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Uniformed Services University of the Health Sciences

Dissertation Title: The Causes and Consequences of Altered Glucose Metabolism in Cancer

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Abstract

Altered glucose metabolism as reflected by lactate accumulation despite adequate oxygen availability is commonly associated with malignant transformation. However, the causes and consequences of this link remain unclear. Here we explore possible causes of this metabolic pattern and how they may promote malignancy. The transcription factor Hypoxia Inducible Factor-1 (HIF-1) controls the expression of many genