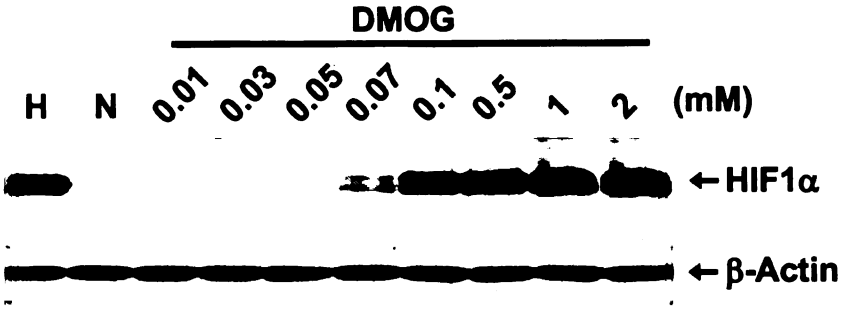

Ctl
shPHD2 #21
PH3MT
CD 31
Factor VIII

dimethyloxallyl glycine (DMOG). If the model is correct, further inhibition of PHD2 in these cells should force them to phenotypically resemble shPHD2-a strains #2 and #5. DMOG was capable of inhibiting PHDs in a dose dependent manner as evidenced by the increasing stability of HIF1α, with a maximal effect observed at 1 mM (Fig. 2-7A). To exam if the inhibition of PHD activity can alter the tumor forming potential of PH3MT cells and shPHD2-a strains #7 and #21, we analyzed their ability to form colonies in an anchorage-independent manner in the presence of DMOG (Fig. 2-7B). As predicted by the model, each of these cell types lost their ability to grow in soft agar when PHD activity was diminished by DMOG. To insure this was not due to DMOG toxicity, cells were plated on standard culture dishes and incubated in the absence or presence of DMOG (1 mM) for 10 days and stained (Fig. 2-7C). There was a reduction in the size of clones on the DMOG treated plates, however, removal of DMOG lead to growth recovery within 5 days suggesting that direct inhibition of PHD activity decreases cellular proliferation. These results suggest that DMOG is capable of inhibiting the anchorage independent growth of the transformed cells and that this inhibition may due to growth inhibition or cellular senescence. Presumably, this is caused by high basal expression of pro-death gene, such as BNIP3, as described for shPHD2-a strains #2 and #5 (Fig. 2-6A). Indeed, inhibition of PHD2 activity by DMOG in tumorigenic shPHD2-a strains #7 and #21 and PH3MT cells led to a significant increase in BNIP3 expression (Fig. 2-7D). These results suggest that

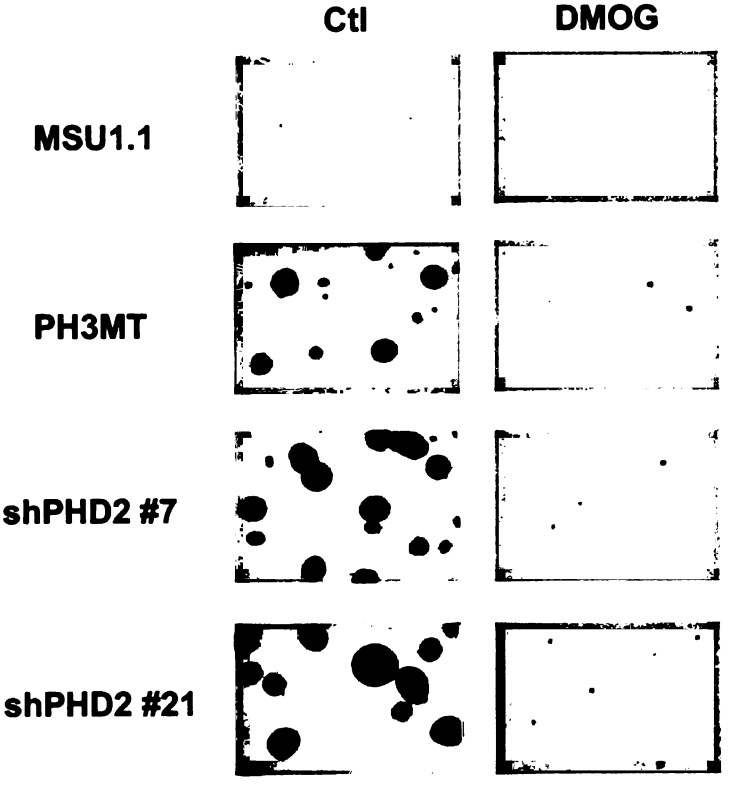
Figure 2-7. Inhibition of PHD2 activity in transformed cells.

(A) HIF1α protein levels were determined in PH3MT cells treated with various concentrations of DMOG by Western blot analysis. HIF1α levels in PH3MT cells exposed to normoxia (N, 20% O_2) or hypoxia (H, 1% O_2) were also analyzed as a control and β-Actin antibody was used as a loading control. **(B)** PH3MT, shPHD2 strains #7 and #21, and MSU 1.1 cells were assessed for anchorage-independent growth using soft-agar assay, in the absence (ctl) and presence of DMOG (1 mM in media). (n=10) **(C)** Cell viability assessment following DMOG treatment. 10^3 cells were plated in 100mm culture dish and cultured in the presence or the absence of DMOG (1 mM) for 10 days and stained. A separate set was exposed to DMOG followed by 5 days recovery in the normal growing media. (n=10) **(D)** BNIP3 mRNA levels were determined in MSU-1.1, shPHD2 strains #7 and #21, and PH3MT cells left untreated (ctl, white bar) or exposed to DMOG (black bar). (n=6, * $p<0.05$)

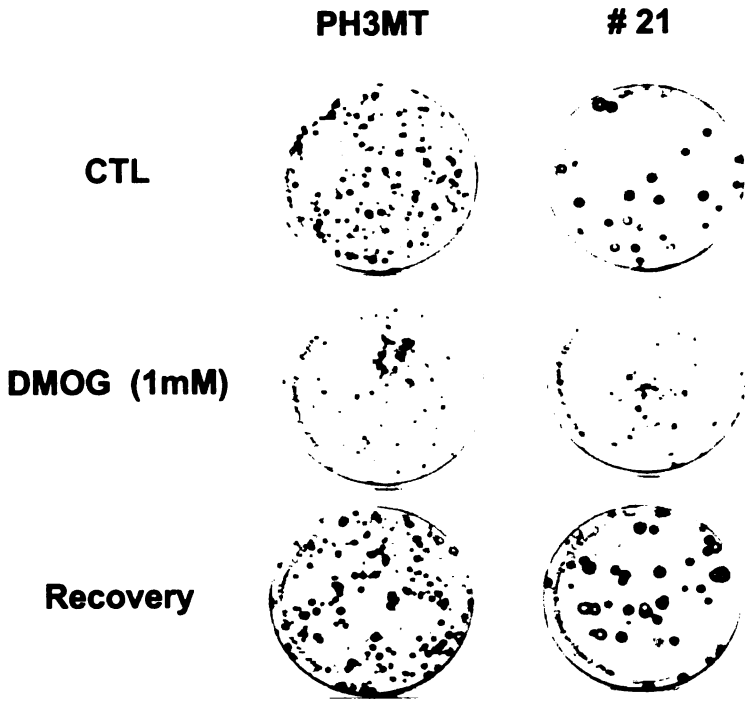
A


DMOG
H
N
0.01
0.03
0.05
0.07
0.1
0.5
1
2
(mM)
←HIF1α
←β-Actin

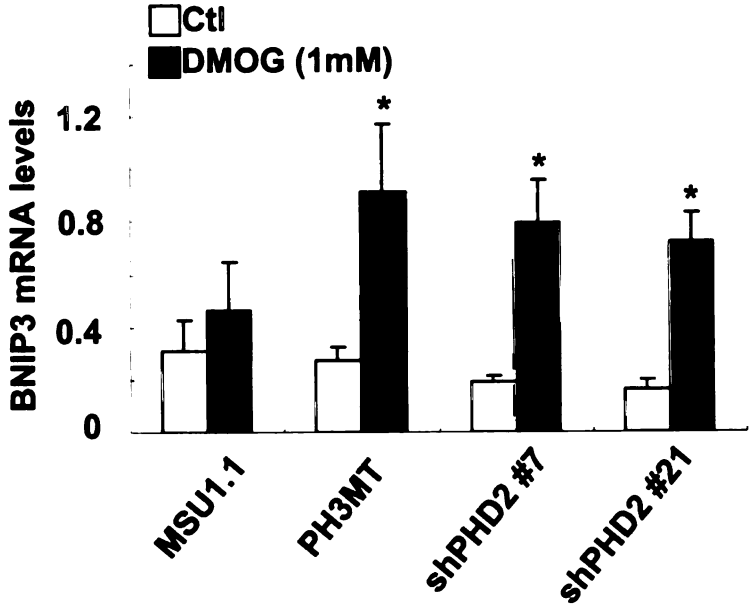
B


Ctl
DMOG
MSU1.1
PH3MT
shPHD2 #7
shPHD2 #21

C


PH3MT
21
CTL
DMOG (1mM)
Recovery

D


Ctl
DMOG (1mM)
BNIP3 mRNA levels
1.2
0.8
0.4
0
*
*
*
MSU1.1
PH3MT
shPHD2 #7
shPHD2 #21

malignantly transformed cells (e.g. shPHD2-a #7 and #21 and PH3MT) can lose their tumor-forming potential in the presence of PHD inhibitors and a pro-death response is involved in this phenotypic alteration.

Effects of alterations in PHD2 activity in the transformed cell strain, PH3MT

The model also predicts that altering the PHD2 activity in malignantly transformed cells would alter the cell's tumor-forming potential. To test this hypothesis, we altered the PHD2 activity in PH3MT cells. First, PH3MT cells were infected with the shPHD2 cassettes described above, and strains were selected. We were unable to expand any of the 25 cell strains, suggesting these PHD2 shRNA expressing cells were prone to premature cell death (data not shown). This observation is supported by the previous results in which DMOG treatment lead to inhibition of anchorage independent growth in malignantly transformed cells with a corresponding increase in pro-cell death factor, BNIP3, expression. Each of these results is evidence that decreasing PHD2 activity within tumorigenic cells lead to a loss in tumor-forming potential.

The model proposed in Figure 2-5 also suggests that by increasing PHD2 levels within these same cells will lead to a similar decrease in tumor-forming potential. To test this, PH3MT cells were infected with the retroviral vector, pZome-1N, encoding the cDNA for PHD2. pZome-1N produces a tagged PHD2 protein and its expression can be distinguished from endogenous PHD2 based on their molecular size. In addition, a cDNA for GFP was also inserted into separate pZome-1N and was used to create a

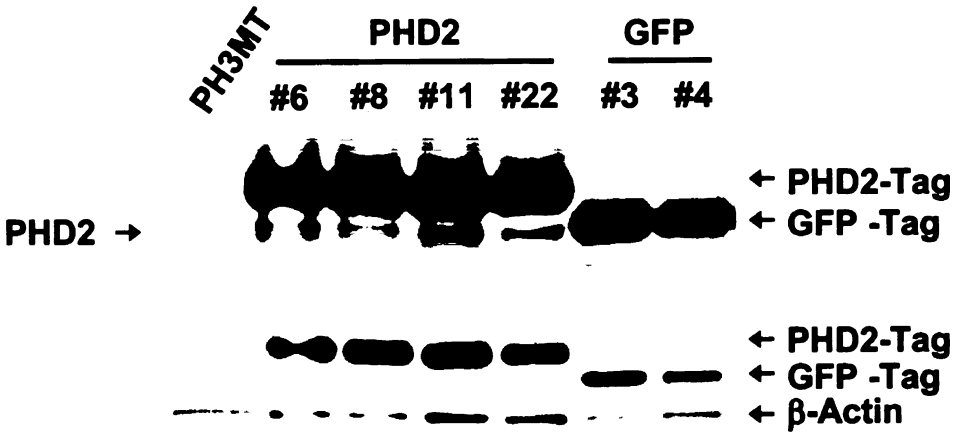
control cell strains. PHD2 cell strains were screened for overexpression of PHD2 using Western blot analysis and four of these (PHD2 strain #6, #8, #11, and #22) were selected for further analysis (Fig. 2-8A). Tagged-PHD2 and GFP are visualized on the β-actin Western blot due to the Protein A motif within the tag. We next examined whether over-expression of PHD2 affects hypoxia-induced HIF1α stabilization (Fig. 2-8B). As expected, HIF1α was undetectable under normoxia and its levels were increased by hypoxia in control, PH3MT and GFP strains, #3 and #4. However, the PHD2 over-expressing cell strains, PHD2 #6, #8, #11 and #22, showed reduced hypoxia-induced HIF1α accumulation. These results are in agreement with previous published reports showing that increased PHD expression can inhibit HIF1α accumulation and HIF1 activity under hypoxic stress (12, 13, 39). In addition over-expression of PHD2 had functional consequences on HIF1-mediated upregulations of GAPDH, LDH and VEGF. The hypoxia-induced expression of these genes was diminished in all PHD2 strains, PHD2 #6, #8, #11 and #22 when compared to hypoxia treated control cells, PH3MT and GFP strains #3 and #4 (Fig. 2-8C). GAPDH and LDH enzyme assays confirmed the mRNA data and showed that PHD2 strains #6, #8, #11 and #21 lost their hypoxia-induced glycolytic activity (Fig. 2-8D).

To determine if PHD2 over-expression can alter a cell's transformed phenotype, each of the PHD2 cell strains was analyzed for its ability to form colonies in soft agar (Fig. 2-9A). The tumorgenic parental PH3MT and control GFP strains, #3 and #4,

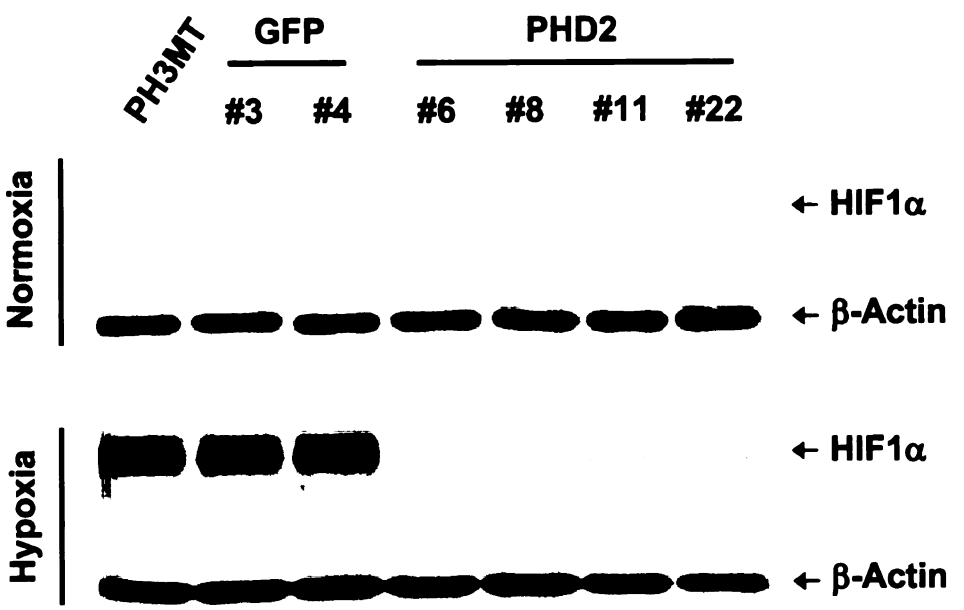
Figure 2-8. Over- expression of PHD2 in PH3MT cells.

(A) PH3MT cells were infected with a retroviral construct that expresses PHD2 cDNA. The expressions of PHD2-TAP were characterized in the parental PH3MT, PHD2 strains (#6, #8, #11 and #22) and GFP cell strains (#3 and #4) by Western blot analysis using a PHD2-specific antibody or β-Actin antbody. The PHD2 and GFP proteins are visible due to the protein A tag. **(B)** HIF1α protein levels in PHD2 strains following exposure to normoxia (20% O_2) or hypoxia (1% O_2) were analyzed by Western blotting with a HIF1α monoclonal antibody or β-Actin antibody. **(C)** mRNA levels of GAPDH, LDH, and VEGF were determined in PH3MT, GFP strains, #3 and #4 and PHD2 strains #6, #8, #11 and #22 using qRT-PCR. Cells were exposed to normoxia (20% O_2, white bar,) or hypoxia (1% O_2, black bar) for 16 hr. (n=8, * $p<0.05$, ** $p<0.01$). **(D)** GAPDH and LDH activity were determined in MSU-1.1, PH3MT, GFP strain #3 and #4 and PHD2 strains #6, #8, #11 and #22. Cells were exposed to normoxia (20% O_2, white bar,) or hypoxia (1% O_2, black bar) for 16 hr and enzymatic activity was normalized to protein concentration. (n=8, * $p<0.05$, ** $p<0.01$).

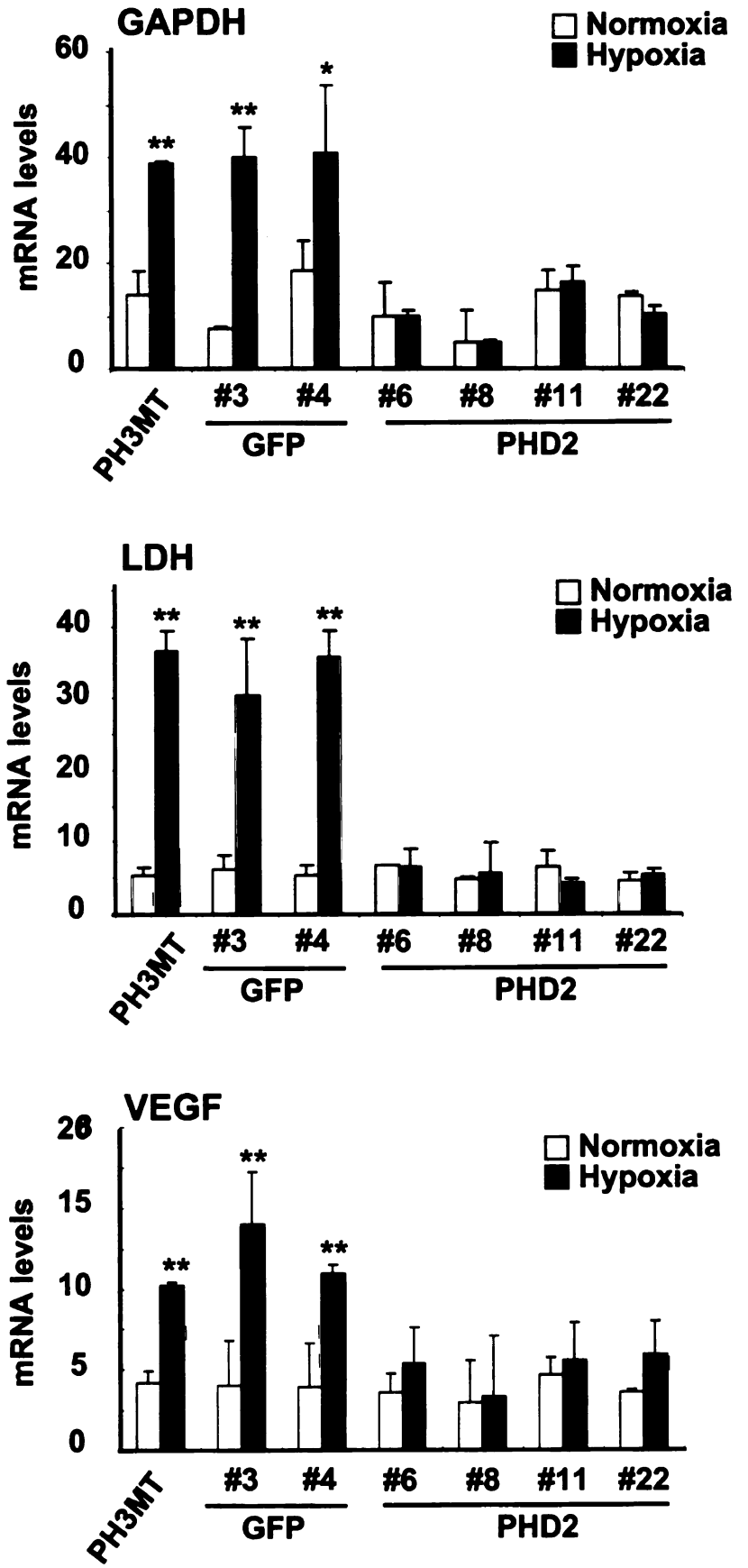
A


PH3MT
PHD2
GFP
#6
#8
#11
#22
#3
#4
PHD2 →
← PHD2-Tag
← GFP -Tag
← PHD2-Tag
← GFP -Tag
← β-Actin

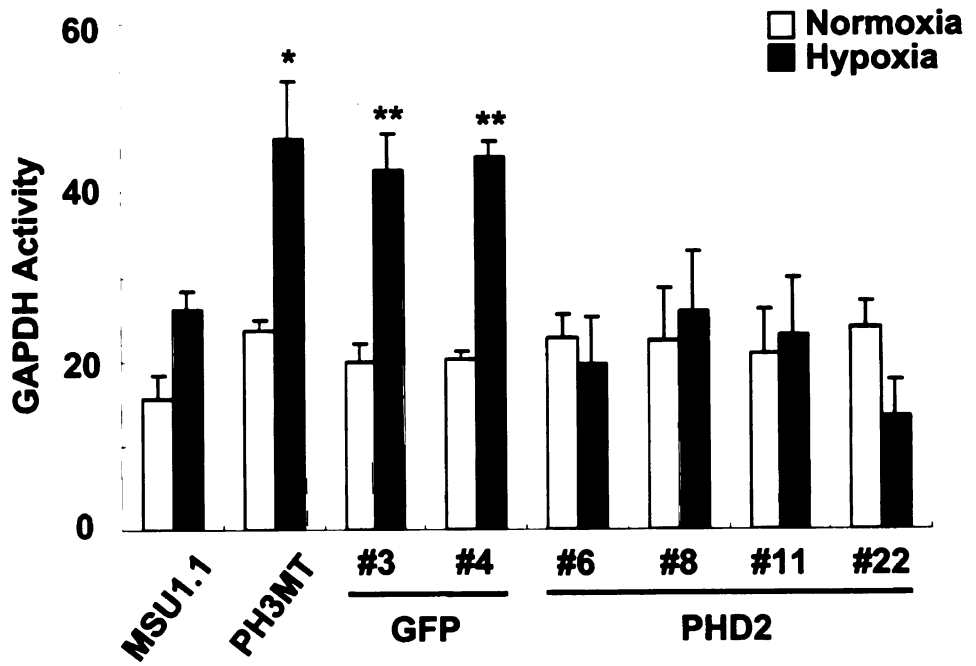
B

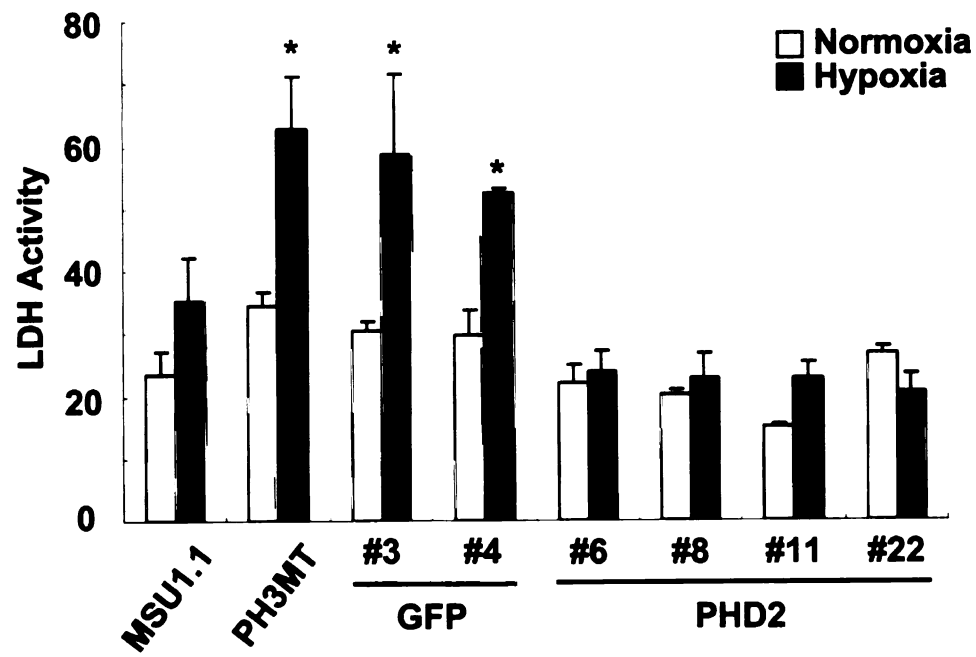

PH3MT
GFP
PHD2
#3
#4
#6
#8
#11
#22
Normoxia
Hypoxia
← HIF1α
← β-Actin
← HIF1α
← β-Actin

C


GAPDH
Normoxia
Hypoxia
mRNA levels
60
40
20
0
**
**
*
PH3MT
#3
#4
#6
#8
#11
#22
GFP
PHD2
LDH
Normoxia
Hypoxia
mRNA levels
40
30
20
10
0
**
**
**
PH3MT
#3
#4
#6
#8
#11
#22
GFP
PHD2
VEGF
Normoxia
Hypoxia
mRNA levels
20
15
10
5
0
**
**
**
PH3MT
#3
#4
#6
#8
#11
#22
GFP
PHD2

D


Normoxia
Hypoxia
GAPDH Activity
60
40
20
0
*
**
**
MSU1.1
PH3MT
#3
#4
#6
#8
#11
#22
GFP
PHD2

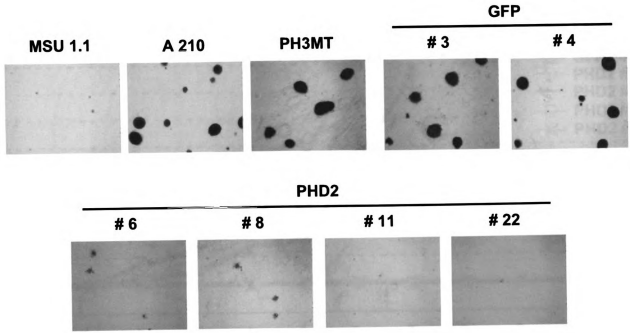

Normoxia
Hypoxia
LDH Activity
80
60
40
20
0
*
*
*
MSU1.1
PH3MT
#3
#4
#6
#8
#11
#22
GFP
PHD2

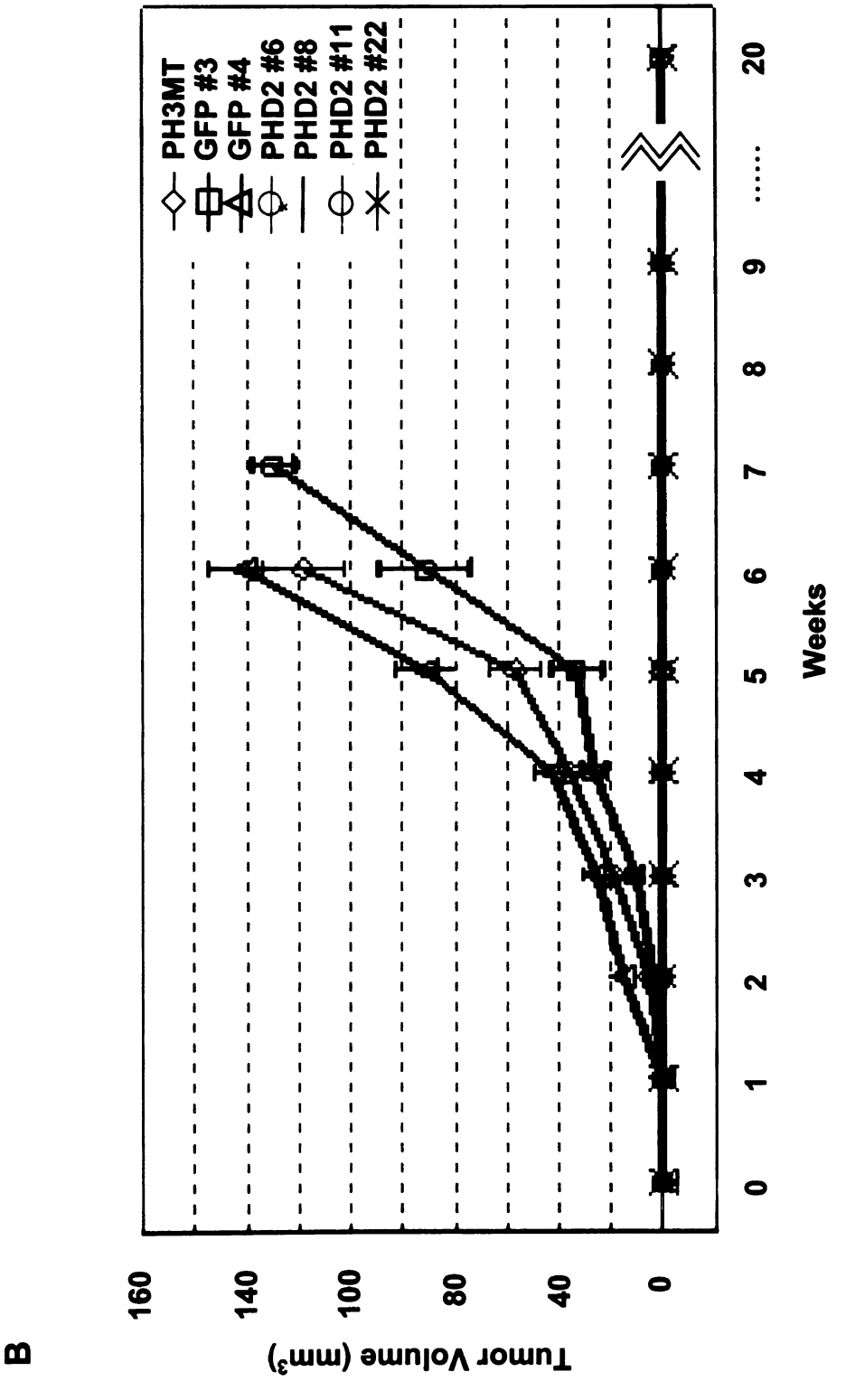
exhibited large colonies when grown in agar, while the PHD2 clonal cell strains, #6, #8, #11 and #22, lost this ability to grow in an anchorage-independent manner. To determine whether over-expression of PHD2 lead to decreases in tumor forming potential, PHD2 strains # 6, #8, #11, and #22 were examined for their ability to form tumors in athymic mice (Fig. 2-9B). Parental PH3MT and GFP strains, #3 and #4, were used as a control and yielded tumors within 6 weeks (Fig. 2-9B). In contrast, all of the PHD2 strains, #6, #8, #11, and #22, were negative for tumor formation after 5 months (Fig 2-9B). These results show that the increase of the PHD2 activity in malignantly transformed cells can inhibit a cells' transformed phenotype and support our biphasic model relating PHD2 with tumor forming potential.

Figure 2-9. Characterization of tumor forming potential in PHD2 strains.

(A) PHD2 strains (#6, #8, #11 and #22) and GFP strains (#3 and #4) were assessed for anchorage-independent growth by forming colonies in soft agar. Parental PH3MT cells and A210 and MSU-1.1 cells were used as positive controls and negative control, respectively. (n=10) **(B)** PHD2 strains (#6, #8, #11 and #22) and GFP strains (#3 and #4) were injected into athymic mice and tumor growth was monitored weekly for 5 months. (n=10)

A




B
Tumor Volume (mm³)
160
140
100
80
40
0
0
1
2
3
4
5
6
7
8
9
.....
20
Weeks
PH3MT
GFP #3
GFP #4
PHD2 #6
PHD2 #8
PHD2 #11
PHD2 #22

DISCUSSION

HIF1 regulated genes are involved in many cellular processes including cell proliferation, angiogenesis, metabolism, migration and many others which are known to be required for adaptive survival of tumor cells (9, 10, 19, 35). Alternatively, severe hypoxia exposure can lead to cell death through HIF1-mediated up-regulation of pro-cell death factors or p53-dependent processes (1, 6, 11, 37). Hypoxia signaling, therefore, regulates a delicate balance between life and death through cellular adaptation and a programmed death response. A cancer cell's survival is dependent upon its ability to maintain cell growth and/or decrease its programmed death response once it is exposed to the hypoxic microenvironment of a tumor. The correlation between PHD2 levels and tumor-forming potential suggests that these hydroxylases might be involved in altering this balance (Fig. 2-1~2-5). Presumably, small decreases in PHD activity would promote an adaptive response without increasing pro-death signals (Fig. 2-6). In addition, the decreased hydroxylase activity and subsequent increase in HIF1-mediated signaling can explain the observation that tumors and corresponding cell strains have an increase in hypoxia signaling, even in the presence of normal oxygen concentrations. The direct link between decreased PHD activity, increased HIF1 signaling, and increased glycolytic activity might also explain the Warburg effect (44). Previous reports have shown that HIF1 is necessary for the Warburg effect, and a cellular decrease in PHD activity would explain a tumor's

increased dependence on aerobic glycolysis (5, 16, 19, 34). It is possible that one step in the transformation process is the sustained decrease in PHD activity, through genetic or epigenetic mechanisms. This would serve to pre-adapt the cells (eg. increased glycolytic rate) to the hypoxic environment found in many, and perhaps all, tumors and gives them a growth advantage upon tumor development. It is also possible that this loss of PHD activity and subsequent increased glycolytic activity causes the malignant transformation, as Warburg had proposed (44).

Interestingly, cells with a severe loss of PHD2 showed no ability to form tumors in athymic mice (Fig. 2-3 and 2-4). These cell strains, both MSU-1.1-derived and PH3MT-derived, displayed growth abnormalities such as signs of premature cell death, increased doubling time and an inability to grow under hypoxic stress (Fig. 2-6). It is hypothesized that this is due to an uncontrolled pro-death response. It is hypothesized that this response is driven by direct HIF1-mediated transcription of genes such as BNIP3 and NIX. The almost complete loss of PHD2 in shPHD2 strains #2 and #5, and subsequent HIF1 activation, would also presumably contribute to p53-mediated cell cycle arrest and apoptosis. Given the overwhelming pro-death response following almost complete loss of PHD2 activity, no amount of adaptive cell signaling can support continued expansion in the tumor microenvironment.

These two groups of cell strains, mild decrease and severe decrease in PHD2 levels, led to the proposed biphasic model presented in Figure 2-5. This type of model

would also predict that transformed cells are within the phase of the curve that supports tumor formation and movement in either direction (more or less PHD activity) will alter the cell's tumor forming potential. The tumorigenic PH3MT cells and modulation of PHD2 levels in these cell strains strongly support our model. First, the PH3MT cells have a decreased level of PHDs when compared to the MSU-1.0 or MSU-1.1 (Fig. 2-2B). Second, PH3MT cells that express the PHD2 shRNA cassette (movement left along the abscissa, Figure 2-5) stop growing after colony selection and cannot be expanded. Third, tumorigenic cells (PH3MT, shPHD2-a #7 and #21) lost their transformed characteristics when PHD activity was drastically decreased by a hydroxylase inhibitor (Fig. 2-7). Fourth, over-expression of PHD2 (movement right along the abscissa, Fig. 2-5) in the PH3MT cell's inhibits the cells ability to grow in an anchorage-independent manner and form tumor in athymic mice, suggesting they have lost a transformed phenotype (Fig. 2-8). Finally, a recent report has shown that over-expression of PHD1 in colon cancer cells inhibits tumor growth (15). Taken together, these results provide evidence for the biphasic model of PHD activity and tumor forming potential and suggest the clinical importance of characterizing the factors that establish the boundaries dictating the separate areas within this model.

The biphasic model will have profound consequences on chemotherapeutic agents that target PHDs and the hypoxia signaling cascade for cancer therapy. Cells that have amassed the cellular mutations necessary to progress towards complete

transformation (e.g. MSU-1.1 cells) might become fully tumorigenic when exposed to a drug that only partially blocks this cascade, while other cells at a different transformation stage (e.g. PH3MT cells) will undergo cell death. Alternatively, new therapies that completely shut down PHD activity will have detrimental consequences on normal cellular homeostasis by promoting an uncontrolled pro-death response. Ideally, any drug targeting this pathway should be specific to precancerous and hypoxic cells thus increasing the chance that decreases in PHD activity within the cell will push these cells toward cell death. The data presented here implies that cells will have a biphasic response to such agents and if this is the case, it will create difficulties when targeting these enzymes for cancer therapeutics.

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CHAPTER 3

THE PROTEIN INTERACTION NETWORK OF HIF PROLYL HYDROXYLASE, PHD2, IN HUMAN FIBROBLASTS

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ABSTRACT

The oxygen-dependent degradation of the alpha subunits of the hypoxia-inducible factors (HIFα) plays an essential role in regulating gene expression system in response to hypoxia. This process requires the prolyl hydroxylase (PHDs)-mediated hydroxylation of conserved prolines within the HIFα, facilitating its interaction with the von Hippel-Lindau protein, the recognition component of the E3 ubiquitin lagase. PHDs belong to the iron- and 2-oxoglutarate-dependent oxygenase family that require dioxygen for activity. The oxygen dependence of PHD and the role of PHD in regulating HIF signaling suggest a role for PHD as an oxygen sensor. Characterizing the precise mechanism and regulation of these enzymes, therefore, is essential to understand physiological and pathological responses to hypoxia. In this study, we characterized the PHD2-protein interaction network (PHD2-PIN) using tandem affinity purification and liquid chromatography coupled tandem mass spectrometric analysis (LC-MS/MS). Our results suggest that PHD2 might have no specific protein interaction under normoxic conditions in this particular cell type.

INTRODUCTION

A cell's ability to respond to changes in oxygen availability is a fundamental property of most aerobic organisms and involves a precisely regulated gene expression system. Hypoxia-inducible factors (HIFs) are the key transcription factors in regulating a wide range of genes responding to decreased oxygen tension (22, 30). HIFs are heterodimers of HIFα and HIFβ subunits, both of which are member of the basic helix-loop-helix Per-ARNT-SIM (bHLH-PAS) protein family of transcription factors (30). HIF1β is constitutively expressed as a nuclear protein and has previously been recognized as the dimerization partner of the aryl hydroxarbon receptor, where it is termed the aryl hydrocarbon receptor nuclear translocator (ARNT). HIFs is are specific to the hypoxic response and constantly synthesized and under normoxia, immediately removed via ubiquitination and proteasomal degradation (23, 30). This process involves the interaction of the HIFα with the von Hippel-Lindau (VHL) tumor suppressor protein (pVHL) which acts as a recognition component of the E3 ubiquitin-ligase complex (23). The interaction between HIFα and pVHL depends on the posttranslational hydroxylation of two conserved proline resides within the oxygen-dependent degradation domain (ODD) of HIFα (13, 14).

A family of prolyl hydroxylase domain containing proteins (PHDs, also known as egg-laying deficient nine-like proteins (EGLNs) and HIF-prolyl-hydroxylases (HPHs)) has recently been identified that can catalyze the hydroxylation of the conserved proline residues within the ODD in an oxygen-dependent manner (9, 20, 23).

Three human PHD isoforms, PHD 1-3, have been identified with distinct characteristics in substrate specificity, intracellular distribution and tissue specific inducibility under hypoxia (5, 7). In mammals, PHD2 appears to be the predominant isoform that regulates HIFα protein stability (4, 15). PHDs belong to an iron and α-ketoglutarate (2-oxoglutarate)-dependent dioxygenase family that require oxygen for proper hydroxylation of prolines within the HIF1α (5, 7, 12). Unlike other members of this class of dioxygenases, H_2O_2 cannot be substituted for molecular oxygen in the PHDs (9, 25). The oxygen-dependence and relatively high Km for O_2 (*in vitro*) of these enzymes suggests a role for PHDs as cellular oxygen sensor for the hypoxia signaling system (9, 10, 18, 26). In addition to oxygen, ferrous iron (Fe^{2+}) and ascorbate are also required for proper PHDs function. The substrate and product, α-ketoglutarate and succinate, respectively, also affect PHD's activity. Succinate functions as a competitive inhibitor for PHD, which can be overcome by increasing α-ketoglutarate concentrations (17, 25). Both α-ketoglutarate and succinate are tricarboxylic acid (TCA) cycle intermediates, suggesting some level of crosstalk of hypoxia signaling with mitochondrial activity and metabolic state. Several recent studies suggested that abnormal levels of TCA cycle intermediates (i.e. succinate and furmarate) and glycolytic metabolites (i.e. pyruvate), can perturbed PHD activity (11, 19, 21, 29). It has also been proposed that mitochondrial generated reactive oxygen species (ROS) can alter PHD activity suggesting a possible mechanisms that links the respiratory chain to HIF signaling (8).

Although several studies have implicated the role of other signals in PHD activity, the mechanisms underlying the link between them have not been characterized and one possibility is that additional proteins and/or pathways may be involved in this crosstalk. In addition, PHD-mediated HIFα degradation in response to oxygen availability is very prompt and specific suggesting possible contribution of other proteins in this process. Indeed recent study reported that OS-9, the protein product of a widely expressed gene, interacts with PHD2 and HIF1α and promotes HIF1α-hydroxylation, pVHL binding, and proteosomal degradation (1). What is more, other upstream signals may exist that can modulate PHD acitivty. Recent reports demonstrate that the Siah protein, a homologue of the *Drosophila* seven-in-absentia protein, post-translationally regulates PHD1 and PHD3 levels and the peptidyl-prolyl cis/trans isomerase, FKBP38, is involved in regulating PHD2 protein stability (2, 27). Creating a comprehensive library of PHD-interacting proteins, therefore, is important to understanding PHD-mediated oxygen sensing mechanism(s) and hypoxia signaling. In this study, we characterize a map for the PHD2-protein interaction network (PHD2-PIN) using tandem affinity purification (TAP) and liquid chromatography coupled mass spectrometric analysis (LC-MS/MS). Using this approach, we did not identified any specific proteins associated with PHD2 within the human fibrosarcomas, PH3MT cells, suggesting that PHD2 might act alone in modulating HIF1α activity under normoxia in this cells.

MATERIAL AND METHODS

Cell culture

Human fibroblasts PH3MT cells (a generous gift from Dr. J. Justin McCormick, MSU., (24)) were cultured in α-MEM (Mediatech Inc., Herndon, VA) and Phoenix ampho cells (a generous gift form Garry Nolan, Stanford Univ., (28)) were maintained in DMEM (Mediatech Inc.), supplemented with 10 % fetal calf serum (HyClone, Logan, UT), 100 unit/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. All supplements were purchased from Invitrogen (Carlsbad, CA). Cells were incubated at 37 °C and 5 % CO_2 (Precision, Winchester, VA).

Plasmid construction and retroviral infection

The cDNA for PHD2 (a generous gift form Dr. Steven McKnight, Univ. of Texas Southwestern (5)) was inserted into pZome1N, a retroviral vector including sequence for TAP-tag and puromycin resistance. A corresponding GFP-pZome1C was also designed to monitor viral packaging and infection rate of the virus. It is also used as the negative control for the TAP-tag protocol. Both viral constructs were sequenced-verified and used to transfect Phoenix-ampho cells (a generous gift from Garry Nolan, Stanford Univ.(28)) utilizing Lipofectamine^{2000} via manufacturer's instructions (Invitrogen). Following a 24 hour incubation, the media was replaced with fresh growth media and the cells were incubated for an additional 24 hours. Viral particle were collected and purified by centrifugation (650g for 5mins), and filtered using a 0.45 μm membrane (Millipore, Billerica, MA). PH3MT cells ($2x10^5$) were plated on 60 mm

tissue culture dishes 24 hours prior to infection. Prepared virus containing polybrene (5μg/ml) (Sigma) were added to the cells and incubate for 24 hours. Media were replaced and infected cells were selected using puromycin (0.4 μg/ml, USBiologicals, Swampscott, MA).

Western blot analysis and quantitative Real Time -PCR analysis

Total proteins were prepared in lysis buffer (50 mM HEPES, pH7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% Glycerin, 1 mM dithiothritol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor tablet (Roche, Indianapolis, IN)). Nuclear proteins were prepared from cells lysed in Buffer A (10mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 2 mM dithiothritol, 0.2 mM PMSF, 2 mM Na_3VO_4, and protease inhibitor tablet (Roche)) and nuclei were collected by centrifugation (2000g, for 5min). Nuclei pellets were then lysed in Buffer C (0.42 M KCl, 20 mM Tris, pH7.5, 1.5 mM $MgCl_2$, 20% (vol/vol) glycerol, 1 mM EDTA, 0.1 mM dithiothritol, 0.2 mM PMSF, 2 mM Na_3VO_4, and protease inhibitor tablet (Roche)). Antibodies used for Western blot analysis; rabbit polyclonal anti-PHD2 (Novus, Littleton, CO), mouse monoclonal anti-HIF1α (Novus), rabbit polyclonal anti-β-Actin (Sigma, St. Louis, MO), goat anti-rabbit and -mouse secondaries (Sigma).

Total RNA was extracted form cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and integrity were analyzed using UV spectrometry and denaturing gel electrophoresis. 1 μg total RNA was primed

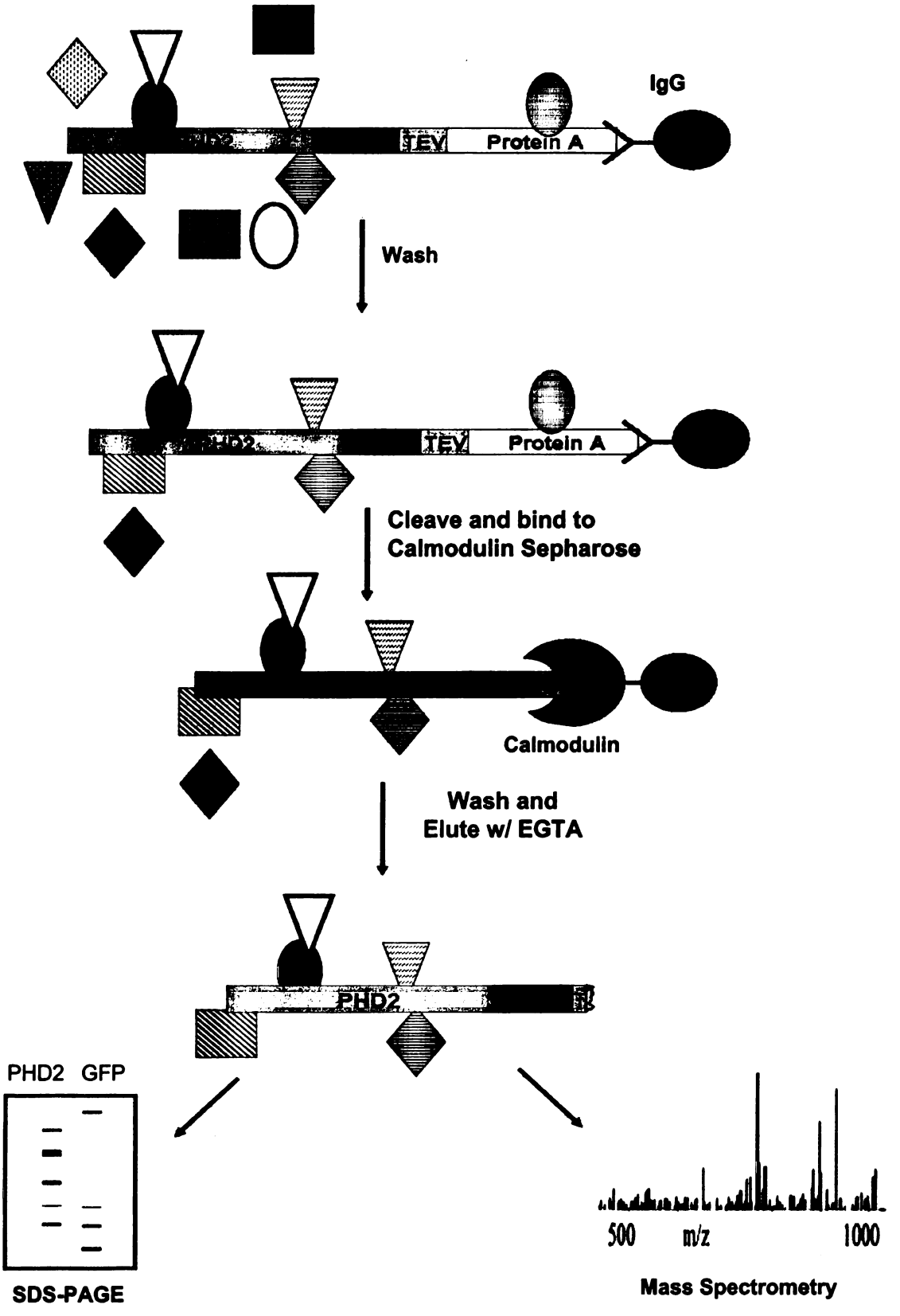
with oligo(dT)$_{18}$ and used for first-strand synthesis with the SuperScript First-Stranded Synthesis System (Invitrogen) as described previously (31). Gene expression was determined using quantitative real time-PCR (qRT-PCR), based on Sybr-Green methodology (Applied Biosystems, Foster City, CA) as previously described (31). Analysis was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers used for qRT-PCR (5' to 3'): VEGF: tcctcacaccattgaaacca and gatcctgccctgtctctctg; GAPDH: cagcctcaagatcatcagca and gtcttctgggtggcagtgat; HPRT: gaccagtcaacaggggacat and cctgaccaaggaaagcaaag. Specificity of each primer set was verified by BLAST and dissociation curve analysis.

Tandem Affinity Purification (TAP)

TAP was performed as outlined in figure 3-1. Briefly, cells were washed three times in ice cold PBS and manually collected from the culture plates in the presence of lysis buffer (10 % glycerol, 50 mM Hepes, pH=8.0, 100 mM KCl, 2 mM EDTA, 0.1 % NP-40, 2 mM DTT, protease inhibitors). Lysates were incubated for 30 min on ice followed by two freeze/thaw cycles and supernatant was purified by centrifugation (10,000g, for 20 min). IgG sepharose was added to cell lysates and incubated for 4 hours (4 °C, agitation). IgG sepharaose was collected by gravity sedimentation and washed 3 times with cold lysis buffer and 3 times with TEV buffer (10 mM Hepes, pH 8.0, 150 mM NaCl, 0.1 % NP-40, 0.5 mM EDTA, 1 mM DTT). The clean beads were then resuspended in TEV buffer containing 1 unit/μl of TEV and incubated for 4 hours at 4 °C. The IgG-separose was separated by centrifugation (600g, for 1min) and the

Figure 3-1. Tandem Affinity Purification.

Cartoon representation of TAP protocol. The TAP-tag PHD2 is purified from total cell lysate using IgG-sepharose. The beads are extensively washed and complex is cleaved from the IgG beads by TEV protease. The released protein complex is then bound to calmodulin-sepharose. Following washing and elution, proteins in the complex are identified by SDS-PAGE and/or mass spectrometry.


IgG
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Wash
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Calmodulin
Wash and
Elute w/ EGTA
PHD2
PHD2 GFP
SDS-PAGE
500
m/z
1000
Mass Spectrometry

supernatant was moved to a clean tube. The IgG-sepharose beads were washed 2 times with calmodulin binding buffer (10 mM β-mercaptoethanol, 10 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazol, 0.1 % NP-40, 2 mM DTT) and the supernatant was collected by centrifugation (600g for 1min) and combined with original sample. Calmodulin-sepharose was washed 3 times with calmodulin binding buffer and added to collected supernatant. $CaCl_2$ was added to a final concentration of 2 mM and incubated for 90 min (4 °C, agitation). The calmodulin-sepharose was washed 3 times with calmodulin binding buffer. Associated proteins were finally eluted from the calmodulin beads either by elution buffer (50 mM NH_3HCO_3, pH=8.0, 25 mM EGTA) or SDS protein sample buffer (0.5 M Tris, pH=6.8, 4.4 % (W/V) SDS, 20 % Glycerol, 2% β-mercapto-ethanol, 0.2 % bromophenol blue).

Trypsinization and Mass spectrometry

The samples being prepared for mass spectrometry were resuspended in 50 mM di-ammonium phosphate (dAP), supplemented with 10 % acetonitrile (final pH =8.0). The solution was supplemented with Tris (2-Carboxyethyl) Phosphine Hydrochloride (TCEP-HCl) and heated to 95 °C for 10 min. The proteins were then incubated in the presence of 15 mM iodoacetamide (1 hour, dark). The iodoacetamide was quenched by the addition of dithiothreitol (final cons.= 5 mM) and the protein mixture was digested with the addition of freshly diluted trypsin (30 ng, 37 °C for 18 hrs). An equal volume of formic acid (5 %) was added to the final peptide mixture and sonicated for 10 mins.

After the digestion has been completed, the peptides were desalted on a 2 cm x

100 μm peptide trap packed with Micron Magic C18 AQ packing material. The bound peptides were flushed onto a 10 c m x 75 mm New Objecteives Picofrit colum packed with a Micro Magic C18 AQ packed material and eluted over 50 mins with a gradient of 5 % B to 70 % B in 45 mins (Buffer A= 0.1 % formic acid, Buffer B= 95 % Acetonitrile 0.1 % formic acid) into a Micromass Qtof Ultima API mass spectrometry with a flow rate of 250 nl/min. The top three ions in each survey scan was subjected to automatic low energy collision induced dissociation (CID) and the resulting uninterpreted MS/MS spectra was searched against an appropriate database using the X!Tandem searching algorithm. Identifications were considered positive if 2 peptides per protein are identified with a significant X!Tandem Score (log(e).-2). If only one peptide is identified, they were subjected to a Mascot cutoff of less than 5 % probability that the match could be considered chance identification. The data were filtered by comparison with the TAP-GFP data set. Those interactions that occur in both data sets were marked as non-specific and removed from further consideration. The screening processes also remove the most common background proteins, such as tubulin, actins and keratins.

RESULTS

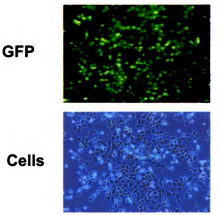
Tandem affinity purification (TAP) was applied as a methodology to characterize the PHD2 protein interaction network. Stable cells expressing TAP-tagged version of PHD2 were created through retroviral infection of PHD2-TAP in human fibrosarcomas, PH3MT cells, tPHD2-3MT cells. GFP-TAP expressing PH3MT cells, tGFP-3MT cells, were also generated to monitor infection (Fig. 3-2A) and to act as a negative control for the TAP procedure. The expression of PHD2- and GFP-TAP protein was verified using Western blot analysis with a PHD2-specific antibody (Fig. 3-2B). TAP-tag proteins are detectable with all antibodies including pre-immune serum because of the presence of the protein A portion at the end of tag. The tPHD2-3MT and tGFP-3MT cells showed a strong band at the appropriate molecular weight of TAP-tagged PHD2 and GFP respectively. In addition, there was a band migrating at the correct molecular weight for endogenous PHD2 across all three cell lines. The expressions of TAP-tagged PHD2 and GFP in tPHD2-3MT cells and tGFP-3MT cells were confirmed by additional Western blot analysis using pre-immune serum (Fig. 3-2B). The pre-immune serum showed a band at the same molecular weight of TAP-tagged PHD2 and GFP as shown in Western blotting with PHD2-specific antibody and it did not show any band in non-infected PH3MT cells. These results show that the newly created tPHD2-3MT and tGFP-3MT cells express significant amount of their respective TAP-tagged proteins.

PHD is a well known controller of HIF1α stability and consequently HIF1-mediated gene expression. To test whether ectopic expression of PHD2 has a functional

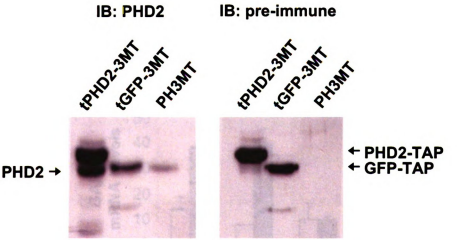
Figure 3-2. Characterization of tPHD2-3MT and tGFP-3MT cells

(A) PH3MT cells were infected with a retroviral construct that expresses the green fluorescent protein (GFP). GFP expressions were observed via fluorescent microscopy. **(B)** The expression of PHD2-TAP and GFP-TAP proteins were characterized in newly created tPHD2-3MT and tGFP-3MT cells by Western blot analysis using PHD2-specific antibodies or pre-immune. The parental PH3MT cell line was included as control. **(C)** HIF1α protein levels were analyzed in tPHD2-3MT and tGFP-3MT cells exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 5 hrs by Western blot analysis. To verify equal loading, the blot was stripped and reprobed with a β-Actin antibody. **(D)** mRNA levels of VEGF and GAPDH were determined in tPHD2-3MT and tGFP-3MT cells using qRT-PCR. Cells were exposed to normoxia (20% O_2, white bar,) or hypoxia (1% O_2, black bar) for 16 hr. (n=8, * $p<0.05$, ** $p<0.01$).

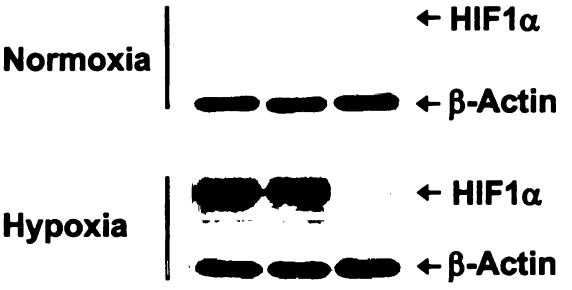
A


GFP
Cells

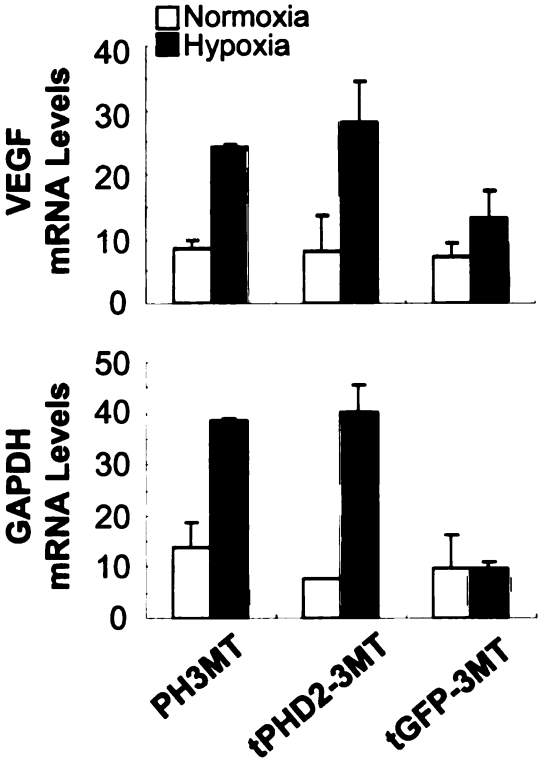
B


IB: PHD2
IB: pre-immune
tPHD2-3MT
tGFP-3MT
PH3MT
tPHD2-3MT
tGFP-3MT
PH3MT
PHD2 →
← PHD2-TAP
← GFP-TAP

C


Normoxia
← HIF1α
← β-Actin
Hypoxia
← HIF1α
← β-Actin

D


Normoxia
Hypoxia
VEGF mRNA Levels
40
30
20
10
0
GAPDH mRNA Levels
50
40
30
20
10
0
PH3MT
tPHD2-3MT
tGFP-3MT

consequence on HIF1 signaling, we first determined HIF1α protein levels (Fig. 3-2C). As expected, HIF1α was not detectable under normoxia and induced by hypoxia in control cells, PH3MT and tGFP-3MT, while hypoxia-induced HIF1α accumulation was inhibited in the tPHD2-3MT cells. In addition, over-expression of PHD2 had a functional consequence on HIF1-mediated transcriptional of VEGF and GAPDH, two classic hypoxia-regulated genes (Fig. 3-2D). The hypoxia-induced expression of these genes was inhibited in tPHD2-3MT cells compared with that in control cells, PH3MT and tGFP-3MT.

To characterize a map for PHD2-protein interaction network, we performed tandem affinity purification (TAP) on each of the newly created cell strains. TAP-tagging was initially developed for use in proteomic analysis of *Saccharomyces cerevisiae* (28). The TAP-tag consists of a *Staphyloccoccus aureus* protein A and a calmodulin binding peptide (CBP), separated by *Tobacco Etch* Virus (TEV) protease site (Fig. 3-1). The TAP strategy has several advantages over conventional purification schemes. It has permitted the rapid identification of protein interacting network with less background contaminants. This approach can also be applied to link proteins of unknown function to known pathways and gain detailed information regarding changes in composition of protein complex that occur in different stimuli, e.g., various oxygen concentrations. To identify the PHD2-PIN, TAP analysis was performed with tPHD2-3MT cells. As a control, tGFP-3MT cells were used to exclude non-specific proteins. Each purification step was verified using Western blot analysis with a PHD2-specific

antibody (Fig. 3-3). GFP-TAP protein can be seen by PHD2-specific antibody due to the presence of the Protein A portion of the TAP-tag. After TEV protease cleavage, tPHD2-3MT cells showed a band (PHD2-CBP) migrating lower than PHD2-TAP and tGFP-3MT cells did not show band due to the loss of Protein A part of the tag (Fig. 3-3).

To profile protein complex associated with PHD2, final elutes were separated using Bis-Tris gradient (4-20%) gel electrophoresis and visualized by silver staining (Fig 3-4A). The PHD2 and GFP band can be seen in tPHD2-3MT and tGFP-3MT cells, respectively, and were confirmed by mass spectrometry. The tPHD2-3MT cells, however, did not showed any significant difference on the profile of protein bands compared with those in tGFP-3MT cells (Fig 3-4A). The individual bands were analyzed by mass spectrometry and the results suggested that most were non-specific contaminants (Table 3-1). In an attempt to limit the amount of non-specific contamination, associated proteins were also eluted under mild condition with optimized elution buffer and results showed no-specific protein band (Fig 3-4B). Since gel electrophoresis and silver staining have certain limitations, we next attempted an on-bead trypsinization protocol for PHD2 and GFP and digests were directly analyzed by MS. Again, no specific, reproducible proteins were identified (Table 3-2 and 3-3). Finally, we also analyzed the supernatant from the post-TEV protease treatment to determine if we might be losing specific proteins with the Calmodulin sepharose. The results from this fraction were similar to the previous attempts (Table 3-4).

Figure 3-3. Verification of Tandem Affinitu Purification (TAP).

Individual steps of TAP protocol were verified using Western blot analysis with PHD2-specific antibody.

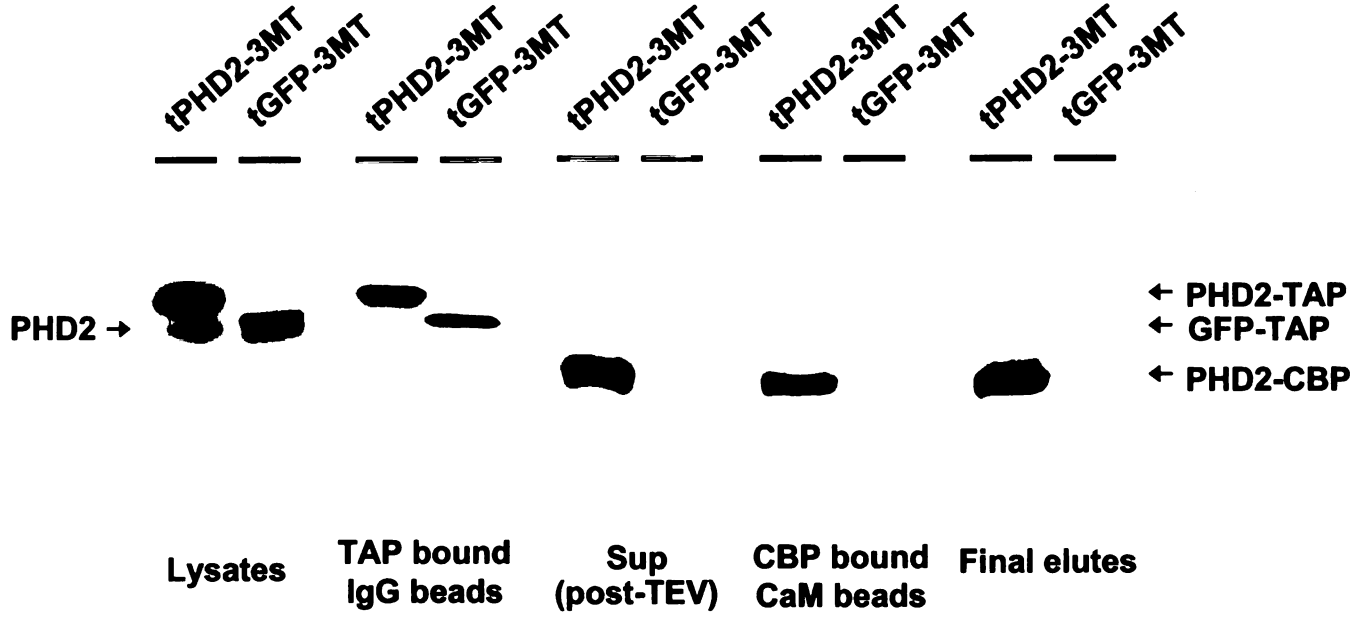

tPHD2-3MT
tGFP-3MT
tPHD2-3MT
tGFP-3MT
tPHD2-3MT
tGFP-3MT
tPHD2-3MT
tGFP-3MT
tPHD2-3MT
tGFP-3MT
PHD2 →
← PHD2-TAP
← GFP-TAP
← PHD2-CBP
Lysates
TAP bound IgG beads
Sup (post-TEV)
CBP bound CaM beads
Final elutes

Figure 3-4. Protein profile of TAP-tagged purified PHD2

(A) PHD2-associated proteins were isolated via Tandem Affinity Purification. Calmodulin beads were eluted with 2xSDS protein sample buffer and proteins were separated with Bis-Tris gradient gel (4-20%) and stained with silver. **(B)** PHD2-protein complex were purified using TAP protocol and eluted with elution buffer.

A
tGFP-3MT
tPHD2-3MT
* 1
* 2
* 3
* 4
* 5
* 6
PHD2
GFP
B
tGFP-3MT
tPHD2-3MT
PHD2
GFP

Table 3-1. Identification of proteins within individual band in Figure 3-4A using mass-spectrometry.

Number in a silver stained gel (Fig. 3-4A)	Protein Identified using Mass-Spectrometry
1	gi_34527698 unnamed protein product (Homo sapiens) gi_34534305 unnamed protein product (Homo sapiens) gi_34526220 unnamed protein product (Homo sapiens) gi_50949554 hypothetical protein (Homo sapiens) gi_34527453 unnamed protein product (Homo sapiens) gi_40795897 hornerin precursor (Homo sapiens) gi_18031805 estrogen-induced tag 6 (Homo sapiens)
2	gi_28317 unnamed protein product (Homo sapiens) gi_6470150 BIP protein (Homo sapiens)
3	gi_28317 unnamed protein product (Homo sapiens) gi_908801 keratin type II gi_21961227 keratin 6B (Homo sapiens) gi_307086 keratin-10 gi_18999435 KRT5 protein (Homo sapiens)
4	gi_55665459 tublin beta 5 (rattus norvegious) gi_11935049 keratin 1 (Homo sapiens) gi_18999435 KRT5 protein (Homo sapiens) gi_10433717 unnamed protein product (Homo sapiens) gi_34527453 unnamed protein product (Homo sapiens)
5	gi_55665459 egl nine homolog 1 (Homo sapiens) **"PHD2"** gi_1346343 keratin type II cytoskeletal 1 (cytokeratin 1) gi_435476 cytokeratin 9 (Homo sapiens) gi_28317 unnamed protein product (Homo sapiens)
6	gi_1373325 Enhanced Green Fluorescent protein **"GFP"** gi_14595132 Raichu404X (Homo sapiens) gi_11935049 keratin 1 (Homo sapiens)

Table 3-2. Protein profile of TAP-tagged purified PHD2 (on-bead trypsinizing)

Number	Description
gi_825635	calmodulin (Homo Sapiens)
gi_66360504	Chain T, Crystal Structure of Anthrax Bdena actor (Ef) In Complex with Calmodulin
gi_61680528	Chain A, Trapped Intermediate of Calmodulin
gi_16974825	Chain A, Solution Structure of Calcium-Calmodulin N-Terminal Domain
gi_31092	unnamed protein product (Homo Sapiens)
gi_55665459	egl nine homolog 1 (Homo Sapiens) **"PHD2"**
gi_56090271	ribosomal protein S20 (Rattus norvegicus)
gi_229585	Ig A1 bur
gi_16554039	unnamed protein product (Homo Sapiens)
gi_1297274	beta-tubulin (Hopmo Sapiens)
gi_16554039	unnamed protein product (Homo Sapiens)
gi_1143492	BIP (Homo Sapiens)
gi_21669491	immunoglobulin lambda light chain TLJ region (Homo Sapiens)
gi_56789800	MOC27165 protein (Homo Sapiens)
gi_11935049	Keratin 1 (Homo Sapiens)
gi_67664801	hypothetical protein bcen2424DRAFT_3653
gi_7648673	Voltage-gated potassium channel Ev4.2 (Homo Sapiens)
gi_34069	unnamed protein product (Homo Sapiens)
gi_46254043	Immunoglobulin heavy chain (Homo Sapiens)
gi_106586	Ig kappa chain V-III (KAU cold agglutinin) - human

Table 3-3. Protein profile of TAP-tagged purified GFP (on-bead trypsinizing)

Number	Description
gi_825635	calmodulin (Homo Sapiens)
gi_16974825	Chain A, Solution Structure of Calcium-Calmodulin N-Terminal Domain
gi_61680528	Chain A, Trapped Intermediate of Calmodulin
gi_1143492	BIP (Homo Sapiens)
gi_66360504	Chain T, Crystal Structure of Anthrax Bdena actor (Ef) In Complex with Calmodulin
gi_62896605	eukaryotic translation elongation factor 1α 1 variant (Homo Sapiens)
gi_11935049	keratin 1 (Homo Sapiens)
gi_1297274	beta tubulin (Homo Sapiens)
gi_20809886	tubulin, beta, 2 (Homo Sapiens)
gi_18088719	tubulin beta polypeptide (Homo Sapiens)
gi_56090271	ribosomal protein S20 (Rattus norvegicus)
gi_229585	Ig A1 Bur
gi_181402	epidermal cytokeratin 2 (Hopmo Sapiens)
gi_42744612	unkown (protein for MOC:70893) (Homo Sapiens)
gi_16554039	unnamed protein product (Homo Sapiens)
gi_1373325	Enhanced Green Fluorescent Protein (Cloning vector pEGFP-N3) **"GFP"**
gi_31874190	hypothetical protein (Homo Sapiens)
gi_386850	keratin K5
gi_31074633	keratin 6L (Homo Sapiens)
gi_230161	Chain X, immunoglobulin heterologous light chain diner (MCG-WEIR Hybrid)
gi_15193213	anti-hepatitis B surface antigen immunoglobulin heavy chain (Homo Sapiens)
gi_90883	keratin type II
gi_6981490	ribosomal protein S29 (Rattus norvegicus)

Table 3-4. Protein profile of TAP-tagged purified PHD2 (TEV sup)

Number	Description
gi_825635	calmodulin (Homo Sapiens)
gi_66360504	Chain T, Anthrax Bdena actor (Ef) In Complex with Calmodulin
gi_61680528	Chain A, Trapped Intermediate of Calmodulin
gi_16974825	Chain A, Solution Structure of Calcium-Calmodulin N-Terminal Domain
gi_62896605	Eukaryotic translation elongation factor 1α variant (Homo Sapiens)
gi_55665459	egl nine homolog 1 (Homo Sapiens) **"PHD2"**
gi_229601	Ig G1 N Nie
gi_134634	Keratin, type II cytoskeletal 1 (cytokeratin 1) (K1) (CK1)
gi_46254043	Immunoglobulin heavy chain (Homo Sapiens)
gi_34526073	unnamed protein product (Homo Sapiens)
gi_1143492	BIP (Homo Sapiens)
gi_56090271	ribosomal protein S20 (Rattus norvegicus)
gi_37492	Alpha-tubulin (Homo Sapiens)
gi_7648673	Voltage-gated potassium channel KV 4.2 (Homo Sapiens)
gi_61680025	Chain I, HIV-1 neutralizing human rab 4e10 in complex with A 13-residue peptide
gi_67664801	Hypothetical protein BCEN2424DRART_3653
gi_37777889	Immunoglobulin heavy chain variable region (Homo Sapiens)
gi_34526391	unnamed protein product (Homo Sapiens)
gi_18031805	Estrogen-induced tag 6 (Homo Sapiens)
gi_62739981	DKFIP547r 072 protein (Homo Sapiens)
gi_34537233	Unnamed protein (Homo Sapiens)
gi_34191054	Hypothetical proteinFIJ 23878 (Hopmo Sapiens)
gi_509798	IgG heavy chain, variable region, rheumatoid factor (Homo Sapiens)
gi_37694545	Immunoglobulin heavy chain variable region (Homo Sapiens)
gi_7513045	Hypothetical protein KIAA0627-human (fragment)
gi_30749422	ChainA, rbe fibrillin-1 cbegt12-13 fair of ca2+ binding epidermal growth factor-like
gi_639910	Heat shock protein
gi_11935049	Keratin 1 (Homo Sapiens)
gi_3451240	2'-2' oligoadenylate synthase (p59OAC) (Homo Sapiens)
gi_67939669	Sulfate transporter.antisigma-factor antagonist Srac:sulfate transporter
gi_67752988	COG0583:Transcriptional regulator (surkholderia pseudonallei Pasteur)
gi_67763452	COG3795:uncharacterized protein conserved in bacteria
gi_18204970	Tubulin tyrosine ligase-like family member 4 (Homo Sapiens)

DISCUSSION

The PHDs regulate HIF-mediated transcriptional response by promoting HIF1α degradation through the ubiquitin-proteasome pathway. PHDs belong to the iron and α-ketoglutarate dependent oxygenase family and require oxygen, iron, α-ketoglutarate, and ascorbate for their proper activity. In mammals, three PHD isoforms, PHD1, 2, and 3, have been identified and PHD2 is recognized as the main functional isoform in regulating hypoxia signaling in humans. Recently, several studies have suggested a role for other proteins and/or signaling pathways in influencing PHDs activity (1, 2, 27). In addition, it has been implicated that mitochondria generated reactive oxygen species (ROS) and several metabolic intermediates contribute to PHD activity (8, 11, 19, 21, 29). However, the mechanisms underlying these links have not been characterized and one possibility is that additional proteins and/or pathways may be involved in this crosstalk. In this study, we characterize PHD2-protein interaction network on the composition of protein complex using tandem affinity purification (TAP) and mass spectrometry. Our result suggested that PHD2 might have no specific interaction with other proteins under normoxia and that PHD2 might act to coordinate the various input signals without the aid of accessory factors.

Previously, it has been reported that two proteins, OS-9 (3) and FKBP38 (2), interact with PHD2 and play a role in regulating its activity. However, both studies were performed using a yeast-two hybrid under certain condition (1, 2). PHD2-mediated oxygen sensing mechanism is susceptible to change of oxygen levels and

should be controlled tightly not to cause any adverse effects mediated by hypoxia signaling. It is possible that the interaction between PHD2 and other proteins might change very rapidly within cells under normoxia to control the influence of the various input signals on PHD activity. This type of rapid turnover would make it difficult to identify the PHD2-PIN *in vivo*. It is also possible that PHD2 might interact with other proteins and/or signaling pathways in a cell type specific manner. The normal oxygen tension varies in cells within and among different tissues (6, 16) and PHD2-mediated regulation of HIF1 signaling may need more complexity in certain cell types than others. This possibility suggests the requirement of analyzing PHD2-PIN in other cell lines. In addition, PHDs hydroxylate HIF1α only under normoxia and are inhibited by hypoxia leading to HIF1α stabilization and activation. Although any specific protein associated with PHD2 have not been identified in human fibrosarcomas, PH3MT cells, under normoxia, it is possible that there may be changes in the compostion of the PHD2-PIN following exposure to different stimuli, e.g., hypoxia. It is therefore necessary to investigate the changes in the PHD2-PIN under various stresses, such as hypoxia, oxidative stress and TCA cycle inhibition. Characterizing the PHD2-PIN in various tissues under different conditions will provide insight on mammalian oxygen sensing mechanism and will also help target this pathway for therapeutic agent.

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CHAPTER 4

SUMMARY

The ability to adapt to a hypoxic environment is critical to a cancer cell's survival and ability to form tumors. Central to this adaptation is the HIF1-mediated signaling system. HIF1 regulates the expression of a wide variety of genes essential to the adaptive response, including cell proliferation, angiogenesis, anaerobic metabolism, migration and many others (6, 16, 17, 19). During tumor development a cell is capable of modulating HIF1 activity to cope with transient changes in oxygen availability. This adaptability is also important for attempting to re-establish normoxia for normal cells within an organ during hypoxic stress. The adaptive response, however, is not always capable of addressing the hypoxic-induced cellular imbalance. In this case, the cells begin modulating various cell death pathway presumably as a mechanism to eliminate irrecoverably stressed cells and maintaining the tissue as a whole (8, 18). Removal of cells under severe hypoxic stress by programmed cell death could increase the chance of survival for neighboring cells by increasing nutrients and oxygen supply and maintaining appropriate tissue architecture. This cell death pathway involves HIF1-dependent transcription of pro-apoptotic B-cell chronic lymphocytic leukemia (CLL)

/lymphoma (BCL) family members, such as BCL2/ adenovirus E2B 19 kDa interacting protein 3 (BNIP3) and NIX (9, 15, 20). The activation of HIF1 is also able to contribute to the p53-mediated cell cycle arrest and cell death (1, 5). This increased "suicide" response seems in opposition to the adaptive response described above. The HIF1 signaling cascade, therefore, can promote either cell survival through adaptation or cell death. One major unanswered question concerning HIF1 signaling is how this balance between adaptation and cell death is established and maintained. How does a cell know when it should survive through adaptive measures or when it needs to die because it cannot cope with the hypoxic environment? The intracellular regulatory system that makes this decision probably involves numerous factors, including oxygen levels of the cell, metabolic state and levels of key metabolites, growth factor, and the endogenous activity of the PHDs.

In this study, we have demonstrated that PHD activity can serve as a master regulator of a cell's fate under hypoxic stress by the same mechanism that it uses to establish normoxia (Fig. 4-1). For example, under normal oxygen concentration, PHDs enzymes are at maintenance levels, that is, their levels are maintained at those required

Figure 4-1. Relationship between PHD activity and HIF1 target gene expression.

At normal oxygen tensions, the PHDs are capable of initiating the degradation of HIF1a ad expression of HIF1 target genes invovlved in glycolysis, angiogenesis, and apoptosis are maintained at low levels (**top panels**). As the oxygen concentration becomes moderately decreased, PHD activity is diminished for lack of available substrate and some HIF1 accumulates and drives the expression of target genes. These genes include angiogenic factors and glycolytic enzymes, levels of which will be recalibrated to the new metabolic conditions and thus encoding a new "normoxia" (**middle panels**). Finally, under severe hypoxia, PHD activity is incapable of compensating for the loss of oxygen and HIF1 signaling becomes hyper-activated, leading to expression of pro-apoptotic factor and cytotoxicity (**bottom panels**).

Activity of the PHD Enzyme
Normoxia
Mild Hypoxia
Severe or Prolonged Hypoxia
Cellular pO2
Time
Glycolytic
Apoptotic
Angiogenic
Under normal oxygen tension the HIF1 target genes are maintained at basal levels
Small decrease in oxygen tension is compensated for by adaptive responses
Large decrease in available oxygen cannot be compensated leading to apoptotic responses

for normal cellular function and oxygen-sensing capacity (Fig. 4-1, top panel). When the cell is exposed to moderate hypoxia, the activity of PHDs is slightly decreased and consequently induced HIF1 signaling provide adaptive responses such as increased glycolysis and agiogenesis to tumor cells to cope with the detrimental environment (Fig. 4-1, middle panel). This adaptive response can also promote normal cells to gain growth advantages and become malignantly transformed. It is possible that cell death factors are upregulated in the short term; however, once the HIF1 activity is increased in the cell and the energy balance is restored, these genes are turned off and cell death will be avoided. It is likely that these genes are not expressed under moderate hypoxia from which the cell can recover. Under severe hypoxic stress, HIF1-mediated adaptive responses are unable to compensate for the loss in available oxygen (Fig. 4-1, bottom panel). At these oxygen levels, PHD is inhibited and this leads to the HIF1α-mediated pro-death response becoming dominant and this unmitigated expression leads to cell death. These observations suggested that tumor development is dependent upon its ability to induce intracellular adaptive response to hypoxia without supporting pro-cell death mechanisms and PHDs play an essential role in setting up the boundary between cell survival and cell-death.

Targeting HIF1 signaling for cancer therapy, therefore, must be done with the appropriate concern as to the impact these new drugs might have on the HIF1-regulated balance between cell and tissue survival and programmed cell death. The research presented here suggests that the boundary between too much and not enough HIF1 activity is an important determinant of our ability to cope with pathological conditions. Moreover, this boundary can shift a cell towards a transformed phenotype or drive a transformed cell towards cell death. Exploiting HIF1 signaling for therapeutics will require an understanding of how this balance will be shifted in the various tissues and cells and under various environmental conditions. For example, increases in HIF1 activity might promote cellular transformation in certain cells that have amassed the cellular mutation necessary to progress toward complete transformation, i.e. MSU 1.1., but a similar increase in another cell might promote cell death, i.e. PH3MT. Alternatively, new therapeutics that completely shut down PHD activity will have detrimental consequence on normal cellular homeostasis by promoting an uncontrolled pro-death response. Ideally, any drug targeting this pathway should be cell type specific and capable of manipulating PHD activity within this target cell with limited chance of driving other cells towards transformation. Our study implies that cells will have a

biphasic response to agents manipulating PHD/HIF1 signaling and if this is the case, it will create difficulties when targeting this pathway for cancer therapeutics. A complete understanding of normal oxygen levels, endogenous HIF1 signaling, metabolic flux, and PHD activity in a specific tissue will be critical to our ability to manipulate the system in a meaningful therapeutic sense.

In this study, we also characterized the PHD2 protein interaction network (PHD2-PIN) to understand the possible influence of other proteins and/or signaling pathway on PHD2 activity. The responses to hypoxia include various cellular mechanisms, for example respiratory chain and TCA cycle in mitochondria and anaerobic metabolism (10, 11, 13, 14). Recently it has been demonstrated that these responses can also affect HIF1 signaling through regulating PHDs activity. In addition, a link between mitochondria generated reactive oxygen species (ROS) and PHD activity has been established. Although several studies have implicated the role of other signaling pathways in PHD activity, the mechanisms underlying the link between them have not been characterized and one possibility is that additional proteins and/or pathways may be involved in this crosstalk. Characterizing the precise mechanism and

regulation of these enzymes, therefore, is essential to understand physiological and pathological responses to hypoxia. Herein, we attempted to characterize a map for the PHD2-PIN using tandem affinity purification (TAP) and liquid chromatography coupled mass spectrometric analysis (LC-MS/MS). Our result suggested that PHD2 might have no specific interaction with other proteins in human fibrosarcomas under normoxia and that PHD2 might act to coordinate the various input signals without the aid of accessory factors. Although any specific protein associated with PHD2 have not been identified in PH3MT cells under normoxia, it is possible that there may be changes in the PHD2-PIN following exposure to different stimuli in a cell type specific manner. Characterizing the PHD2-PIN in various cell types and under different conditions, such as hypoxia, oxidative stress and TCA cycle inhibition, will provide insight on mammalian oxygen sensing mechanism for HIF1 signaling and will also help target this pathway with therapeutic agents for certain diseases that have hypoxia as a major component of their pathophysiological progression, such as cancer.

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APPENDIX A

IDENTIFICATION AND CHARACTERIZATION OF GENES SUSCEPTIBLE TO TRANSCRIPTIONAL CROSS-TALK BETWEEN THE HYPOXIA AND DIOXIN SIGNALING CASCADES

This chapter represents a manuscript that was published in Chem. Res. Toxicol. 19: 1284-1293 (2006). Authors included: KangAe Lee, Lyle D. Burgoon, Laura Lamb, Edward Dere, Timothy R. Zacharewski, John B. Hogenesch, and John J. LaPres.

ABSTRACT

The aryl hydrocarbon receptor (AHR) and hypoxia inducible factors (HIFs) are transcription factors that control the adaptive response to toxicants such as dioxins and decreases in available oxygen, respectively. The AHR and HIFs utilize the same heterodimeric partner, the aryl hydrocarbon nuclear translocator (ARNT) for proper function. This requirement raises the possibility that cross-talk exists between these critical signaling systems. Single gene and reporter assays have yielded conflicting results regarding the nature of the competition for ARNT. Therefore, to determine the extent of cross-talk between the AHR and HIFs, a comprehensive analysis was performed using global gene expression analysis. The results identified 767 and 430 transcripts that are sensitive to cobalt chloride and 2,3,7,8-tetrachlorodibenzo-F-dioxin (TCDD) stimulation, respectively, with 308 and 176, respectively, exhibiting sensitivity to crosstalk. The overlap between these two sets consists of 33 unique transcripts, including the classic targetgenes CYP1A1, carbonic anhydrase IX, and those involved in lipid metabolism and coagulation.Computational analysis of the regulatory region of these genes identified complex relationships betweenHIFs, AHR, and their respective response elements as well as other DNA motifs, including the SRF, Sp-1, NF-kB, and AP-2 binding sites. These results suggest that HIF-AHR cross-talk is limited to genes with regulatory regions that contain specific motifs and architectures.

INTRODUCTION

The PAS (named for founding members; PER, ARNT1, SIM) family of transcription factors act as sensors for various environmental stimuli, including hypoxia and specific classes of pollutants (1). As transcription factors, their principal reaction involves the modulation of gene expression that ultimately promotes an adaptive response to these stimuli. PAS transcription factors generally function as heterodimers that can have both cytosolic and nuclear components. The cytoplasmic component generally acts as a sensor for environmental stimuli and includes the aryl hydrocarbon receptor (AHR) and the alpha subunit of the hypoxia inducible factors (HIF1-3) (2, 3). Once activated, these factors translocate to the nucleus and interact with the second class of the superfamily, the nuclear component, such as the aryl hydrocarbon nuclear translocator (ARNT, also known as HIF1α) (4, 5). ARNT/HIF1α is the predominant binding partner for the AHR and HIF1α, and therefore, ARNT might act as a point of competition or cross-talk following the activation of the AHR and HIF1α.

The AHR is a ligand activated transcription factor that responds to a broad range of planar aromatic hydrocarbons (6). Classic AHR ligands include environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-F-dioxin (TCDD), naturally occurring compounds such as indole-3-carbazole, and endogenous ligands such as tryptophan metabolites. In the absence of a ligand, the cytosolic AHR is bound to the immunophilin-like protein, aryl hydrocarbon receptor-associated protein (ARA9) and a dimmer of heat shock protein 90 (Hsp90) (7, 8). Upon binding a ligand, the AHR translocates to the nucleus where it heterodimerizes with ARNT. The transcriptionally

active AHR-ARNT complex drives the expression of genes containing dioxin response elements (DREs, core sequence = GCGTG) such as the canonical AHR-responsive gene, cytochrome P450, family 1, subfamily A, polypeptide 1 (Cyp1A1) (9).

Hypoxia is defined as a decrease in available oxygen reaching the tissues of the body. The cellular response to hypoxia is a fine balance between adaptation and cell death and is primarily controlled by HIF1α (10). HIF1α is a cytosolic protein whose stability is regulated by a family of prolyl hydroxylases (11, 12). These hydroxylases are oxygen dependent sensors for the hypoxia-signaling cascade either directly or indirectly through changes in reactive oxygen species generated from complex III of the electron transport chain (13-16). In the presence of oxygen, HIF1R is hydroxylated and degraded via the Von Hippel Lindau tumor suppressor protein and the 26S proteosome pathway (17). In the absence of sufficient oxygen, the hydroxylase is inactive and the HIF1α protein becomes stabilized and translocates into the nucleus where it interacts with ARNT to drive the expression hypoxia response elements (HREs, core sequence = (G/A)CGTG) containing genes (18). Classic hypoxia inducible genes include vascular endothelial growth factor (VEGF), erythropoietin, and most of the glycolytic enzymes (19, 20).

The ability of ARNT to act as the heterodimeric partner for both HIF1α and AHR raises the possibility of cross-talk between these signaling cascades. In addition, the similarities between the HRE and DRE core sequences suggests HIF1R-ARNT and AHR-ARNT might compete for the same regulatory sequence (21). Consequently, hypoxia-AHR cross-talk might have profound consequences on treatments aimed at hypoxic targets such as tumors and might influence the ability of the AHR to regulate

the expression of various drug metabolizing enzymes. Previous *in vitro* and *in vivo* studies have shown varying degrees of competition between AHR and HIF signaling systems, presumably because of competition for ARNT or another critical cofactor (22-*26*). We hypothesized that this cross-talk may only apply to target genes that harbor select response element combinations that include but are not limited to DREs and HREs. Global gene expression analysis was performed in the human hepatoma HepB3 cells following exposure to TCDD, cobalt chloride (CoCl2), and their cotreatment. A set of genes responsive to cobalt or TCDD, with a subset influenced by cotreatment was identified. Computational analysis of the regulatory regions of a subset of these genes, influenced by individual treatment, identified motifs associated with TCDD or cobalt chloride modulated gene expression. The results suggest that cross-talk between the AHR and HIF1 signaling systems in Hep3B cells is not regulated by ARNT levels and is limited to a group of genes with specific promoter architecture.

EXPERIMENTAL PROCEDURES

Cell Culture. Hep3B cells were maintained in αMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 20 mM L-glutamine, 1 mM MEM nonessential amino acids, 100 mM Hepes (pH 7.4), 1000 units/Ml penicillin G and 1000 ίg/mL streptomycin sulfate (Invitrogen). Cells were approximately 70% confluent at the time of treatment. Cells were maintained at 37 °C, 5% CO_2, and 21% O_2 prior to treatment. 2,3,7,8-tetrachlorodibenzo-F-dioxin (TCDD, 10 nM) and cobalt chloride (100 μM) treatments were performed for 20 h at 37 °C, 5% CO_2, and 21% O_2. These doses for cobalt and TCDD were chosen for their ability to activate HIF1α and the AHR, respectively. Cobalt chloride has an established history as a hypoxic mimic and the chosen dose has a demonstrated ability to stabilize HIF1α and activate all classic HIF1α target genes, including VEGF and the glycolytic enzymes. The dose of TCDD was chosen for its ability to maximally activate the AHR (several times its *K*d). Each treatment group was supplemented with dimethyl sulfoxide (DMSO) to a final concentration of 0.01%. Samples were compared to vehicle control (0.01% DMSO).

RNA Extraction. Following treatment, duplicate cell samples were removed from the tissue culture dish by trypsinization and washed in PBS (4 °C). RNA was extracted by homogenization (Polytron, Kinematica, Lucerne, Switzerland) in TRIzol reagent (GIBCO/BRL, Gaithersburg MD) (added to cell pellet) at maximum speed for 90-120 s. Following a 5 min room temperature incubation, a 1/5 volume of chloroform was added, agitated, and subjected to centrifugation at 12 000*g* for 15 min. The aqueous phase was removed, and the RNA was precipitated upon the addition of a half

vol of isopropanol. The RNA was furthered purified with the RNAeasy Total RNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's specifications. Finally, the purified total RNA was eluted in 10 μL of diethylpyrocarbonate (DEPC) treated H2O, and quantity and integrity were characterized using a DU640 UV spectrophotometer (Beckman, Fullerton, CA) and a Bioanalyzer 2100 (Agilent, Palo Alto, CA).

RNA Labeling. Five micrograms of total RNA from two separate biological replicates were used to make first strand cDNA using the Superscript Choice system (Gibco/BRL) and a T7 promotor/ oligo-dT primer (Gibco/BRL). Second strand cDNA was also made with the Superscript Choice system (Invitrogen). The resulting cDNA was subjected to phenol/chloroform purification, ammonium acetate precipitation and used as a template to make biotinylated amplified antisense cRNA using T7 RNA polymerase (Enzo kit, Affymetrix, Santa Clara, CA). Twenty micrograms cRNA was fragmented to a range of 20 to 100 bases in length using fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) and heating for 35 min at 94 °C. The quality of cRNA and the size distribution of fragmented cRNA were examined by both agarose and polyacrylamide gel electrophoresis.

Hybridization. cRNA (20 μg) was hybridized to a U95A version 1 gene chip (Affymetrix) with 1 x 4-morpholineethanesulfonic acid (MES) hybridization buffer using standard protocols outlined in the Gene Chip Expression Analysis technical manual (Affymetrix). Hybridization was conducted in a GeneChip Hybridization Oven for 16 h at 45 °C. Following hybridization, the arrays were washed on a Genechip Fluidics Station 400 according to manufacturer's instructions (Affymetrix). The arrays

were scanned using a Hewlett-Packard 2500A Gene Array Scanner, and the raw images were visually inspected for defects, proper grid alignment, and converted into CEL files using the MAS5 Software Suite (Affymetrix). Finally, the quality of cRNA was assessed by examining 3′/ 5′ ratios for GAPDH oligonucleotides present on the arrays.

Data Analysis. Background subtraction and single intensity measure for each transcript was derived from multiple probe sets by means of the GCRMA algorithm using the full model tag in R (http://www.r-project.org). The GCRMA algorithm was chosen for its performance in reporting low and high level expression over other methods as well as its dynamic range for single probe sets (27-29). Differentially expressed genes that are statistically significant were determined by analysis of variance (ANOVA). Fold change calculations were performed in Excel on data that was median-scaled to a global intensity target value of 100. For each treatment versus control condition, genes that changed were assigned on the basis of a P value of <0.05 and an absolute fold change value of >1.5.

Quantitative Real-Time PCR (qRTPCR) Analysis. Changes in gene expression were observed by microarray analyses and verified by real-time PCR performed on an Applied Biosystems Prism 7000 Sequence detection System (Foster City, CA) as previously described (30). Analysis was performed on triplicate samples that were treated in a way identical to those used in the GeneChip experiments. Briefly, cDNA was synthesized from total RNA (1 μg per sample per treatment, $n = 6$) in a reverse transcriptase reaction in 20 μL of 1x First Strand Synthesis buffer (Invitrogen) containing 1 μg of oligo (5′-T21VN-3′), 0.2 mM dNTPs, 10 mM DTT, and 200 IU of

Superscript II reverse transcriptase (Invitrogen). The reaction mixture was incubated at 42 °C for 60 min and stopped by incubation at 75 °C for 15 min. Amplification of cDNA (1/20) was performed using SYBR Green PCR buffer (1 x AmpliTaqTM Gold PCR Buffer, 0.025 U/mL AmpliTaqTM Gold (Perkin-Elmer, Wellesley, MA), 0.2 mM dNTPs, 1 ng/μL 6-carboxy-*X*-rhodamine, 1:40 000 diluted SYBR Green Dye and 3% DMSO) and 0.1 μM primers. The thermal cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Prior to analyzing the samples, standard curves of purified, target-specific amplicons were created. Briefly, gene specific oligonucleotides were used to PCR amplify the gene product from a pooled sample of prepared cDNA, the concentration of the amplicons was determined by UV spectophotometery, and a standard curve was created (102-108 copies). The mRNA expression for each gene was determined in comparison to its respective standard curve. This measurement was controlled for RNA quality, quantity, and RT efficiency by normalizing it to the expression level of the β-Actin gene. Each primer set produced a single product as determined by melt-curve analysis, and amplicons were of the appropriate size, as analyzed by agarose gel electrophoresis. Statistical significance was determined using normalized fold changes and ANOVA. Primers were designed using the web-based application Primer3 (http://www-genome.wi.mit.edu/cgi-bin/ primer/primer3_www.cgi) biasing toward the 3′ end of the transcript to maximize the likelihood of giving a gene specific product. The settings used in Primer3 were 125 base-pair amplicon, 20mers, 60 °C melting temperatures, and all other as defaults. Primer sequences were analyzed by BLAST. Gene names, accession numbers, and forward and reverse primer sequences are listed

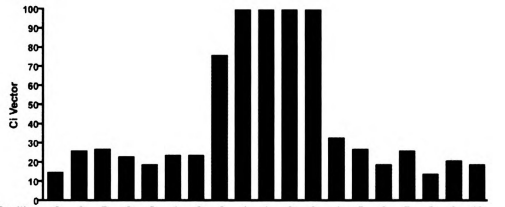
in Table A-1.

Computational Scanning for DREs and HREs. Computational identification and matrix similarity (MS) scores (31) of putative HREs and DREs were performed using 19 base-pair position weight matrices (PWMs) (Fig. A-1A and -1B) as previously developed by Sun et al. (32). The PWMs were constructed using the sequences of reported functional response elements that were positive in either electrophoretic mobility shift or transient transfection assays. In total, 14 DREs (Fig. A-1C) and 38 HREs (Fig. A-1D) were used in the development of the PWMs. Each matrix consists of the 5 base-pair core HRE or DRE, (G/A)CGTG (Fig. A-1A) and GCGTG (Fig. A-1B), respectively, and the adjacent variant 7 base-pair flanking sequences of the bona fide functional response elements. The sequences of these known response elements were subsequently scanned using the PWMs to determine the threshold MS scores (0.818 for DREs and 0.813 for HREs) (*32*). The MS scores for the 14 functional DREs and 38 HREs are listed in Figure A-1C and -1D, respectively. Furthermore, the consensus index (*C*i) vector was calculated for both the HRE and DRE PWMs, where the *C*i vectors represents the conservation of the individual nucleotide positions in the matrices (*31*). A complete list of information regarding the sequences used in creating the PWMs can be found in Supporting Information, Table A-1 and Sun et al. 2004. The genomic sequences (-5000 base-pair to the transcriptional start site (TSS)) and the 5′ untranslated region (UTR)) of the 33 RefSeq genes were extracted from the University of California, Santa Cruz (UCSC) Genome Browser (http://www.genome.ucsc.edu) (Build #35) and scanned for exact matches to the DRE and HRE core sequences on both the positive and negative strands

Figure A-1. HRE and DRE position weight matrices and the sequences used in their construction.

The position weight matrices were created as described in Experimental Procedures. The HRE (**A**) and DRE (**B**) PWMs are displayed relative to the G of the second half of the binding site. The genes and sequences used for the creation of the DRE (**C**) and HRE (**D**) PWMs and their respective MS scores are listed. Sequence information regarding HREs used in construction of PWM can be found in Supporting Information, Table 1.

A



Position	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10
A	31	8	10	20	20	23	8	90	0	0	0	0	10	10	10	2	23	15	15
C	18	23	10	28	44	31	15	0	100	0	0	0	62	23	28	31	28	31	34
G	28	51	49	44	18	41	41	8	0	100	0	100	18	54	36	31	26	41	36
T	23	18	31	8	18	5	36	2	0	0	100	0	10	13	26	36	23	13	15
CI	15	26	27	23	19	24	24	76	100	100	100	100	33	27	19	26	14	21	19

B

Position	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10
A	7	21	43	21	0	0	21	0	0	0	0	0	43	21	64	57	14	29	7
C	57	43	14	14	50	7	7	0	100	0	0	0	57	29	7	7	29	43	29
G	21	36	36	50	29	21	7	100	0	100	0	100	0	50	7	21	57	29	21
T	14	0	7	14	21	21	64	0	0	0	100	0	0	0	21	14	0	0	43
CI	31	34	26	23	36	53	38	100	100	100	100	100	58	36	38	31	41	33	23

C

Gene	DRE Sequence	MS Score
m_Cyp1a1	caagctcGCGTGagaagcg	0.946
m_Cyp1a1	cctgtgtGCGTGccaagca	0.921
m_Cyp1a1	gaggctaGCGTGcgtaagc	0.899
m_Cyp1a1	cggagttGCGTGagaagag	0.954
m_Cyp1a1	ccagctaGCGTGacagcac	0.920
m_Cyp1a1	cgggtttGCGTGcgatgct	0.965
r_Cyp1a1	cggagttGCGTGagaagag	0.954
r_Cyp1a1	ccagctaGCGTGacagcac	0.920
m_Cyp1b1	ccccctGCGTGcggagct	0.953
r_Aldh3a1	tgatgggGCGTGcattagt	0.818
r_Nqo1	tcccctGCGTGcaaaggc	0.940
r_Sod1	gaggcctGCGTGcgcgcct	0.874
r_Gsta2	gcatgttGCGTGcatccct	0.897
r_Ugt1a6	agaatgtGCGTGacaaggt	0.895

D

Gene	HRE Sequence	MS Score
h_TFR	cgagcgtACGTGcctcagg	0.957
r_VEGF	agtgcatACGTGggcttcc	0.951
r_AFP	ttcacccACGTGgctttgt	0.895
h_PAI-1	tgtgtgtACGTGtgtaaga	0.927
m_LDH	ccagcggACGTGcgggaac	0.954
m_LDH	agcctacACGTGggttccc	0.920
m_HO-1	agagaggACGTGccacgcc	0.946
m_HO-1	agagcggACGTGctggcgt	0.956
m_GLUT1	tccacagGCGTGccgtctg	0.827
m_FLT	aggaacaACGTGgaattag	0.905
m_EPO	gggccctACGTGctgcctc	0.956
h_VEGF	agtgcatACGTGggctcca	0.946
h_TF	aagaaatACGTGcgcttgt	0.946
h_TF	tgtgtgtACGTGcaggaaa	0.941
h_RTP801	gttgcttACGTGcgcccgg	0.956
h_RORa	tgggtggACGTGtgtgtgc	0.954
h_PGK1	tagtgagACGTGcggcttc	0.937
h_PGK1	actgccgACGTGcgctccg	0.970
h_pfkfb3	atgcgggACGTGagcgacg	0.934
h_pfkfb3	gtgagcgACGTGtggcagc	0.932
h_iNOS	agtgactACGTGctgccta	0.942
h_IGFBP1	tggcaggACGTGctctggg	0.963
h_GAPDH	ctgagctACGTGcgcccgt	0.949
h_FLT	aggaacaACGTGgaattag	0.905
h_EPO2	gctgcagACGTGcgtgtgg	0.966
h_EPO1	gggccctACGTGctgtctc	0.958
h_Eno	tcggagtACGTGacggagc	0.931
h_Eno	gctgagtGCGTGcgggact	0.854
h_Eno	gggccggACGTGgggcccc	0.963
h_Eno	cggggccACGTGcgccgcc	0.965
h_CRLR	atgttagGCGTGtgtgtgt	0.813
h_CA9	gggctgtACGTGcattgga	0.953
h_BNIP3	gcgccgcACGTGccacacg	0.942
h_AldoA	ccctcggACGTGactcgga	0.899
h_AldoA	cctcttcACGTGcggggac	0.925
h_leptin	ggtagcgACGTGccgggcg	0.950
h_Dec2	gttccgcACGTGagctggg	0.929
h_Dec1	tggccagACGTGcctggag	0.956

Table A-1. Primer information for qRTPCR

Descriptions	Gene Abbrev.	REF Seq	Amplicon Size	Forward	Reverse
adrenomedullin	ADM	NM_001124	127	ggtgcagaagaatccgagtg	acgccgtgagaaatcagttt
BCL2/adenovirus E1B 19kDa interacting protein 3-like	BNIP3L	NM_004331	121	agcatgacccaacctaccac	tcttcaggccaaaaagggta
chemokine (C-C motif) ligand 20	CCL20	NM_004591	124	gtggcttttctggaatggaa	caagtccagtgaggcacaaa
cyclin-dependent kinase inhibitor 1C	CDKN1C	NM_000076	129	agagatcagcgcctgagaag	tgggctctaaattggctcac
glycoprotein hormones, alpha polypeptide	CGA	NM_000735	129	cccactccactaaggtccaa	cgtgtggttctccactttga
ceruloplasmin (ferroxidase)	CP	NM_000096	128	cccatggggtacaaacagag	agcccatggaatacaagcag
cytochrome P450, family 1, subfamily A, polypeptide 1	Cyp1A1	NM_000499	122	cttccgacactcttccttcg	ggttgatctgccactggttt
cytochrome P450, family 1, subfamily B, polypeptide 1	Cyp1B1	NM_000104	119	atggcctcatcaacaaggac	gagccaggatggagatgaag
dual specificity phosphatase 5	DUSP5	NM_004419	133	cctgctaaaactgggatgga	ctatctcactggggcagcat
fibrinogen alpha chain	FGA	NM_000508	140	agccgatcatgaaggaacac	tactggatcccggtagcttg
fibrinogen beta chain	FGB	NM_005141	126	gatgggagaaaacaggacca	catccaccaccgtcttcttt
potassium inwardly-rectifying channel, subfamily J	KCNJ8	NM_004982	138	gatcatctgccacgtgattg	gttcgtgcttgtgtggtgat
lysyl oxidase	LOX	NM_002317	133	gcacacacacagggattgag	ccaggtagctgggggtttaca
lumican	LUM	NM_002345	124	atcagcaacatccctgatga	caaccagggatgacacattg
metallothionein 1X	MT1X	NM_005952	124	gcaaatgcaaagagtgcaaa	cagcagctgcacttgtctga
v-myc myelocytomatosis viral oncogene homolog	MYC	NM_002467	122	ccgaggagaatgtcaagagg	ggccttttcattgttttcca
phosphoenolpyruvate carboxykinase 2	PCK2	NM_004563	137	gggtgctagactggatctgc	gagggagaacagctgagtgg
SMAD, mothers against DPP homolog 5	SMAD5	NM_001001419	120	atctcaggtctcccagagca	tgcagaagaaatgccttcaa
UDP glucuronosyltransferase 1	UGT1A1	NM_007120	133	gtgcctttatcacccatgct	tccagctcccttagtctcca
beta-Actin	ACTB	NM_001101	116	ctcttccagccttccttcct	agcactgtgttggcgtacag

using the PWMs. The MS score for each match was computed, and those with scores greater than the threshold values are expected to have both a greater probability of possessing a measurable binding affinity and presumable biological relevance. **Identification of Over-Represented Short Sequence Motifs in Gene Regulatory Regions.** Gene regulatory regions, defined as starting -5000 kb relative to the transcription start site through the 5′ UTR, were obtained from the UCSC Genome Browser for all known genes assigned a mature RefSeq mRNA accession. These sequences were stored in the Gene Regulatory Subsystem of our toxicogenomic database, dbZach (http://dbzach.fst.msu.edu, (33)), to facilitate further analysis. All 5-10 nucleotide short sequence motifs were identified using a sliding window method (34). An empirical Bayes implementation of the Wilcoxon's Rank Sum Test, similar to those used for microarray analysis (35, 36), was used to identify 5-10 nucleotide motifs that are over-represented in one population compared to that in another. This method computes a posterior probability, which represents the likelihood of that result occurring (i.e., a posterior probability of 0.90 means that there is a 90% probability that the result is true). The Transfac database (37) was queried to annotate the motifs and identify potential binding proteins. Absolute numbers of individual motifs that were determined to be over-represented in the cobalt or TCDD treatment groups were then analyzed by hierarchical clustering (unweighted pair-group method using arithmetic averages using correlation distances; http://gepas.bioinfo.cnio.es/cgi-bin/cluster (38)). These values were clustered with the GeneChip expression data found in Fig. A-4.

RESULTS

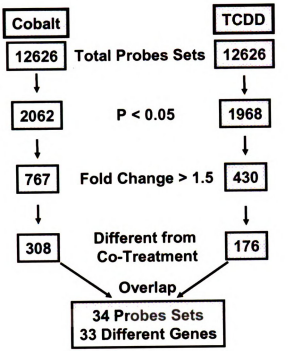
Identification of genes potentially susceptible to cross-talk between the AHR and HIF signaling cascades was performed on a high-density oligonucleotide array. Hep3B cells were treated with DMSO (0.01%, vehicle control), cobalt chloride (100 mM), TCDD (10 nM), or cobalt chloride and TCDD (100 mM and 10 nM respectively) for 20 h, and global gene expression patterns were analyzed. The procedure to identify these genes is outlined in Fig. A-2A. Briefly, significant ($p < 0.05$) gene expression changes following a single treatment were identified and further pared using a 1.5-fold cutoff leaving 767 and 430 probe sets for cobalt and TCDD treatment, respectively. These genes were then analyzed for significant differences between single treatment and co-treatment as determined by paired student's *t*-test. With these criteria, 308 and 176 probe sets were identified for cobalt- and TCDD-treated gene groups, respectively. These two groups constitute the genes that can be influenced by cross-talk from either single treatment. Of these, 34 probe sets, corresponding to 33 genes were represented in both lists (Fig. A-2A). These 33 genes represent a group of targets that are affected by both single treatments (i.e., AHR and HIF responsive) and whose expression is modulated by cross-talk.

Because the analysis was performed with no bias toward direction of change, it is possible that some of the changes would be in the same or in opposite directions. Analysis showed that a majority of these genes displayed the same direction changes, either both up or both down. Only 33% of the genes exhibited an opposite pattern of expression (Fig. A-2B). These results suggest a more complex interaction between the AHR and HIF signaling cascades and identify genes where this interaction may lead to

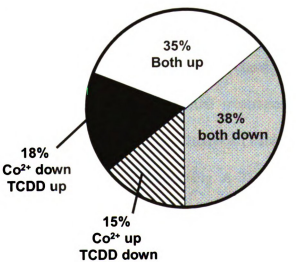
Figure A-2. Analysis of genomic data and comparison of 33 target genes.

The genomic data was analyzed in a four step process (**A**). First, genes were screened for significance ($p < 0.05$). Second, these genes were filtered for those that displayed greater than 1.5-fold change. Third, the list was analyzed for those that were significantly altered ($p < 0.05$) when single treatment was compared to co-treatment. Finally, the cobalt and TCDD lists were compared for overlap. (**B**) Direction of expression changes was analyzed for the 34 probe sets identified in the screen. A complete list of cobalt and TCDD influenced genes can be found in Supporting Information, Tables 5 and 6).

A


Cobalt
TCDD
12626
Total Probes Sets
12626
2062
P < 0.05
1968
767
Fold Change > 1.5
430
308
Different from Co-Treatment
176
Overlap
34 Probes Sets
33 Different Genes

B


35%
Both up
38%
both down
18%
Co2+ down
TCDD up
15%
Co2+ up
TCDD down

antagonism (i.e., less than additive) or synergy (i.e., more than additive).

The list of 33 genes include prototypical dioxin and hypoxia responsive genes, notably Cyp1A1 and heme oxygenase-1. These genes exhibit a classic cross-talk expression pattern. For example, Cyp1A1 is induced approximately 100-fold in the presence of TCDD, whereas it is repressed almost 6-fold after cobalt treatment. Following co-treatment, gene expression is only induced 65-fold (Table A-2). A similar pattern is seen for heme oxygenase-1, where it is induced by cobalt, repressed by TCDD, and only partially induced by co-treatment. This type of competition is not the only type of regulation observed. SOX-9 and CDKN1C displayed clear evidence of additive regulation in which individual treatments modify expression in the same direction, and co-treatment yields an additive expression value (Table A-2). In addition, UGT1A1 displays a level of synergy between treatments. These results suggest that simple regulation or competition for ARNT or other cellular factors cannot fully explain all of the expression changes seen in the genomic screen.

The expression patterns of 19 different genes, including 11 from Table 2, were verified by qRTPCR. These genes were a mixture of classic and novel cobalt- or TCDD-inducible genes identified in the individual treatment groups (Supporting Information, Table A-3 and A-4) and cross-talk analysis (Table A-2). In most cases, these results verified those of the Gene Chip data (Fig. A-3). Forty-nine of the 57 gene expression changes (19 different genes under 3 different treatments) were verified by qRTPCR (Fig. A-3B). Interestingly, in the gene chip data, the fibrinogen alpha subunit (FGA) was up-regulated upon cotreatment, whereas the fibrinogen beta subunit (FGB) was downregulated under similar conditions (Table A-2). qRTPCR results showed that

Table A-2. Microarray fold change and information for 33 cross-talk genes

probe set	fold change			REF seq	descriptions	abbrev.
	Co	TCDD	TCDD+Co			
1025g_at	-5.9	99.6	64.8	NM_000499	cytochrome P450, family 1, subfamily A	CYP1A1
33436_at	-2.9	-3.9	-5.7	NM_000346	SRY (sex determining region Y)-box 9	SOX9
1787_at	-2.6	-2.3	-5.6	NM_000076	cyclin-dependent kinase inhibitor 1C	CDKN1C
37319_at	-2.3	-1.8	-4.3	NM_000598	insulin-like growth factor binding protein 3	IGFBP3
39545_at	-2.2	-2.3	-3.9	NM_000076	cyclin-dependent kinase inhibitor 1C	CDKN1C
40385_at	-2.2	-1.8	-2.1	NM_004591	chemokine (C-C motif) ligand 20	CCL20
35303_at	-2.0	1.9	1.1	NM_005542	insulin induced gene 1	INSIG1
38519_at	-1.9	-1.6	-3.0	NM_005123	nuclear receptor subfamily 1, group H, member 4	NR1H4
39352_at	-1.9	-11.9	-4.8	NM_000735	glycoprotein hormones, alpha polypeptide	CGA
41424_at	-1.9	-1.7	-3.1	NM_000940	paraxonase 3	PON3
33701_at	-1.8	-1.8	-2.6	NM_000277	phenylalanine hydroxylase	PAH
37235g_at	-1.8	-2.1	-3.6	NM_000893	kininogen 1	KNG 1
38586_at	-1.7	1.8	-3.1	NM_001443	fatty acid binding protein 1	FABP1
38178_at	-1.7	-2.8	-3.9	NM_002153	hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2
36135_at	-1.6	1.8	1.1	NM_006824	EBNA1 binding protein 2	EBNA1BP2
37188_at	-1.6	-2.1	-1.8	NM_004563	phosphoenolpyruvate carboxykinase 2	PCK2
31792_at	-1.5	-3.1	-2.2	NM_005139	annexin A3	ANXA3
33260_at	1.7	11.4	6.8	NM_005633	son of sevenless homologue 1	SOS1
38789_at	1.8	1.9	2.3	NM_001064	transketolase	TKT

37019_at	1.8	-4.0	-1.6	NM_005141	fibrinogen beta chain	FGB
39070_at	1.9	1.9	2.0	NM_003088	fascin homolog 1	FSCN1
38376_at	2.0	1.7	1.3	NM_000018	acyl-Coenzyme A dehydrogenase	ACADVL
31824_at	2.4	2.1	4.7	NM_002395	malic enzyme 1	ME1
39425_at	2.5	1.6	3.2	NM_003330	thioredoxin reductase 1	TXNRD1
38545_at	2.5	-2.1	1.1	NM_002193	inhibin, beta B	INHBB
38825_at	2.9	-1.9	5.9	NM_000508	fibrinogen alpha chain	FGA
38637_at	3.6	-3.3	-1.0	NM_002317	lysyl oxidase	LOX
39008_at	4.5	1.5	2.2	NM_000096	ceruloplasmin	CP
33802_at	6.0	-1.9	3.9	NM_002133	heme oxygenase (decycling) 1	HMOX1
1232s_at	10.0	2.2	3.6	NM_000596	insulin-like growth factor binding protein 1	IGFBP1
39072_at	10.4	1.6	7.1	NM_005962	MAX interactor 1	MXI1
34777_at	33.1	1.8	11.0	NM_001124	adrenomedullin	ADM
40309_at	39.4	2.2	20.3	NM_001216	carbonic anhydrase IX	CA9
32392s_at	80.7	77.7	295.4	NM_007120	UDP glucuronosyltransferase 1	UGT1A1

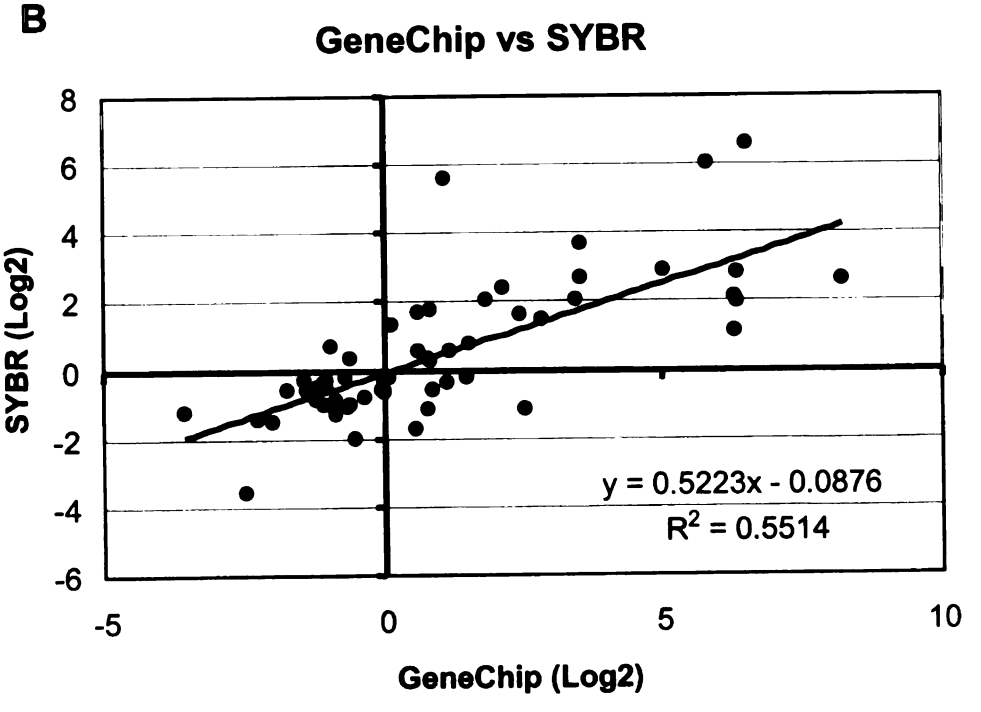
Figure A-3. qRTPCR verification of expression changes for selected cross-talk genes.

qRTPCR was performed on 19 genes using β-actin for normalization. **(A)** Gene abbreviations, accession numbers used in primer design, and fold changes relative to vehicle control are listed. **(B)** A direct comparison between qRTPCR and Gene Chip results was performed and analyzed by linear regression.

A

Gene Abbrev.	Genbank Accession	Fold Change		
		Cobalt	TCDD	Cobalt+TCDD
CDKN1C	AF079221	-1.9	-8.5	-13.8
CCL20	NM_004591	-1.8	-3.8	-2.9
SMAD5	NM_001001419	-1.8	-2.0	-4.0
Cyp1B1	U03688	-1.6	6.8	1.4
KCNJ8	D50312	-1.5	1.3	-2.3
LUM	U21128	-1.5	2.7	-1.2
PCK2	NM_004563	-1.3	-1.6	-2.4
MYC	NM_002467	-1.2	2.5	1.4
Cyp1A1	NM_000499	-1.1	102.9	70.5
DUSP5	U15932	1.0	1.3	-1.5
CGA	NM000735	1.0	-3.2	-4.1
FGA	M64982	1.7	-2.1	-2.3
CP	NM_000096	1.9	-7.6	-3.0
MT1X	NM_005952	2.7	-1.2	3.8
LOX	L16895	3.1	-2.9	-2.0
FGB	J00129	3.3	-2.8	-2.1
UGT1A1	NM_007120	5.0	2.8	7.8
BNIP3L	AF079221	6.4	-1.5	3.0
ADM	NM001124	6.8	-1.1	3.8

B



these two subunits were expressed in a similar pattern under each treatment condition (Fig. A-3A). Because different pools of RNA were used for the qRTPCR and microarray experiments, cell line or culture differences cannot be ruled out as an explanation for this difference. One possible explanation for the different expression patterns exhibited by the 33 genes (Table A-2) that display cross-talk regulation is promoter context of response elements. To initially characterize differences between these promoters, genomic sequences 5000 base pairs upstream of the transcription start codon and the 5′ UTR were analyzed for homology, HREs, DREs, and GC content. The promoter and 5′ UTRs from the 33 genes were aligned using the ClustalW algorithm. Interestingly, the sequences clustered on the basis of their expression patterns with four clusters containing four or more sequences (A-D, Fig. A-4). Group A was predominantly up-regulated in both treatments to an equal level, and several were additive. Group B was primarily inhibited and showed a tendency for competition, where the co-treatment group was somewhere between the two individual expression levels. Group C was predominantly down-regulated in both treatments, and Group D showed higher expression in the cobalt treatment compared to that of TCDD (Fig. A-4). This pattern was not unsystematic or due to some inherent base pair composition found within the promoters and/or 5′ UTR of the genes analyzed because randomization (http://www.cellbiol.com/cgi-bin/randomizer/sequence_randomizer.html) of the promoter sequence yielded no consistent pattern with relation to expression. In addition, random sequences exhibited no relation to the alignment seen in Fig. A-4 (data not shown). These results suggest that motifs within the analyzed

Figure A-4. Alignment and sequence analysis of the promoters and 5′ UTR of core genes.

5000 bp of upstream sequences and the 5′ UTRs were aligned by ClustalW and compared to expression patterns from oligonucleotide chips. The promoters were also analyzed for HREs and DREs using a PWM (Experimental Procedures). Finally, the GC content was also calculated. Up-regulated genes are shown in white, and down-regulated genes are shown in dark gray. Four distinct clusters are also noted (A-D).

Co
TCDD
TCDD + Co
DRE
HRE
Gene Abbreviation
% GC Content
39.4 2.2 20.3 0 5 CA9 48.1
-1.6 1.8 1.1 1 10 EBNA1BP2 42.3
-2.2 -1.8 -2.1 1 6 CCL20 38.7
80.7 77.7 295.4 1 3 UGT1A1 43.7
2.4 2.1 4.7 3 4 ME1 41.3
1.8 1.9 2.3 0 7 TKT 54.0
1.9 1.9 2.0 1 10 FSCN1 58.4
2.5 1.6 3.2 1 6 TXNRD1 44.3
-1.7 1.8 -3.1 0 5 FABP1 47.7
-1.5 -3.1 -2.2 1 4 ANXA3 43.3
-1.7 -2.8 -3.9 0 12 HSD17B2 44.8
33.1 1.8 11.0 1 6 ADM 48.4
1.8 -4.0 -1.6 0 1 FGB 36.5
-1.9 -11.9 -4.8 0 1 CGA 35.1
-1.8 -2.1 -3.6 1 3 KNG 1 44.7
2.9 -1.9 5.9 0 2 FGA 38.5
-1.9 -1.6 -3.0 0 4 NR1H4 42.8
-1.9 -1.7 -3.1 0 2 PON3 43.3
-1.6 -2.1 -1.8 1 9 PCK2 49.7
-2.0 1.9 1.1 5 13 INSIG1 51.9
1.7 11.4 6.8 3 12 SOS1 53.9
-1.8 -1.8 -2.6 0 8 PAH 39.4
-2.9 -3.9 -5.7 1 4 SOX9 54.5
2.5 -2.1 1.1 3 8 INHBB 62.6
-2.3 -1.8 -4.3 2 5 IGFBP3 48.4
10.0 2.2 3.6 2 5 IGFBP1 49.8
3.6 -3.3 -1.0 0 12 LOX 41.1
4.5 1.5 2.2 2 6 CP 39.8
6.0 -1.9 3.9 3 4 HMOX1 50.3
10.4 1.6 7.1 2 3 MXI1 48.7
2.0 1.7 1.3 2 7 ACADVL 58.4
-2.6 -2.3 -5.6 3 10 CDKN1C 60.9
-5.9 99.6 64.8 7 10 CYP1A1 58.5
A
B
C
D
294.5
250
200
150
100
50
0
Nucleotide Substitutions (x100)

regions contained the regulatory information that influenced the cross-talk expression patterns observed. Differences in the regulation patterns of the various genes could also be explained by the number of hypoxia or dioxin response elements located within the regulatory region. Comparison of these motif sequences to HRE and DRE PWMs suggests that DREs were better represented in Group D and that this bias did not correlate with an increase in GC content (Fig. A-4). The analysis did identify more HREs than DREs, primarily due to the fact that the HRE PWM was only exclusive at four positions, compared to five positions in the DRE (Fig. A-1 and A-4). Given the redundancy within the core sequences of the DREs and HREs, it is also possible that several sequences could register as both DREs and HREs. To determine the level of overlap, each DRE and HRE was further scored using the opposing PWM. For example, each DRE listed in Figure 4 was scored with the HRE PWM, and a similar cutoff (the MS score had to be higher than the lowest HRE used to create PWM) was applied. The results show that 40% of the DREs also scored positive as HREs, whereas only 14% of the HREs scored positive as DREs (Supporting Information, Tables 8 and 9). However, there was no relationship between HRE-DRE overlaps and the type of cross-talk observed. Finally, it is possible that base-pairing relationships, G/C versus A/T, may bias the association of a promoter into one group or another. On the basis of GC content, there was no correlation between base-pairing relationships and gene expression patterns or groupings, suggesting that other information within the promoter regulated expression (Fig. A-4).

Given that it is not the absolute number of HREs or DREs within the regulatory region or their basic composition that dictates expression, promoter analysis for other

Figure A-5. Promoter analysis for over-represented motifs and correlation with expression.

The promoters of 65 different genes from the cobalt- and TCDD-treatment groups were analyzed for motifs that were over-represented as described in Experimental Procedures. Information about the motif, its corresponding response element as determined by TRANSFAC, and the statistical values are represented for the Cobalt group (**A**) and the TCDD group (**B**) as defined in Experimental Procedures. The total number of response elements listed in A and B (including HREs and DREs) were then identified in the regulatory regions of the 33 genes from Figure 4 and correlated with expression data via hierarchical clustering. A dendrogram correlating the GeneChip expression data (Co2+, TCDD and TCDD+Co2+) with the various response elements is displayed (**C**). A complete Table of motifs identified in the analysis and the table used to create the dendrogram can be found in Supporting Information, Tables 2-4.

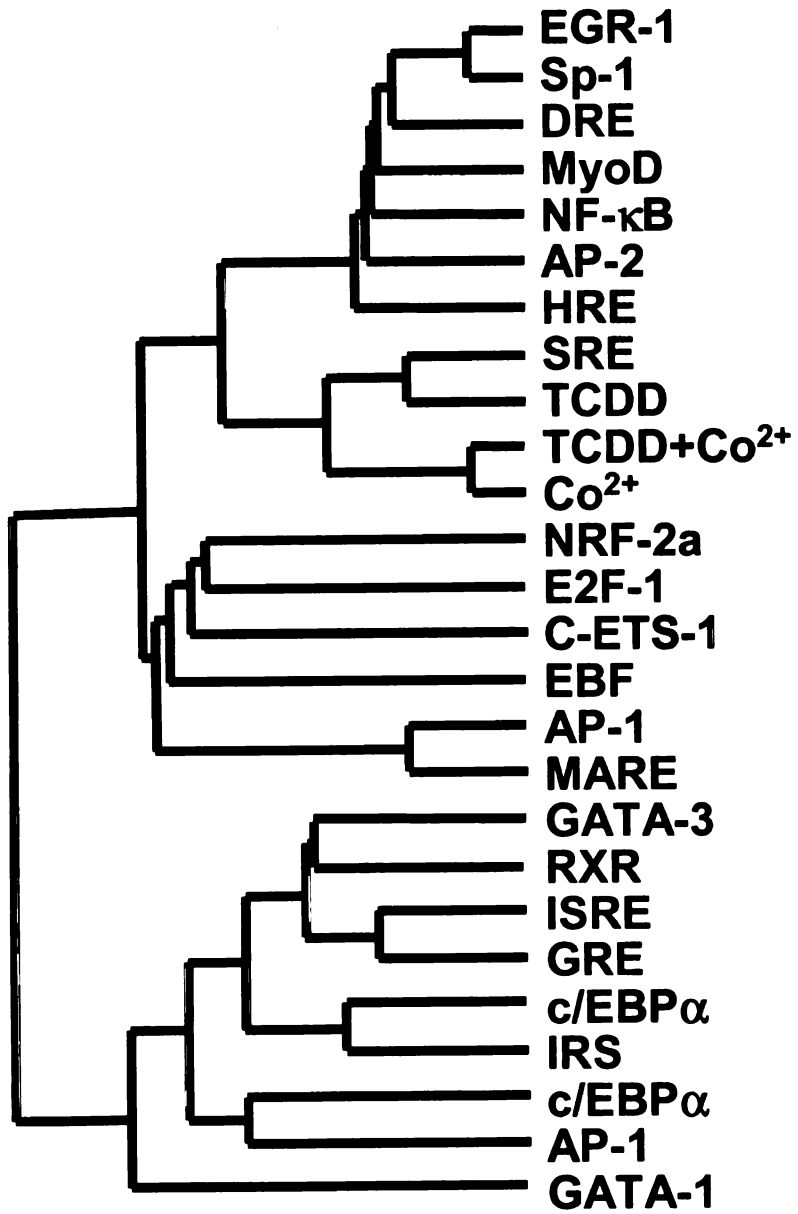
A

Site	word	adjusted p-value
AP-1	gagac	9.58
AP-1	tgaga	9.56
AP-2	tggggc	9.52
c/EBP alpha	gatttt	9.59
c-ETS-1	gctct	9.56
E2F-1	cttggc	9.50
EBF	cttga	9.61
Egr-1	cgccc	9.61
Egr-1	cccac	9.48
IRS	ttttg	9.56
MARE	gctgag	9.58
MyoD	gtggc	9.50
MyoD	gcagc	9.59
MyoD	ctggc	9.59
NF-kB	cctct	9.50
NF-kB	ggctt	9.58
NF-kB	ttcca	9.61
NRF-2 alpha	tcttcc	9.50
RXR	ttcat	9.50
Sp1	tgggc	9.50
Sp1	ccccc	9.58
Sp1	gcgcg	9.61
Sp1	ggggc	9.56
Sp1	gcact	9.48
Sp1	cgcgg	9.52
Sp1	tccct	9.52
SRE	tcctca	9.58

B

Site	word	adjusted p-value
GRE	taaac	0.91
ISRE	ggaaa	0.89
ISRE	aaact	0.88
c/EBP alpha	aagag	0.87
AP-1	agttt	0.85
GRE	ataaac	0.85
c/EBP alpha	gttga	0.85
GATA-3	gatat	0.85
GATA-1	tctca	0.85

C


EGR-1
Sp-1
DRE
MyoD
NF-κB
AP-2
HRE
SRE
TCDD
TCDD+Co2+
Co2+
NRF-2a
E2F-1
C-ETS-1
EBF
AP-1
MARE
GATA-3
RXR
ISRE
GRE
c/EBPα
IRS
c/EBPα
AP-1
GATA-1

motifs that might correlate with the expression pattern in Fig. A-4 were also examined. A computational word search was performed to identify over-represented motifs/elements 5000 base pairs upstream of the start codon and in the 5′ UTR. The 33 genes from Figure 4 were not sufficient to perform a word analysis; therefore, the analysis was conducted on a nonredundant set of 65 active genes from each of the cobalt and TCDD treatment groups for gene regulatory sequences. The lists of overrepresented motifs in cobalt and TCDD groups were combined, and the frequencies of each motif occurrences were calculated for each gene. Twenty-seven different words, corresponding to 15 different known transcription response elements, including Sp1, serum response elements (SRE) and NF-kB sites, were over-represented in the cobalt treated group. Conversely, nine different words, corresponding to six different elements were over-represented in the TCDD treated group. These included a c/EBP-a, GREs, and two types of GATA response elements (Fig. A-5).

The regulatory regions of the 33 genes identified in Figure 4 were analyzed for the over-represented response elements, and the correlation between expression patterns from the microarray, and the frequency of occurrence of these 23 major response elements (15 from the cobalt-treated group, 6 from the TCDD group and HREs and DREs), using hierarchical clustering. All of the expression data clustered together with the SRE (Fig. A-5C). A second cluster showed strong correlation with the expression data, which included EGR-1, Sp-1, DRE, MyoD, NF-kB, AP2, and HRE elements. These results suggest that the expression patterns in Figure 4 are well correlated with the number of SREs in the respective promoters and to a lesser extent

to a group of seven other response elements, including the HREs and DREs.

DISCUSSION

Understanding the nature and extent of the cross-talk between the hypoxia and dioxin signaling cascades is important for determining the role of each signaling cascade in directly modulating the function of the other. The results presented here suggest a gene-specific cross-talk. In some instances, the crosstalk was shown to be additive (e.g., IGFBP3, UGT1A1, and CDKN1C), whereas in others, it was competitive (e.g., P450, IHBB, and LOX). To establish a possible mechanism for the selective nature of the cross-talk, a small portion of the putative regulatory region of a subset of these genes was analyzed for HREs and DREs. There was no apparent correlation between the number of these types of response elements and the nature of the cross-talk observed. Computational analysis of the regulatory regions proximal to the transcription starting site (TSS) identified over-represented motifs within these regions, suggesting that AHR-HIF1 cross-talk cannot be explained completely by competition for ARNT and most likely involves other cofactors, response elements, and promoter context.

Differences in expression patterns for those genes affected by TCDD and cobalt cross-talk suggest that a variety of mechanisms are involved. If the cross-talk was solely dependent upon competition for ARNT, then evidence of interactions would be widespread and would not include additive responses. In contrast, only 33 genes exhibited an interaction, with approximately 30% displaying additive tendencies, including ME1, SRY, CDKN1C, and NR1H4. This pattern suggests that ARNT is not limiting and that alternative mechanisms are involved. These mechanisms probably involve competition and/or synergy between other co-activators/co-repressors or other

signaling systems that are being influenced, and the cross-talk is a result of secondary signaling. For example, TCDD might inhibit the expression of a transcription factor necessary for cobalt-induced expression of another TCDD target gene. Alternatively, cobalt exposure might activate a signal that leads to post-translational modification of the AHR, thus altering its activity. Comparable interactions have been described between steroid receptors and other signaling cascades (ref 39 and references theirein). Finally, these alternative pathways likely involve other cascades that influence the ability of AHR and HIF1 to alter expression patterns. The predominance of SREs present in the regulatory region of genes exhibiting cross-talk suggests that one of these alternative inputs is the serum response cascade.

The presence of the SRE motif correlates with the expression patterns elicited by various treatments, suggesting a regulatory role in gene expression. Surprisingly, the number of SREs across the 33 genes in Table 2 is more predictive of cross-talk than the number of HREs or DREs (Fig. A-4). The 33 cross-talk genes were identified after meeting two criteria: their expression was influenced by each individual treatment, and this expression was further altered upon co-treatment. The original hypothesis that cross-talk was due to ARNT competition meant that this two step process would identify the subset of genes most prone to cross-talk. Given the different types of interactions (e.g., additive and competitive), simple competition for ARNT does not satisfactorily explain cross-talk in Hep3B cells, and these requirements might have biased the results to select for other transcription factor pathways such as the SRE.

The serum response factor (SRF) binds the SRE and regulates the expression of a variety of genes, including plasminogen activator inhibitor 1 (PAI-1), early growth

response factor-1 (EGR-1), and glucose transporter-1 (Glut-1) (40-42). Interestingly, each of these genes is a target for hypoxia-mediated transcription. The over-representation of SREs in the cobalttreated group suggests that HIF1 and SRF signaling pathways may converge upon a select group of genes, and the correlation of SREs with the set of cross-talk genes from Table A-2 might suggest that these genes are important for the response to these two signaling pathways

Genes exhibiting cross-talk fall into a variety of ontological categories, with several distinct processes and functions being over-represented, including blood coagulation, cell proliferation, fatty acid oxidation, metal binding, and oxidoreductases (Supporting Information, Table A-7), suggesting that HIF-AHR interactions may alter these endogenous processes. For example, hypoxia influences the disposition of a variety of drugs through multiple mechanisms, including altering the expression of phase 1 enzymes and several oxidoreductases, and a patient who has been pre-exposed to AHR ligands might be further compromised in his or her ability to dispose of the drugs (43). The putative role of cross-talk might be most evident during development of the liver and heart. Mice lacking functional AHR have aberrant liver development and are prone to hypertension and cardiac hypertrophy (44). Liver development is abnormal in AHR null mice as a result of decreased peripheral profusion (44), possibly due to an imbalance in hypoxia-induced fibrin expression that ultimately influences angiogenesis. Over-production of fibrin and its subsequent deposition into the local circulation might explain the excess hematopoeitic cells in the AHR -/- mouse liver and the subsequent increase in peripheral resistance (2). Similarily, cardiac hypertrophy in AHR -/- mice appears to be correlated with endothelin-1, angiotensin II,

and HIF1α (45-47). Therefore, AHR-HIF1α interactions may adversely affect heart and liver development following signaling cross-talk.

The ability of HIF1α and AHR to influence each other's transcriptional activity on a genome scale appears to involve multiple factors, and our results suggest that HREs, DREs, and promoter context play a role in mediating this cross-talk. It is also likely that promoter context and other transcription factors play a major role in determining gene expression behavior following hypoxia and/or TCDD treatment. In fact, the direction and magnitude of change following exposure to either hypoxia or TCDD compared to the exposure to hypoxia and TCDD is probably more dependent upon additional transcription factors than previously suspected. Therefore, understanding the role of cross-talk between hypoxia and the AHR will require a more thorough characterization of HIFs and the AHR interactions with other transcription factors and their respective ability to influence basic transcriptional machinery.

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APPENDIX B

HYPOXIA, DRUG THERAPY AND TOXICITY

This chapter represents a manuscript that was published in Pharm &Ther. 113: 229-246. 19: 1284-1293 (2006). Authors included: KangAe Lee, Robert A. Roth and John J. LaPres.

ABSTRACT

Hypoxia is defined as a decrease in available oxygen reaching the tissues of the body. It is linked to the pathology of cancer, cardiovascular disease, and stroke, the leading causes of death in the United States. Cells under hypoxic stress either induce an adaptive response that includes increasing the rates of glycolysis and angiogenesis or undergo cell death by promoting apoptosis or necrosis. The ability of cells to maintain a balance between adaptation and cell death is regulated by a family of transcription factors called the hypoxia inducible factors (HIFs). HIF1, the most widely studied HIF, is essential for regulating the expression of a battery of hypoxia-responsive genes involved in the adaptive and cell death responses. The ability of HIF1 to balance these two responses likely lies in the regulation of HIF1α stability and transcriptional activity by post-translational hydroxylation and its ability to respond to other cellular factors including key metabolites and growth factors. Targeting HIF1 signaling for therapeutics, therefore, requires an understanding of how these various signals converge upon HIF1 and regulate its role in maintaining the balance between adaptation and cell death. In addition, one must understand how this balance can be perturbed during toxicant-induced tissue damage. This review will summarize our current understanding of hypoxia signaling as it applies to drug therapy and toxicity and describe how these processes can influence the HIF-mediated balance between adaptation and cell death.

INTRODUCTION

The reaction of Complex IV of the electron transport chain (ETC) utilizes greater than 95% of the oxygen we breathe. This reaction, catalyzed by cytochrome oxidase, is required for maintaining proper ATP production within the cell. This coupling of energy production to the consumption of oxygen through cytochrome oxidase has made the ability to sense and cope with low oxygen tension essential for survival. Decreases in oxygen reaching the tissues of the body, a condition known as hypoxia, is detrimental to the cells and tissues because it decreases their ability to maintain energy production and thereby disrupts their ability to maintain proper function. In fact, hypoxia and hypoxia-related signaling has been linked to the pathology of all the major causes of death, including cardiovascular disease, stroke and cancer. The fact that hypoxia signaling is directly linked to a tumor's ability to thrive has made it and several hypoxia-activated genes the focus of intense drug discovery research. To target hypoxia signaling successfully for therapeutics or to appreciate fully its role in xenobiotic toxicity, it is necessary to understand the cellular events following loss of normal oxygen tensions and to characterize the proteins and pathways involved.

This review summarizes current thinking with regard to causes of hypoxia under normal and pathophysiological conditions and the programmed response to decreases in oxygen availability. This includes a detailed discussion of the proteins involved and

the cellular consequences of hypoxia-activated signaling, and also outlines a balance between hypoxia-induced cellular adaptation and death. Finally, this balance and its role in therapeutics and toxicity are discussed.

2. Hypoxia Signaling

2.1. Normoxia vs. Hypoxia

Oxygen is transported from the air that we breathe throughout the body via a respiratory system. The primary function of this system is to deliver inspired oxygen to peripheral tissues and remove carbon dioxide that cells produce as a byproduct of normal cellular function. When a breath is taken, air passes from the airways into microscopic air sacs called alveoli. The exchange of oxygen and carbon dioxide between the alveoli and blood is called external respiration. Although the oxygen carrying capacity of the blood is increased markedly by the presence of hemoglobin, it is the gradient of oxygen partial pressure (pO_2) between the blood plasma and the locus of oxygen consumption in the mitochondrion that drives O_2 into the tissues by passive diffusion. The pO_2 in the inspired air is 150 mmHg, whereas the pO_2 in the alveoli and arterial blood is around 100 mmHg. In the tissues, oxygen dissociates from hemoglobin and diffuses through capillary endothelium into parenchymal cells, so that the pO_2 of the blood draining tissues (ie, venous pO_2) is far less than arterial pO_2, averaging about 40 mmHg. Thus, the pO_2 in the cells of tissues is much lower than that of the arterial blood.

The normal partial pressure of oxygen varies in cells within and among different tissues. For example, the normal pO_2 of skeletal muscle has been reported to be 20-30 mmHg and that of brain is 45-65 mmHg (33, 66). Even within a single organ, cells are exposed to a wide range of oxygen tensions in different locations (64). For example, the pO_2 of the blood in liver varies by site, so that pO_2 in hepatic artery, portal vein, sinusoids and central vein are estimated to be 95-105 mmHg, 50-65 mmHg, 35-45 mmHg and 30-40 mmHg, respectively (21, 64, 94). Although estimates of values for "cellular pO_2" have been made in numerous tissues, uncertainty exists due to limitations of methodologies used for the measurements. Moreover, cellular pO_2 must vary with the location of parenchymal cells along the length of the capillary (or sinusoid), and gradients must also exist intracellulary due to the continuous consumption of O_2 in the mitochondria. Thus, cells in the portal triad, where arterial and portal venous blood mixes upon entering the liver, are exposed to a more oxygen-rich environment than cells close to the central vein, since the blood has lost O_2 by diffusion into upstream cells. Nevertheless, each of the cells along the sinusoid considers the degree of oxygen to which they are usually exposed to be "normal". How cells establish or perceive this level of oxygen as "normoxic" is not entirely clear; however, when the pO_2 in the tissues and individual cells drops below this normal level, a state of hypoxia is said to exist.

2.2. Occurrence of hypoxia

Hypoxia arises in biological systems for a wide array of reasons, including normal physiological variation and pathological conditions. During embryonic and fetal development, hypoxia occurs as the process progresses and the cellular requirements throughout the developing organism display varying demands for oxygen. Studies *in vitro* have demonstrated that the optimum oxygen tension in a mammalian embryo is approximately 23-38 mmHg, and this oxygen environment is an important physiological factor for vasculogenesis, angiogenesis, and tubulogenesis (71, 124, 157). In addition, normal organogenesis in the developing fetus takes place in a relatively oxygen-poor environment (relative to adult tissues), and increasing oxygen tension during this time is deleterious for heart, kidney, and lung development (74, 140, 157).

Hypoxic conditions can also occur in the adult under various conditions. For example, during exercise muscle demand for energy can outpace the supply of oxygen, causing localized hypoxia. This type of stress leads to a buildup of lactic acid as the body turns to anaerobic metabolism to address the energy debt. These normal biological processes highlight the balance that is established between oxygen levels and energy production and the importance of a programmed response to disruptions in this balance. Imbalances can occur either through reduced oxygen availability, as in the developing embryo, or increased energy demand, as in the exercising muscle. More importantly, this balance is also central to hypoxia's role in pathological conditions.

Recent interest in hypoxia and its effects in biological systems has stemmed

from its role in a wide variety of pathological conditions, most notably cancer. Hypoxia is an important feature of most solid tumors, arising from the process of tumor formation and progression that is characterized by rapid cell growth and drastic changes in the local oxygen supply. Rapid cellular expansion quickly outpaces the ability of a tumor to create new blood vessels for oxygen delivery, leading to a hypoxic or even anoxic microenvironment within certain portions of the tumor. The hypoxic microenvironment occurs very early during tumor development, beginning as the tumor reaches approximately 2-3 millimeters in diameter. To this the tumor must respond, primarily by increasing its glycolytic rate and angiogenic potential (53, 54, 119). The resultant newly formed vasculature differs from that in normal tissues, displaying abnormal structure and function. For example, the new tumor vessels are highly disorganized and usually leaky, with incomplete endothelial lining, qualities that lead to irregular blood flow and diminished nutrient and oxygen supply (22, 69). Newly developed vasculature does not always address the oxygen debt of the tumor since many portions remain further than 150 μm from the nearest blood vessel, which, due to tissue O_2 consumption, is the approximate diffusion limit of oxygen (22). Hypoxia, therefore, remains a constant feature of tumors even after neovascularization. For many years, tumor hypoxia has been considered a challenge for cancer therapy because of its adverse impact on the effectiveness of radiation and chemotherapy. Moreover, low oxygen availability has recently emerged as a major factor that

enhances malignant progression, because tumor cells that have adapted to hypoxia gain various advantages in growth. In most cases, this is a sign of poor prognosis (9).

Disruption in oxygen homeostasis also occurs during other pathophysiological conditions, such as cardiovascular disease, stroke, and chronic pulmonary disease (113). Atherosclerosis often leads to arterial stenosis which ultimately impairs perfusion of the vascular bed and further disrupts oxygen and nutrient flow to regions of the heart muscle (110). When this ischemia persists, it can irreversibly reduce myocardial viability (105, 113). Stroke is a well known cause of cerebral hypoxia because it disrupts the normal flow of blood in the brain and leads to localized loss of oxygen and nutrient supply. In brain tissues, even if this disruption only lasts for a short period of time, it can lead to neuronal cell death and disability (60, 113). Chronic obstructive pulmonary disease (COPD) is the most common form of pulmonary dysfunction and can be subcategorized as asthma, chronic bronchitis, and emphysema. Alveolar hypoxia is common in COPD because of the difficulties in expelling air from the lungs, and it can lead to pulmonary hypertension, pulmonary arteriolar remodeling, and ultimately right heart failure (154, 155). The hypoxia-induced injury and tissue remodeling seen in these pathophysiological conditions and others, including diabetes and wounding, can be considered a response to the inability to maintain the balance between oxygen delivery and energy production. The injurious process takes place because the tissue cannot adapt to the change in oxygen tension. As will be discussed

later in this review, toxicant-induced cellular damage that involves the hypoxia signaling system can also be considered in a similar way.

2.3. The hypoxia-inducible factor (HIF) system

Aerobic metabolism provides a significant advantage to multicellular organisms, especially in the area of energy production. When oxygen supply cannot meet the energy demand of the cells, however, an adaptive response must compensate for the energy imbalance to maintain tissue function. A primary mechanism for this adaptive response is the transcriptional regulation of a battery of hypoxia-responsive genes. In fact, genomic screens have shown that thousands of genes are influenced by exposure to hypoxia (127, 143, 144). The most well studied mechanism identified in this process is an interaction of a family of transcription factors, called hypoxia-inducible factors (HIFs) with a cis-acting element, called the hypoxia-responsive element (HRE), located in regulatory regions of target genes. This mechanism was first demonstrated for the erythropoietin gene, the expression of which was upregulated more than 100-fold by hypoxia via HIF-induced transcription (6, 96). Since then, more than 70 target genes regulated directly by HIFs have been identified, and expression of over several hundred genes are known to be directly or indirectly influenced by HIF.

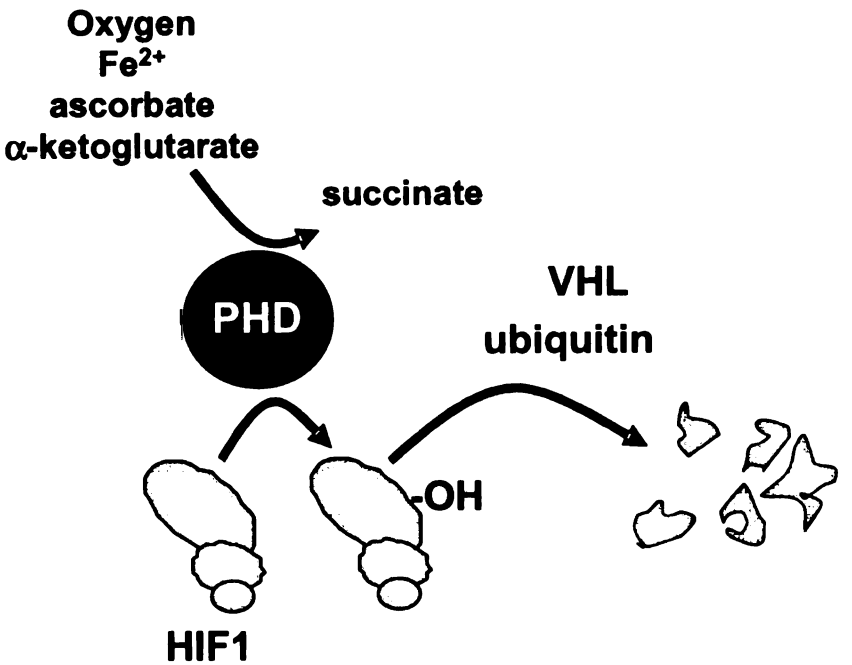
The family of HIFs belong to the Per-ARNT-SIM (PAS) superfamily of transcription factors is characterized by the presence of the PAS domain that controls dimerization (55, 56, 73). HIFs are heterodimeric proteins comprising an α and

β subunit. The alpha class is composed of HIF1α, HIF2α, and HIF3α (for the purpose of this review, thesewill be collectively referred to as HIFα). The beta subunit includes the aryl hydrocarbon nuclear translocator (ARNT, also known as HIF1β) and ARNT2. HIF1 is the most widely studied HIF heterodimer and is the combination of HIF1α and ARNT (146). HIF1 is the principal regulator of the hypoxic response in most mammalian cells (116). ARNT and ARNT2 are constitutive nuclear proteins, whereas the expression and activity of the α subunit is tightly regulated by oxygen concentration. It rapidly accumulates upon exposure to hypoxia and on reoxygenation is quickly degraded with a half-life of less than 5 minutes (153). Given how detrimental hypoxia is to a cell, this rapid response time highlights the speed at which a cell must elicit a reaction to loss of oxygen tension. Equally important is the short half life of the HIF protein following reoxygenation, and this time frame suggests that prolonged HIF activation might not be beneficial to the cell. Our understanding of the mechanism a cell uses to sense hypoxia and convey this signal to the alpha subunit has greatly increased over the last several years. This is due to the recent identification of a family of hydroxylases that modify the α subunit in an oxygen-dependent manner.

Three hydroxylases, known as prolyl hydroxylase domain containing proteins (PHDs, also known as egg laying abnormal (EGL) 9 homologues [EGLNs] and hypoxia prolyl hydroxylases [HPHs]), control HIF1α stability by modifying conserved prolines within the oxygen-dependent degradation domain (ODD) of HIFα under

Figure B-1. PHD-mediated hydroxylation of HIF1

Prolyl hydroxylase (PHD) requires oxygen, iron, ascorbate and α-ketogluturate for activity. During the enzymatic process, α-ketoglutarate is decarboxylated, yielding succinate, and the HIF1α ODD is hydroxylated on proline residues. Once hydroxylated, HIF1α is quickly degraded in a VHL-ubiquitin-26S proteosome-dependent manner.

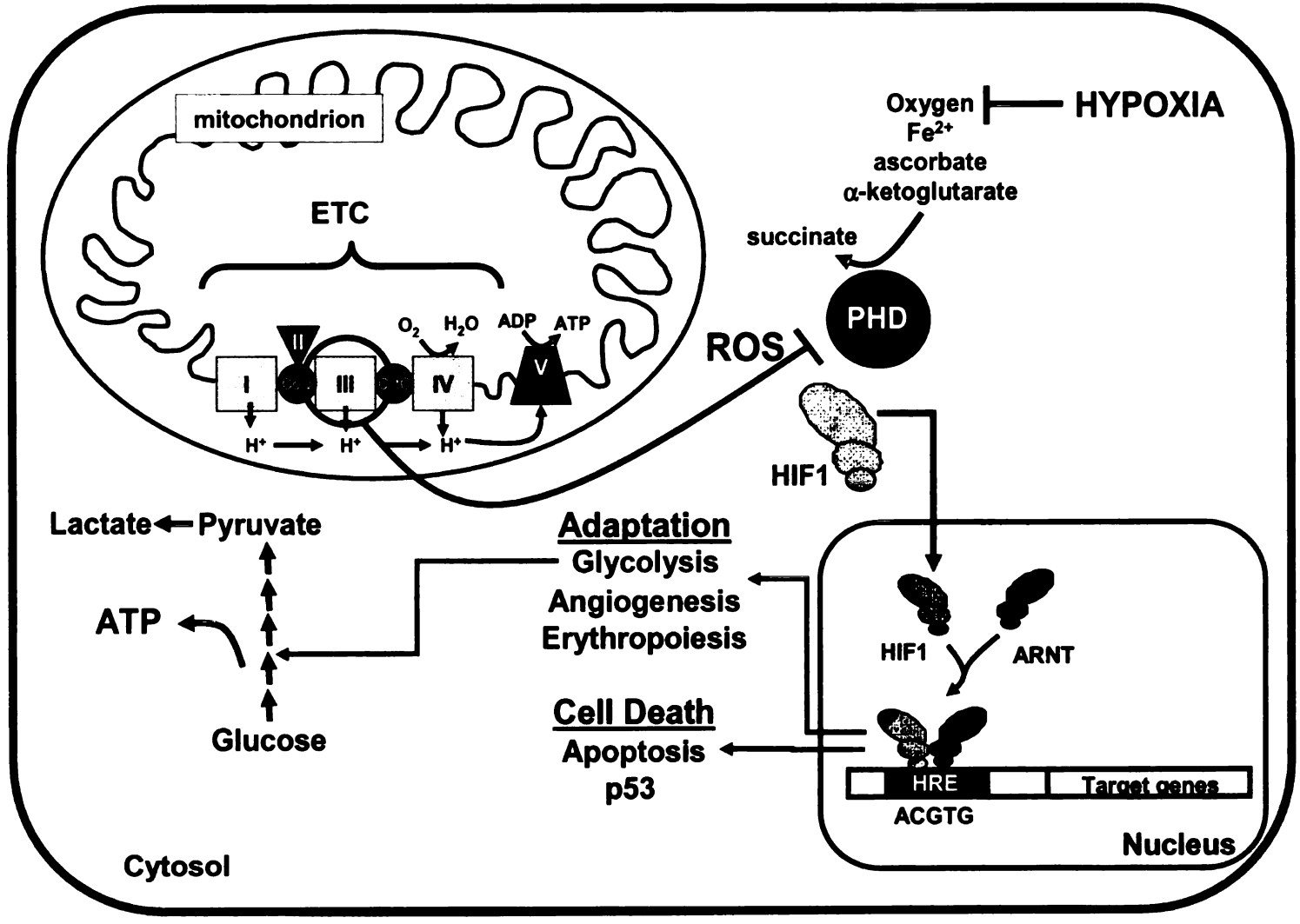

Oxygen
Fe2+
ascorbate
α-ketoglutarate
succinate
PHD
VHL
ubiquitin
-OH
HIF1

normoxia (Fig. B-1) (10, 34). The proline hydroxylations are required for the interaction of HIFα with the von-Hippel Lindau tumor suppressor gene product (pVHL). pVHL serves as the recognition component of E3 ubiquitin-ligase that leads to HIFα ubiquitination and proteosomal degradation (10, 34). The transcriptional activity of HIF1α is also reduced under normoxia by oxygen-dependent hydroxylation of asparagine 803 within the C-terminal transactivation domain (CTAD) of this transcription factor (82). Factor inhibiting HIF (FIH) mediates this modification of the conserved Asn residue, and the hydroxyl-asparagine prevents the binding of co-activator p300/CBP to HIF1α (72). This effectively removes the ability of HIF1α to direct p300-dependent transcription; however, it remains possible for the transcription factor to modulate transcription in a p300-independent manner, though this possibility has not been explored. These hydroxylases require four things for activity, oxygen, iron, α-ketoglutarate (αKG) and ascorbate. The enzymic process involves decarboxylation of the αKG to yield succinate and concomitant hydroxylation of the targeted residue (Fig. B-1). The oxygen, iron and enzymic reaction products play a critical role in regulation of the hydroxylase.

The hydroxylation of HIF1α, both PHD and FIH mediated, are inhibited under low oxygen conditions, leading to HIF1α accumulation and consequent induction of HIF1 activity (10, 34, 63, 72). Currently, there are two theories as to how the PHDs sense the decrease in cellular oxygen tension (Fig. B-2). First, since these enzymes

Figure B-2. The hypoxia signaling system

The hypoxia signaling cascade begins in the cytosol where the PHDs are continuously responding to local oxygen concentrations. Once the available oxygen concentration reaches some critical point, the PHD becomes inhibited. This can involve direct inhibition of the PHD due to loss of its molecular oxygen substrate or indirect inhibition due to changes in reactive oxygen species (ROS) produced at complex III of the electron transport chain (ETC). This change in ROS is caused by the inhibition of the ETC due to decreased oxygen, the terminal electron acceptor at complex IV, and subsequent accumulation of ubisemiquinone at complex III. Once the PHD is inhibited, HIF1α translocates to the nucleus where it interacts with ARNT and becomes transcriptionally active. HIF1 target genes include adaptive and cell death genes. The cellular increase in anaerobic metabolism leads to the overproduction of lactate and a decrease in cellular and local tissue pH.


mitochondrion
ETC
I
II
III
IV
V
O_2
H_2O
ADP
ATP
H^+
H^+
H^+
Oxygen
Fe^{2+}
ascorbate
α-ketoglutarate
HYPOXIA
succinate
PHD
ROS
HIF1
Lactate
Pyruvate
ATP
Glucose
Adaptation
Glycolysis
Angiogenesis
Erythropoiesis
Cell Death
Apoptosis
p53
HIF1
ARNT
HRE
Target genes
ACGTG
Nucleus
Cytosol

require oxygen for activity, hypoxia acts as a loss of substrate, and this might be all that is required for PHD inhibition. The loss of available oxygen would inhibit the enzyme's ability to modify HIF1α and thereby lead to increased transcription factor stability. The disparity between the oxygen requirement for these enzymes and the level of hypoxia needed to induce HIF1α stability in cells has led others to consider alternative mechanisms. In enzyme preparations, a progressive decrease in PHD activity from just below 20% O_2 induces an increasing stability of HIF1α. In contrast, HIF1α stability is not increased in most cell types until the oxygen levels are decreased to less than 7% O_2 (34, 139). In the stabilization of HIF1α, several laboratories have reported a role for reactive oxygen species (ROS) generated in the mitochondria. These experiments have documented an inability of mitochondria-deficient cells to induce HIF1α stabilization during hypoxic stress, suggesting a role for this organelle in the process (15). Recent reports have elaborated on this, establishing complex III as the likely site of action(11, 16, 51, 83). The proposed mechanism accounts for evidence implicating the mitochondrial respiratory chain in regulation of HIF activity (Fig. B-2). In respiration, electrons donated by NADH and $FADH_2$ flux through complexes I-IV of the electron transport chain (ETC) and are finally transferred to molecular oxygen at complex IV. Hypoxia causes ETC inhibition and the generation of ROS from a number of potential sites, such as complex I and the ubisemiquinone site of complex III. It has been proposed that this oxidative stress

within the cell disrupts the catalytic activity of the PHDs, possibly through inhibiting the ability of iron to cycle between oxidation states. In recent years, it has become evident that changes in ROS levels within the cytosol results in PHD inhibition, ultimately leading to the induction of HIF transcriptional activity (17, 48, 52). Regardless of which mechanism is involved in the inhibition of the PHD, hypoxia leads to HIF stabilization, and this begins the cell's attempt to adapt to the decrease in available oxygen.

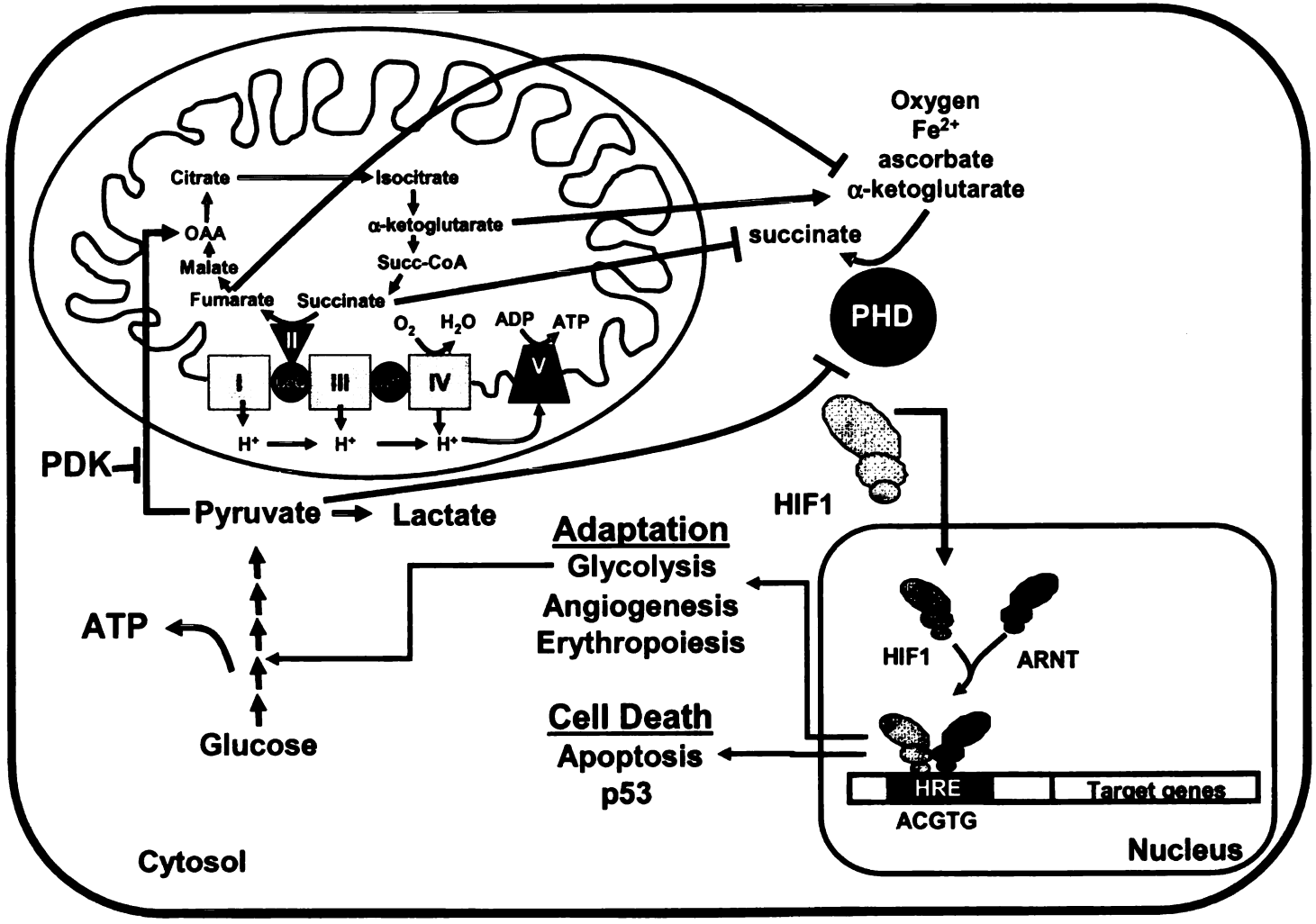
Hypoxia is not the only signal that can lead to HIF1α stabilization. It has long been known that a number of chemicals, such as cobalt, deferoxamine and the αKG analog, dimethyloxaloglutarate (DMOG), can induce the stabilization of HIF1 . experimentally (34, 84). They act by directly inhibiting PHD activity by either removing or competing with iron or αKG. More recently, a direct link has been established between key metabolites and HIF1α stabilization. As discussed above, PHDs require αKG for activity and, during the catalytic process, generate succinate. Recently, people with mutations in their succinate dehydrogenase enzyme (SDH, complex II of the ETC) have been demonstrated to display characteristics of a pseudo hypoxic response (8, 27, 111, 112). These people have a gene expression profile similar to that induced by hypoxia in normal humans, and affected people are prone to pheochromocytomas. In addition, people with mutation in fumarate hydratase (FH), a citric acid cycle enzyme, also display signs of aberrant hypoxia signaling (59). People

with FH mutations are also prone to certain types of cancer, and presumably this involves direct inhibition of the PHD through modulation of the decarboxylation step within the enzyme. The mutation in SDH would tend to increase intracellular concentrations of succinate, leading to PHD dysfunction via product inhibition (Fig. B-3). The FH mutation would be expected to lead to increased fumarate levels within the cell, and fumarate can act as a competitive inhibitor for the αKG binding site in PHD. Finally, the glycolytic end product, pyruvate, regulates HIF1α stability, and this activation might be responsible for the Warburg effect (77).

In the early part of the last century, Otto Warburg described a tumor's increased dependence upon fermentation or anaerobic glycolysis (148). He suggested that the increased glycolytic activity and metabolic adaptation might be the reason for the cellular transformation. The demonstration that pyruvate can activate HIF1α raises the possibility that it can participate in feed-forward activation of HIF1α, leading to a prolonged hypoxic signal. Recent reports have shown that the pyruvate dehydrogenase kinase (PDK) gene is a HIF1 target gene (67, 143). PDK is responsible for inactivating the pyruvate dehydrogenase complex, thus trapping pyruvate in the cytosol. This would further serve to prolong the hypoxic signal by maintaining the cytosolic concentration of pyruvate. The resultant extended signal might explain the Warburg effect and directly links HIF1α activation to increased glycolytic activity. These combined results suggest that a better characterization of the metabolic state of the cell,

Figure B-3. Link between intermediary metabolism and HIF1 signaling

Recent reports have established a direct link between key metabolic intermediates and HIF1 signaling. These metabolites include α-ketoglutarate (α-KG), which is necessary for PHD activity. Recently, it was demonstrated that patients with mutations in succinate dehydrogenase (Complex II of the ETC) display characterics of a pseudo-hypoxic state. Another link was established between fumarate disregulation and HIF1 signaling, and it was suggested that fumarate competes for α-KG binding within the PHD enzyme. Finally, pyruvate acts in a feed-forward mechanism to stabilize HIF1α in the absence of hypoxia. The recent characterization of pyruvate dehydrogenase kinase (PDK) as a HIF1 target gene might amplify this linkage between pyruvate and hypoxia signaling, since PDK over-regulation would serve to trap pyruvate in the cytosol.


Oxygen
Fe2+
ascorbate
α-ketoglutarate
succinate
PHD
Citrate
Isocitrate
α-ketoglutarate
Succ-CoA
Succinate
Fumarate
Malate
OAA
O2
H2O
ADP
ATP
II
I
III
IV
V
H+
H+
H+
PDK
Pyruvate
Lactate
HIF1
Adaptation
Glycolysis
Angiogenesis
Erythropoiesis
ATP
Glucose
Cell Death
Apoptosis
p53
HIF1
ARNT
HRE
Target genes
ACGTG
Nucleus
Cytosol

as well as oxygen and PHD levels, is required if the HIF signaling cascade is to be understood and targeted successfully by therapeutic agents.

Other modifications, in addition to hydroxylation, have been linked to hypoxia signaling. Acetylation of HIF1α at lysine 532 by arrest defective-1 (ARD1) acetyltransferase has been suggested to regulate HIF1α stability by enhancing the interaction between hydroxylated HIFα and pVHL; however, these interactions might be cell type specific (63,40). Phosphorylation is also involved in regulating HIF activity. During hypoxia, p42/p44 mitogen activated protein kinase (MAPK) phosphorylates HIF1α and enhances the transcriptional activity of HIF1 (97, 109). HIF activity is also regulated in an oxygen-independent manner at the HIF1α expression level by certain growth factors that ensure the maintenance of oxygen homeostasis in normal growing tissues. These responses result from the activation of signaling pathways involving phosphoinositide 3-kinase (PI3K)/AKT/mTOR that lead to the increases in the translation factor elF-4E, which in turn enhances HIF1α mRNA translation (109, 117). In summary, there are many ways in which HIF activity can be regulated, some of which are oxygen-dependent and others of which are not. The relative importance of these various conditions on normal and pathophysiology remains to be determined.

2.4. The integrative response to hypoxia

Multicellular organisms have developed sophisticated physiological

infrastructures to maintain oxygen homeostasis. Hemoglobin, a major constituent of red blood cells, is responsible for carrying oxygen to peripheral tissues, and the number of circulating erythrocytes is a major determinant of tissue oxygenation (12, 114). Decreased oxygen availability results in compensatory stimulation of erythropoiesis by upregulating the production of erythropoietin (EPO) (114). EPO enhances the production of red blood cells, and its hypoxia-induced transcription is regulated by HIF through an HRE site in the 3' flanking region in the EPO gene (6, 96). The hypoxia-induced expression of the EPO gene is thought to be part of a systemic response that an organism initiates to cope with the decrease in available oxygen. Angiogenesis is a tissue-oriented response to hypoxia and is critical to restoring oxygen transport to ischemic areas by improving blood supply to the affected tissues. Vascular endothelial growth factor (VEGF) is a central factor controlling physiological and pathological angiogenesis, and, in most cases, it is induced in response to hypoxia (138). Similar to EPO, the expression of VEGF is regulated by binding of HIF to HREs located in the 5'-flanking region of VEGF (125). In addition, VEGF receptors and the proangiogenic cytokine interleukin-8 are upregulated in response to hypoxia (37, 88).

The cellular response to hypoxia is focused on addressing the energy deficit created by the decrease in available oxygen. When aerobic metabolism is diminished due to the lack of molecular oxygen, the cell utilizes the HIF signaling cascade to

increase the expression of the complete battery of glycolytic enzymes, since it is the one place the cell can turn to address its energy debt. The glycolytic process is not as efficient as the ETC and ATP synthase in producing ATP, generating only 2 moles of ATP per mole of glucose, and this inefficiency can lead to impairment of physiological function (115). The increased dependence upon anaerobic glycolysis is coupled to the upregulation of various glucose transporters and other growth factors in an attempt to supply the cell with the necessary catabolic reagents to carry out this adaptive response (18). The importance of HIF in this switch to anaerobic metabolism has been well demonstrated with *in vivo* and *in vitro* models. Cells with a loss of HIF activity display reduced hypoxia-induced expressions of 13 different glucose transporters and glycolytic enzymes, compared with their wild type counterparts (18, 60, 144).

Finally, this adaptive response also involves HIF1-mediated transcription of the PHDs themselves (7, 20, 35, 144). This ability is PHD isoform- and cell type-specific. PHD2 and PHD3 are upregulated in response to hypoxia in a wide variety of cells, whereas PHD1 is only marginally regulated by loss of oxygen tension in most cells. There has been speculation that this HIF1-mediated upregulation of PHD functions as a shut-off mechanism for the hypoxic response once normal oxygen tension is restored. This might be one role for such feedback inhibition, however, there may be a more subtle role for this regulatory loop, i.e. that this feedback loop actually establishes the set-point for "normoxia" within the various cell types of the body.

The cells in various tissues and even within a single tissue are exposed to a wide range of oxygen tensions. For example, in the liver cells close to the portal vein and hepatic artery are exposed to a greater oxygen tension than those near the central vein, and yet each cell type perceives the oxygen concentration to which it is exposed as normal. It is possible that the decreasing oxygen concentration along the sinusoid leads to a partial activation of HIF1-mediated transcription of the appropriate PHD. The increased PHD levels along the sinusoidal oxygen gradient could compensate for reduced delivery of oxygen, similar to the way an increased receptor concentration can compensate for lack of available ligand in signal transduction. In this manner, the HIF1:PHD-linked transcription can establish the required level of HIF1 signaling necessary for the cell's microenvironment and normal cellular function.

2.5. The balance between adaptation and cell death

The adaptive response, including altered PHD levels and upregulation of EPO, VEGF and anaerobic glycolysis, is not always capable of addressing the hypoxia-induced metabolic imbalance. In these cases, the cell begins modulating various cell death pathways presumably as a mechanism to eliminate irrecoverably stressed cells (22, 123). This pathway involves HIF1-dependent transcription of various pro-apoptotic B-cell chronic lymphocytic leukemia (CLL)/lymphoma (BCL) family members, such as BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) and NIX (32, 93, 144). The activation of HIF is also able to contribute to the p53-mediated

cell cycle arrest and cell death by stabilizing p53 caused by cross-talk with alpha HIFs (1, 14). This increased "suicide" response seems in opposition to the adaptive response described above. It is currently thought that this pathway to cytotoxicity is an attempt by the cells to maintain the tissue as a whole. Removal of cells under severe hypoxic stress by programmed death could increase the chance of survival for neighboring cells by increasing nutrient and oxygen supply and maintaining appropriate tissue architecture.

One major, unanswered question concerning hypoxia is how this balance between adaptation and cell death is established and maintained. How does a cell know when it should survive through adaptive measures or when it needs to die because it cannot cope with the hypoxic environment? The intracellular regulatory system that makes this decision probably involves numerous factors, including oxygen levels of the cell, metabolic state, levels of key metabolites and growth factors, and the endogenous activity of the PHDs. The latter can serve as a master regulator of a cell's fate under hypoxic response by the same mechanism that it uses to establish normoxia (Fig. B-4). For example, under normal oxygen concentrations, PHD and glycolytic enzymes are at maintenance levels, i.e., their levels are maintained at those required for normal cellular function and oxygen-sensing capacity.

When the cell is exposed to moderate hypoxia, the expression of PHDs and glycolytic enzymes is increased to cope with the detrimental environment (Fig. B-4).

Figure B-4. Relationship between oxygen concentration and HIF1 target gene expression

At normal oxygen tensions, the PHDs are capable of initiating the degradation of HIF1α and expression of HIF1 target genes, including glycolytic enymes, PHDs and apoptotic genes, are maintained at low levels (**top panels**). As the oxygen concentration becomes moderately decreased, PHD activity is diminished for lack of available substrate and some HIF1 accumulates and drives the expression of target genes. These genes include PHDs and glycolytic enzymes, levels of which will be recalibrated to the new metabolic conditions and thus encoding a new "normoxia" (**middle panels**). Finally, under severe hypoxia, PHDs activity is incapable of compensating for the loss of oxygen and HIF1 signaling becomes hyper-activated, leading to expression of pro-apoptotic factors and cytotoxicity (**bottom panels**).

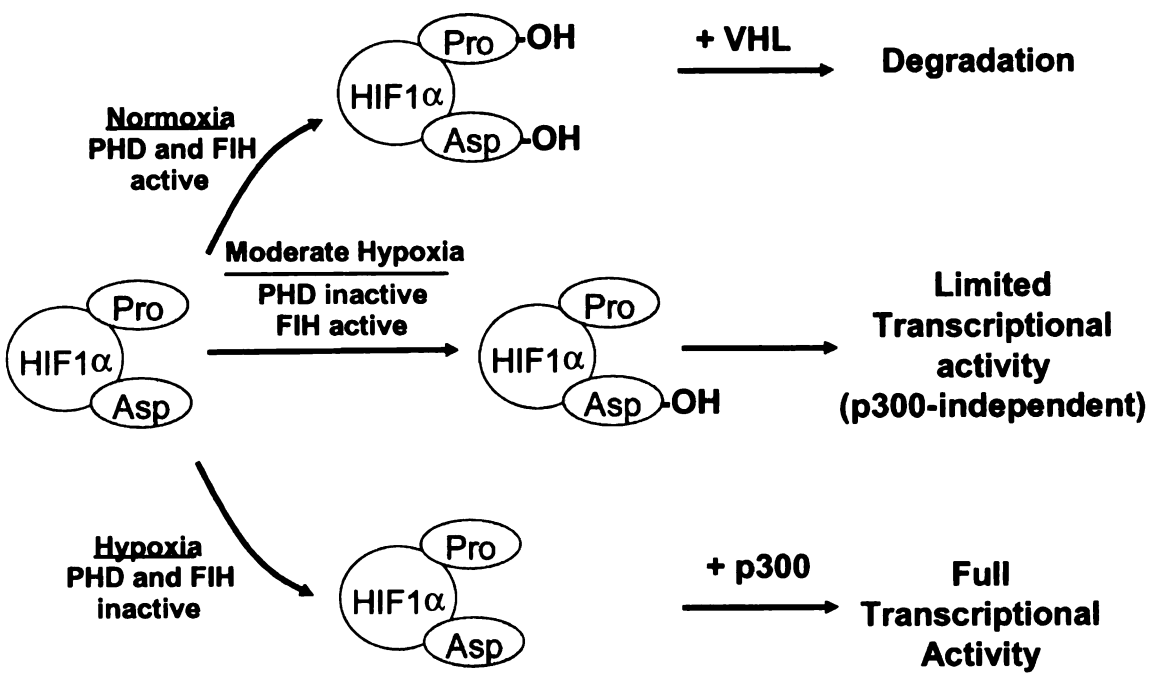
Activity of the PHD Enzyme
Normoxia
Mild Hypoxia
Severe or Prolonged Hypoxia
Cellular pO2
Expression of Three Gene Groups
PHD
Glycolytic
Apoptotic
Time
Under normal oxygen tension the three classes of genes are maintained at basal levels (PHD and FIH active)
Small decrease in oxygen tension is compensated for by increases in PHD levels and mild adaptive response (PHD inactive, FIH active)
Large decrease in available oxygen cannot be compensated leading to a full blown apoptotic response (PHD and FIH inactive)

It is possible that cell death factors are upregulated in the short term; however, once the PHD activity is increased in the cell and the energy balance is restored, these genes are turned off and cell death will be avoided. It is likely that these genes are not expressed under moderate hypoxia from which the cell can recover. If so, this would suggest that HIF1-target genes require complex transcriptional regulatory mechanisms. It can be hypothesized that these transcriptional mechanisms could involve the different oxygen requirements of the PHD and FIH enzymes, as PHD requires a greater oxygen concentration for function than FIH (Fig. B-5) (130). This raises the possibility that, at certain lowered oxygen tensions, HIF1α could be stabilized because the PHD is inhibited but unable to interact with p300, while FIH is still capable of hydroxylating the critical asparagine (Fig. B-5). This would establish a stable HIF1α transcription factor that regulates p300-independent transcription, thereby serving as the selective switch. Indeed, using p300 mutant mice, Kasper et al. demonstrated that HIF1α is capable of modulating transcription in a p300-independent manner (65). In further support of this model, Dayan et al. have recently established that FIH regulates distinct genes sets and characterized a number of genes that are susceptible to oxygen dose-dependent transcription (30).

Under severe hypoxic stress, increased HIF1-mediated transcription of PHDs is unable to compensate for the loss in available oxygen (Fig. B-5). At these oxygen levels, PHDs and FIH are inhibited and this leads to HIF1α-mediated regulation of its

Figure B-5. HIF1α stability and transcriptional activity is controlled by hydroxylation

HIF1α contains conserved prolines (Pro) and asparagines (Asp) that are important for its regulation. Prolyl hydroxylation, via PHD, controls the ability of VHL to direct HIF1α degradation (**top**). PHDs require more oxygen than FIH for activity. Therefore, it is possible that HIF1α could be found in a stable form (with asparagine hydroxylation but without proline hydroxylation) and unable to bind to p300 (**center**). Finally, once the hypoxia becomes severe, both FIH and PHD may be inhibited, and HIF1α would become fully activated (**bottom**).


Pro
OH
HIF1α
Asp
OH
+ VHL
Degradation
Normoxia
PHD and FIH
active
Moderate Hypoxia
PHD inactive
FIH active
Pro
HIF1α
Asp
Pro
HIF1α
Asp
OH
Limited
Transcriptional
activity
(p300-independent)
Hypoxia
PHD and FIH
inactive
Pro
HIF1α
Asp
+ p300
Full
Transcriptional
Activity

complete library of target genes, including those expressing cell death factors (Fig. B-4). This unmitigated expression leads to cell death. Obviously, this concept is simplified since the HIF-mediated adaptive responses are probably more predominant in certain tissues and cell types under various environmental conditions. For example, macrophages seem to survive and function in hypoxic tissue under conditions in which death of parenchymal cells occurs, and this ability to survive depends on adaptive responses mediated by HIFs (13, 25). Conversely, hypoxia-stimulated cell death may be more important beyond the boundary of oxygen deprivation. Understanding the factors involved in how HIF signaling establishes this balance is critical to our ability to manipulate the system in a meaningful therapeutic sense. Drugs that target this pathway must consider the target tissue and the factors involved within this tissue in establishing this balance. Finally, this balance will also be critical to toxicity of any compound that disrupts blood flow and to the pathology of any disease, such as cancer, that unnaturally creates a hypoxic environment.

3. Hypoxia and pharmacological implications

The role of hypoxia and HIF1 in cancer, stroke and cardiovascular disease has made it an attractive target for drug design. As discussed above, however, the HIF1-regulated balance between adaptation and cell death contains many inputs, such as PHD activity, oxygen concentration and metabolite concentrations. Targeting this pathway for viable drug candidates must take these inputs and their effects on this

balance into consideration. For example, given that HIF1 is upregulated in various cancers, inhibiting HIF1 signaling directly might eliminate the ability of tumors to promote an adaptive response. Conversely, patients at risk of stroke-induced hypoxia/reoxygenation injury might benefit from HIF1 activation to promote adaptive or neuroprotective effects. Moreover, the input signals that regulate HIF1 activity are likely to vary among tissues and even among cells within the same tissue. Finally, though HIF1 is the focus of most of the research related to this field and the focus of this review, it can be assumed that HIF2α and HIF3α are also targeted by these same pharmaceutical paradigms. A successful drug candidate must be able to cope with these differences and still give the expected pharmacologic outcome within the target tissue.

3.1. Hypoxia-related drug therapy

Most investigations have focused on targeting hypoxia and the HIF signaling cascade for cancer therapeutics. The goal of these new therapies is to manipulate the HIF signaling pathway to inhibit tumor growth or to exploit the hypoxic microenvironment of a tumor to make the therapies more specific and efficacious. There are four major areas of research in this field: 1) designing drugs that directly inhibit HIF1 signaling, 2) influencing other signaling cascades that indirectly alter HIF signaling, 3) exploiting the hypoxic microenvironment to increase specificity and decrease toxicity, 4) altering regulation of HIF target genes that are critical for tumor

growth or for the function of their protein products (Table B-1).

Designing drugs that directly inhibit HIF1 signaling is not as easy as targeting enzymatic processes. There are five principal ways that HIF1 can be selectively targeted by therapeutics. The first direct mechanism involves specifically inhibiting HIF1α transcription or translation (75, 133). PX-478, a drug developed by ProlX Pharmaceuticals is billed as the first drug that selectively targets HIF1α and has shown efficacy in antitumor assays (81, 150). The second direct mechanism is to inhibit HIF1α's ability to translocate to the nucleus. Currently, there are no drugs that have exploited this avenue, and this is most likely because the process is not well understood. Third is to block HIF1α's ability to interact with ARNT. This strategy has been used experimentally in the design of HIF-dominant negative constructs. These constructs lack a transactivation domain but are still capable of binding ARNT. This sequesters the ARNT pool in a transcriptionally deficient complex, thereby inhibiting normal hypoxia-induced gene transcription (41). Eliminating HIF1's ability to bind DNA would eliminate its ability to regulate its target genes transcriptionally (98). Echinomycin, a quinoxaline family antibiotic, inhibits HIF1α DNA binding; however, it has not been determined if this is the only effect of echinomycin that makes it an attractive cancer chemotherapy test compound, since it also induces apoptosis in certain cell types (68). Finally, inhibiting HIFs ability modulate transcription by blocking its ability to interact with the basic transcriptional machinery would also

Table B-1. Drugs that target the hypoxia signaling cascade

Class of drug	Name	Action
Direct inhibitor	PX-478	decreases HIF expression
	Echinomycin	inhibits DNA binding
	103D5R	decreases HIF expression
	Dominant negative	inhibits ARNT binding
	siRNA	decreases HIF expression
	Chetomin	inhibits HIF1α/p300 interaction
Indirect inhibition	BAY-43-9006 (Sorafenib)	Raf Kinase inhibitor increased
	Berberine	HIF1α protein degradation
	CCI-779 (Temsirolimus)	mTOR inhibitor
	Dibenzoylmethane and others	iron chelators
	dimethyloxaloylglycine (DMOG)	non-selective prolyl hydroxylase inhibitor
	FG-4095	selective PHD inhibitor
	Flavopiridol	cyclin-dependent kinase inhibitor
	Geldanamycin, Apigenin, Radicicol and others	Hsp90 inhibitors
	OSI 774 (Tarceva(erlotinib))	EGFR inhibitor
	PD98059	MEK inhibitor
	PS-341 (Bortezomib)	proteosome Inhibitors
	SN38 (CPT-11 (irinotecan))	topo I inhibitor
	STI571 (Gleevac (Imatinib))	c-KIT inhibitor
	trastuzumab (Herceptin)	HER2 inhibition
	Trichostatin A	histone deacetylase
	Vincristine	microtubule disruption
	YC-1	soluble guanylate cyclase activator
	ZD-1839 (Iressa® or gefitinib)	EGFR inhibitor
Hypoxia-regulated genes	2-Methoxyestradiol	anti-angiogenic
	AG013736	VEGFR inhibtor
	Bevacizumab (Avastin)	VEGF inhibitor

	CEP-7055	VEGF inhibitor
	CP-547,632	VEGFR inhibtor
	hypericin	anti-angiogenic
	PTK787 (Vatalanib)	VEGFR, c-Kit, PDGFR inhibitor
	SCH66336 (farnesyltransferase inhibitor)	anti-angiogenic
	SU11248 (Sutent)	VEGFR, c-Kit, PDGFR inhibitor
	ZD6474 (Zactima)	VEGFR, EGFR inhibitor
Hypoxia-activated agents	2-Cyclopropylindoloquinones	hypoxia selective cytotoxin
	AQ4N	hypoxia selective cytotoxin
	KIN-847(2-nitroimidazole 1-acetylhydroxymate)	hypoxia selective cytotoxin
	KS119	hypoxia selective cytotoxin
	NLCQ-1	hypoxia selective cytotoxin
	Quinoxaline 1,4 dioxides	hypoxia selective cytotoxin
	Tirapazamine	hypoxia selective cytotoxin
	TX-402	hypoxia selective cytotoxin

serve to eliminate hypoxia-mediated activity. For example, chetomin, a natural product from the fungus *Chaetomium,* disrupts the interaction of HIF1α with p300 (Kung et al., 2004). Therapies that directly influence HIF signaling comprise a relatively small category for hypoxia-centered drug design.

The second and largest category of drugs that target hypoxia signaling influence HIF1 signaling indirectly. Most of these compounds were not originally intended to influence HIF1 signaling but have been found to modulate HIF1 signaling at some level. Recent work in this area has surrounded mammalian Target of Rapamycin (mTOR) and its role in HIF1α translation. Inhibition of mTOR by CCI-779 decreases HIF1α and other protein levels and is linked to decreased growth in certain cancer models (46, 135). This indirect-acting class also includes compounds that influence HIF1 by inhibiting Hsp90 function. Geldanamycin, apigenin and others can increase HIF1α signaling at low doses but decrease HIF1-mediated transcription at larger concentrations (36, 58, 79, 87, 91). The results suggest that Hsp90 might play a crucial role in several different facets of HIF1 signaling, and the success of targeting Hsp90 for cancer therapy might depend upon how much drug can be delivered to target (57). The final group of drugs that fall within this indirect category are the prolyl hydroxylase inhibitors. Like Hsp90 inhibitors, these drugs increase HIF1 signaling and include direct prolyl hydroxylase inhibitors, such as dimethyloxaloylglycine, and iron chelators, such as dibenzoylmethane (3, 80). These drugs increase HIF1 signaling by

removing the enzymic activity necessary to direct HIF1α degradation. The hydroxylase inhibitors work by competing for the α-ketoglutarate binding site within the PHD enzyme, whereas iron chelators remove the metal necessary for catalytic activity. These types of inhibitors are neuroprotective in models of hypoxia/reoxygenation injuries (31, 158).

Though this class of indirect inhibitors is currently the largest, it is not the one making the most impact for patients. Drugs designed to inhibit HIF1 target genes have gained FDA approval and are currently in various phases of clinical trials, alone or in combination therapy, for the treatment of a wide range of cancer types. These drugs focus on the tumor's dependence on angiogenesis for oxygen and nutrients. Several critical growth factors necessary for this vascularization response are hypoxia target genes. Most notable is VEGF. The most widely recognized of these drugs is bevacizumab (Avastin), a recombinant antibody designed to inhibit VEGF function (39). Bevacizumab is currently the only approved drug in this class and has shown promise in the treatment of colon cancer but only limited efficacy in breast cancer when used as a single agent. Efficacy seems to be consistent across this class of compounds, and they all may prove efficacious when used in conjunction with other therapeutics. The majority of these anti-angiogenic drugs fall under the class of receptor tyrosine kinase (RTK) inhibitors (38, 100). These RTK inhibitors have various degrees of selectivity toward the receptors necessary for neovascularization, such as

VEGF receptor and the PDGF receptor (103).

One extension of this class of drugs that target HIF1-controlled genes is the use of HRE-driven suicide genes as gene therapy agents (24). These agents utilize knowledge of genomic structure of the regulatory regions of hypoxia-inducible genes to engineer gene therapy vectors to deliver cytotoxic genes, such as herpes simplex virus thymidine kinase (121, 147). These vectors contain multiple copies of HREs derived from the VEGF or EPO gene promoters and in some cases additional regulatory sequences. This type of approach is not limited to suicide constructs for the treatment of cancer; it has also been explored to deliver angiogenic factors to the ischemic heart in a hypoxia-inducible fashion (24, 131).

The final class of drugs actually exploits the hypoxia signal itself. These compounds are hypoxia-activated cytotoxins and come in three main varieties, including oxides, quinones and nitroheterocyclics (19, 92, 118). Each of these drugs are nontoxic at physiological oxygen tension because cells are capable of reoxidizing the reduced intermediate with available molecular oxygen. Under hypoxic conditions, however, this futile cycle is inhibited due to the lack of available oxygen, and the reduced intermediate reaches concentrations that are able to cause cellular damage by interacting with various macromolecules, especially DNA. These drugs require activation by cytochromes P450 and P450 reductases. The most widely studied of these is tirapazamine, an aromatic N-oxide that is currently in clinical trials in

combination therapy for treatment of various solid tumors (70).

Taking advantage of the hypoxic environment as a drug target has its limitations. The efficacy of these therapeutics depends upon the level of hypoxia, the cells exposed, the tissue's normal pO_2, and levels of P450 and P450 reductases and the activation range of the drug itself. To begin to cope with these limitations, these bioreactive compounds are now being used in conjunction with gene therapy vectors (24, 99, 145). This type of gene-directed enzyme prodrug therapy (GDEPT) couples the bioreactive drug, such as tirapazamine, with gene delivery vectors that express different P450-related enzymes, such as NADPH cytochrome P450 reductase (24). These therapies have also exploited HRE-driven gene constructs, as described above, to insure that the enzyme is only expressed in hypoxic tissues, where the drug should be activated.

Development of each of these compounds as drugs has utilized our understanding of the hypoxic environment and the HIF1 signaling system to target hypoxic tissues. These therapies, however, are still restricted by our limited knowledge of the ability of HIF1 to regulate metabolic flux and how the resulting changes in metabolism ultimately link back to HIF1 activity. In addition, these compounds might also be limited in their efficacy by several factors that make hypoxic tissues refractory to conventional therapies, such as poor circulation, decreased cell cycle progression, and changes in drug detoxifying enzyme profiles.

3.2. Hypoxia and drug disposition

Hypoxia influences drug effectiveness on both systemic and local levels. For example, hypoxic patients have different clearance rates for a variety of drugs compared to normoxic patients (42). A decrease in blood oxygenation influences the distribution and effectiveness of drugs in part by changing the expression pattern of many phase I drug metabolizing enzymes. These effects vary for each particular enzyme, and the pattern of enzyme expression ultimately influences how each particular drug will be affected. A wide range of studies have been performed on human patients suffering from diseases that influence blood oxygenation, including chronic obstructive pulmonary diseases. These studies have documented hypoxia-related changes in drug clearance for theophylline, tolbutamide, and ethanol, to name a few (26, 45, 128, 129). All of this research points to potential toxicity or inadequate pharmacological effectiveness if hypoxia-associated diseases are not taken into consideration in the determination of dosing regimen.

Hypoxia can also influence drug disposition by altering systemic blood flow distribution. Since drugs are delivered to the liver in the blood, changes in hepatic blood flow will influence the rate at which toxicants or drug substrates reach the liver to be metabolized. Indeed, the hepatic metabolism of many drugs is limited by liver blood flow rather than the amount of enzyme(s) in the liver that are responsible for their metabolism (90). Breathing atmospheres of lowered pO_2 ("hypoxic hypoxia") causes redistribution of blood flow away from the splanchnic area, resulting in a

decrease in liver perfusion. Thus, in addition to reducing metabolism by limiting O_2 as a cofactor for drug oxidation, hypoxia can diminish drug clearance through its propensity to reduce drug delivery to the liver by decreasing hepatic blood flow. Altered hepatic blood flow has its greatest effect on drugs with high extraction ratios. For example, the hepatic clearance of hexobarbital, a drug eliminated by cytochrome P450 oxidation, was markedly reduced by exposure of rats to 8% O_2, resulting in prolongation of the anesthetic effect of the drug (101). This was associated with a marked reduction in liver blood flow (102). Interestingly, an equal decrease in blood oxygenation caused by breathing a carbon monoxide-containing atmosphere did not result in reduced hepatic blood flow and caused a less pronounced decrease in hexobarbital clearance. Thus, hypoxia can influence drug disposition by altering delivery of the drug to the liver for metabolism, and the means by which hypoxia is produced can influence its effectiveness in decreasing drug oxidations *in vivo*.

3.3. Hypoxia and Cancer Chemotherapy

It has long been known that the hypoxic nature of solid tumors limits the efficacy of chemo- and radiotherapies. The decreased level of molecular oxygen within the central portion of the tumor limits the ability of radiation therapy to create oxygen free radicals, which is the basis of the pharmacological efficacy. It has been estimated that hypoxic cells are approximately 3 times less susceptible to radiation-induced damage (134).

The limitations of chemotherapy are influenced both by the decrease in available oxygen at the level of the organism as well as site-specific loss, such as in the central portion of the tumors. A decrease in tissue oxygen tension can influence therapies in at least four major ways. First, as mentioned above, changing expression of the P450s within hypoxic tissues can serve to increase or decrease a drug's effectiveness. In addition, low oxygen concentration can influence the catalytic activity of the P450s by removing a required cosubstrate. Second, distribution of the drug can be decreased within the affected tissue due to inadequate vascularization and perfusion (95). For example, within a tumor, the main cause of the localized hypoxia is an inability of the angiogenic process to keep up with the fast-growing tumor cells. The rapid growth of the tumor also leads to misformed and leaky vessels that cannot support proper function. Third, the decreased oxygen tension can lead to a decrease in cell cycle progression. In fact, hypoxic conditions can lead to complete cell cycle arrest (44). A wide range of cancer therapeutics require rapidly dividing cells for efficacy, and hypoxia-induced removal of cells from the cell cycle will limit their effectiveness. Finally, the changes in intermediate metabolism that occur as a result of hypoxia can also influence drug metabolism and effectiveness. For example, as discussed above, the HIF1 signaling system is a metabolic switch that can lead to tissue lactic acidosis, especially within a tumor. Changes in local pH can influence drug delivery and metabolism. Each of these four factors will influence a drug's effectiveness in the

hypoxic microenvironment and, in the case of a tumor, they might ultimately determine prognosis.

The hypoxic microenvironment within a tumor might also select for a more aggressive phenotype. It has been shown that hypoxia increases the rate of mutations within a cell (156). This increased mutation rate might promote an already partially compromised cell to full transformation, or it might start a normal cell down the path to full tumorigenic potential. It has also been suggested that hypoxia can select for mutant forms of p53 (49). This selective pressure within the tumor could lead to tumor cells that have removed themselves from one principal regulator of cellular homeostasis. In addition, prolonged hypoxia can select for cells that are resistant to pro-apoptotic signals, thus eliminating one potential mechanism of drug-induced cell removal (152). Finally, the inability of therapy to kill all of the cells within a tumor due partly to a combination of the systemic and localized effects of hypoxia could produce cells refractory to later treatment. Indeed, each of these factors might play a role in the ability of hypoxia to promote more aggressive tumors and ultimately increase the chance of a poor prognosis.

4. Hypoxia and toxicity

It is well known that many toxicants act by producing tissue hypoxia. These include agents that cause respiratory depression, such as CNS depressants or convulsant chemicals, irritant gases that injure the respiratory tract and impair the

ability of the lungs to transfer O_2 to the blood, and chemicals that cause tissue ischemia, such as cocaine and ergot alkaloids. Chemicals that impair the ability of hemoglobin to act as an oxygen carrier also lead to tissue hypoxia. These include agents such as carbon monoxide, which competes with O_2 for hemoglobin binding, and methemoglobin-forming chemicals, such as nitrites. Although they act by different mechanisms, all of these agents are toxic because they reduce tissue pO_2 and thereby impair delivery of oxygen to the mitochondrial electron transport chain (50).

Other agents act at the level of the mitochondrion by interfering with electron transport, inhibiting ATP synthase or dissipating mitochondrial membrane potential. For example, cyanide is toxic because it binds to cytochrome oxidase, thereby inhibiting the transfer of electrons to O_2. All of these agents reduce ATP biosynthesis, so that many of their clinical effects resemble those of hypoxia. For this reason, their effects have often been called "chemical hypoxia." This is an inappropriate term that should be avoided, since tissue pO_2 during intoxication is not decreased. For example, in cyanide intoxication, tissue pO_2 does not decrease, but rather increases due to the inability of cells to consume oxygen as the terminal electron acceptor in the respiratory chain. Accordingly, cyanide and other such agents could have markedly different effects on hypoxia signaling than true hypoxia because there is no decrease in cellular oxygen concentration to drive PHD-mediated stabilization of HIFs.

Although it is clear that severe hypoxia from toxicant exposure can cause tissue

injury by itself, nontoxic decreases in cellular pO_2 can promote or unveil harmful effects of other agents. For example, panadiplon is a drug that causes idiosyncratic liver injury in humans. *In vitro*, this drug was only weakly toxic to hepatocytes, unless the hepatocytes were made hypoxic, in which case cytotoxicity became pronounced (5). By now, many drugs and other chemicals have been shown to have hypoxia as a component of their direct toxicity or as a progression factor that promotes worsening of tissue lesions and functional impairment. Indeed, much has been written on hypoxia as a factor in chemical intoxication. The remainder of this section will focus primarily on the role of HIF signaling in toxic responses.

To analyze the roles of hypoxia and HIF1 in the toxicities of various agents, careful consideration must be made of the balance that is established within the cell with regard to hypoxia signaling and the life and death decision. As described above, HIF1 mediated signaling can promote an adaptive response, which promotes cell and tissue survival through the upregulation of genes such as glycolytic enzymes and VEGF-induced angiogenesis. At the same time, HIF1 can promote cell injury by upregulating cell death genes such as BNIP3 and NIX and by influencing p53-mediated processes. Very little is known about how this balance is maintained within the cell or how it is initially established. Any toxicant-induced upset of this balance can lead to adverse cellular events by either overstimulation of the prodeath response or inhibition of the adaptive response or both. In addition, toxicity might be a result of

direct toxicant-induced HIF1 activation or an indirect action that alters HIF signaling. For example, a toxicant might stabilize HIF1α, leading to overstimulation of the prodeath response. Alternatively, a toxicant might indirectly overstimulate the hypoxic signaling system by influencing phosphorylation events or cofactors required for HIF signaling.

4.1. Link between HIF1 activation, metal-induced toxicity and the prodeath response

One example of how direct activation of hypoxia signaling can cause cytotoxicity involves divalent metals. Three metals, cobalt, nickel and manganese, are recognized as hypoxia mimics, and recent research has focused on the role of hypoxia signaling in the ability of these metals to cause cellular injury (106, 108, 142). These metals inhibit PHD function and lead to HIF1α stabilization. Currently, two different mechanisms have been proposed for their inhibitory activity. First, it is suggested that these metals directly compete for the iron binding site within the enzyme and render it inactive (47). Second, it has been proposed that they chelate free ascorbate within the cell, leading to PHD inhibition (107). Regardless of their mechanism of action, it is clear that exposure to these metals leads to HIF1α stabilization and to the regulation of a battery of genes similar to the changes caused by hypoxia. The expression profile following exposure to these metals does not completely overlap that of hypoxia (143). In the case of nickel and cobalt, however, there is enough similarity with the hypoxia

profile to suggest that a significant portion of the toxic effects of these metals may involve HIF1 signaling (106, 142). These experiments utilized engineered mouse embryonic fibroblasts that were incapable of expressing HIF1α to show that the hypoxia signaling cascade plays a critical role in these processes. These experiments further identified a set of gene products that might be responsible for this pheonotype, including several pro-apoptotic factors (144). These results support the idea that unregulated HIF1 signaling can promote cytotoxicity by altering the balance between the adaptation and cell death responses. The established link between HIF1 over-activation and toxicity suggests a need for caution when considering prolyl hydroxylase or iron chelators for therapeutic purposes. These types of drugs could have inherent toxicity because of their ability to promote a similar response to that of cobalt and nickel.

4.2. Indirect toxicity and unregulated hypoxic signaling

A more common mode for hypoxia to influence toxicity is through secondary mechanisms. Drugs or toxicants that produce tissue injury can cause disruption of blood flow. This disruption can lead to tissue injury downstream of the initial insult caused, in part, by local hypoxia. This type of injury is thought to play a central role in a phenobarbital/carbon tetrachloride-induced model of liver cirrhosis (141). Moreover, carbon tetrachloride-induced liver injury can be potentiated by hypoxia cotreatment (120). Oxygen deprivation is also thought to play a role in ethanol-induced

hepatoxicity (137). Ethanol-induced hypoxia involves an increase in liver oxygen consumption and metabolic activity ("hypermetabolic state") and results in pericentral liver hypoxia and necrosis (2). Interestingly, the mode of action involves Kupffer cell activation, since pre-treatment with gadolinium chloride ($GdCl_2$) inhibits the hepatocellular injury. $GdCl_2$, an agent known to destroy Kupffer cells, inhibits ethanol induced changes in intracellular metabolism and oxygen utilization, suggesting that Kupffer cell activation drives the localized hypoxia and is necessary for ethanol-mediated liver injury (136). Endotoxin-induced liver damage also involves disruption of blood flow. An animal model of sepsis-induced liver damage was studied and shown to involve inadequate oxygenation of the tissue (62). Moreover, endotoxin hepatotoxicity is potentiated by breathing a low pO_2 atmosphere (122).

The role of HIF signaling in drug toxicity seems to be emerging as an area of interest. A recent study found that HIF1α in liver increases during acetaminophen hepatotoxicity in mice (61). This activation begins before the onset of hepatocellular injury and also occurs in isolated hepatocytes incubated with acetaminophen in an oxygen replete atmosphere. *In vitro*, inclusion of cyclosporine A, an inhibitor of the mitochondrial permeability transition and the oxidative stress that occur in this model, prevented the increase in hepatocellular HIF1α.

The hepatoxicities of other medicinal agents also appear to involve hypoxia and/or HIF-signaling. For example, pyrrolizidine alkaloids are hepatotoxic constituents

of certain herbal medicines. One of these, monocrotaline, caused vascular disruption, hemostasis and hypoxia in livers of exposed rats (Copple. et al., 2006). Moreover, expression of the HIF-regulated cell death factor, BNIP3, was increased (23). Nitrofurantoin is an antibiotic that causes idiosyncratic hepatotoxicity in human patients. In rats, treatment with this drug increased HIF-1 binding to DNA in liver, suggesting that HIF signaling is activated during nitrofurantoin exposure (132).

Small, nontoxic doses of bacterial endotoxin (lipopolysaccharide, LPS) that cause modest inflammation potentiate the toxicity of many hepatotoxicants and lower the threshold for toxicity of several drugs (43). For example, ranitidine is a drug that causes idiosyncratic hepatotoxicity in humans but is not hepatotoxic in rats. However, rats develop liver injury when cotreated with ranitidine and LPS at doses that are not hepatotoxic when given alone (78). Accordingly, a modest inflammatory stress appears to be a susceptibility factor for drug hepatotoxicity, and this might be the basis for some idiosyncratic adverse drug reactions in people (43). In livers of LPS/ranitidine-cotreated rats, hemostasis occurs around the time of onset of liver injury, and this is accompanied by liver hypoxia and increased expression of HIF1α (Luyendyk et al., 2004). Moreover, the hepatic expression of hypoxia-regulated cell death factors such as BNIP3, EGLN3, Nr4a1 and RTP801 was selectively enhanced (Luyendyk et al., 2006). Heparin administration reduced both the hepatocellular injury and liver hypoxia, suggesting the possibility that hypoxia could be an important factor in the pathogenesis

(Luyendyk et al., 2005). These results suggest that certain drugs interact with an inflammatory stress to precipitate liver hypoxia and HIF signaling that leads to expression of proapoptotic factors and death of hepatocytes. Together, these studies with drugs have revealed associations between hepatotoxcity and HIF activation in livers of drug-treated animals; however, much remains to be learned about the role of HIF signaling, if any, in the pathogenesis of adverse drug responses.

Drug-induced decreases in blood oxygenation can also affect development. The toxicity of phenytoin, an antiepileptic drug and human teratogen, is linked to hypoxia-induced malformations during development (28). Fetuses from phenytoin-treated rats show a drastic decrease in heart rate and increased risk for various abnormalities, including craniofacial defect and impaired growth. These effects could be partially ameliorated by treatment with hyperoxia, suggesting that systemic hypoxia is involved (86). Similar results were found with other antiepileptic drugs that affect human ether-a-go-go related gene (hERG) channels, and this has led to the suggestion that cardiac arythmias and hypoxia are the cause of teratogenicity of this class of compounds (4, 29). These results emphasize the critical role of oxygen homeostasis during development and suggest that disruption of the balance between adaptation and cell death has severe consequences during fetal maturation.

5. Neuronal protection by stimulation of the hypoxia adaptive response

The balance between adaptation and cell death can be manipulated to offer

protection against subsequent hypoxic challenges. Most of this work has involved neuronal protection against severe ischemic injury by pre-exposure to low-level hypoxia ("hypoxia preconditioning"). *In vitro* and *in vivo* data suggest that upregulation of HIF1α signaling is involved in this protection (76). VEGF and EPO, two primary HIF1 target genes, play a critical role in this process (104, 151). In fact, EPO offered some protection on its own. EPO is a growth factor that has been studied for its hematopoietic regulatory function; however, recent studies have suggested a broader role for this hypoxia target gene. Neuroprotection from ischemia by EPO requires pretreatment, lasts for approximately three days and might involve activation of phosphoinositide-3-kinase or mitogen activated protein kinases (149). In addition, using primary neurons, Ruscher et al. have shown that EPO is critical to establishing a paracrine signaling pathway that mediates this protection (104). These studies suggest that prior adaptation to hypoxia can increase cell survival that likely involves HIF1α-mediated transcription. These treatments have extended to iron chelation and prolyl hydroxylase inhibition for neuroprotection (89, 126).

Inhibition of this adaptive response could limit a cell's survival chances under stress. In a neuronal model of ischemic damage, the glycolysis inhibitor, iodoacetate, potentiated glutamate-induced damage (85). The action of iodoacetate, a glyceraldehyde-3-phosphate dehydrogenase inhibitor, could be prevented by pyruvate cotreatment, suggesting that maintenance of metabolic flux into the mitochondria is

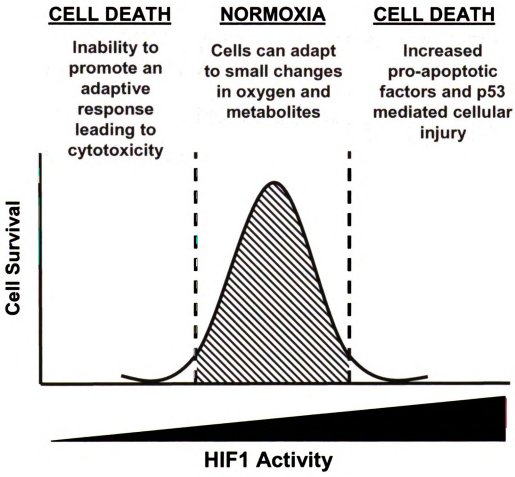
important for this function (85). Though this model does not directly deal with hypoxic stress, it does entail one downstream consequence of cerebral ischemia, i.e. accumulation of excitatory amino acids. All of these results, when taken together, demonstrate that inhibition of the hypoxia-induced adaptive response can have detrimental consequences. Additionally, they support the notion that there is a delicate balance between adaptation and death, and manipulation of this balance can influence a cell's survival under stress.

6. Conclusions

The ability to adapt to a hypoxic environment is critical to survival, and HIF1 signaling has been demonstrated to be important to this adaptation. HIF1-mediated signaling drives the expression of a wide variety of genes essential to the adaptive response, including VEGF, EPO and the glycolytic enzymes, but can also enhance cell death signaling. The HIF1 signaling cascade, therefore, can promote either adaptation or cell death. Which of these two outcomes happens in a given condition likely rests with the control of HIF1α stability and thus its activity (Fig. B-6). Under normoxic conditions and during normal cellular processes, a cell is capable of modulating HIF1α activity to cope with small transient changes in oxygen availability. This adaptability is also important for attempting to re-establish "normoxia" for cells within an organ during hypoxic stress. Exposure to severe hypoxia is capable of driving the expression of prodeath genes, such as BNIP3, as well as p53-mediated signaling which promotes

Figure B-6. Relationship between HIF1 activity and cell survival under stress

During normal cellular processes, HIF1 activity can be modulated to adapt depending upon cellular conditions (hatched section). Under severe hypoxia, however, HIF1 can promote cell death through upregulation of pro-death genes and p53 mediated processes. Conversely, if the cell is unable to promote HIF1 activity and adaptation during hypoxic stress, either through drug intervention or metabolic disruption, cell death may also occur.


CELL DEATH
Inability to promote an adaptive response leading to cytotoxicity
NORMOXIA
Cells can adapt to small changes in oxygen and metabolites
CELL DEATH
Increased pro-apoptotic factors and p53 mediated cellular injury
Cell Survival
HIF1 Activity

cell death (Fig. B-6). Under these conditions, such as during cerebral ischemia and severe drug-induced tissue damage, cells within an affected region undergo necrotic or apoptotic cell death. Finally, if the HIF-mediated adaptive process is inhibited through physiological or exogenous means, then even small stresses can promote cell injury through an inability to cope with the energy debt that results from hypoxia. The balance between cell survival through adaptation and cell death is probably determined differently for various cellular conditions. The boundaries between too much and not enough tissue oxygen and oxygen's influence on HIF1α activity are important determinants of our ability to cope with pathological conditions that alter oxygen homeostasis and our ability to design drugs that target HIF1 signaling.

HIF1 signaling and hypoxia are components of the pathological progression of various diseases, including stroke, cardiovascular disease, and cancer. Targeting hypoxia signaling for therapeutic purposes, therefore, has garnered considerable interest. Current therapeutic strategies involve modulating hypoxia signaling through four distinct mechanisms: directly inhibiting HIF, indirectly modulating HIF activity through secondary signaling, specifically influencing the expression or activity of HIF1 target genes and utilizing HRE-mediated therapies to influence the hypoxic cell (Table B-1). Each new approach utilizes our expanding understanding of hypoxia biochemistry and signaling. This understanding has also raised consciousness about cautions needed when targeting this system for drug design and determining what role

these drugs will have on the HIF1-regulated balance betweencell and tissue survival and programmed cell death. Exploiting HIF1 signaling for therapeutics will require an understanding of how this balance will be shifted in the various tissues of the body by drugs and under various pathological conditions. For example, increases in HIF1α activity might promote neuronal protection, but a similar increase in another tissue might prompt cellular transformation or cell death. In addition, as recent research has shown, upsetting this balance can alter the toxicity profile of drugs, both directly and indirectly. A complete understanding of the metabolic flux, normal oxygen levels, growth factor exposure and endogenous HIF-signaling will be critical to our ability to develop therapies that are both efficacious and safe.

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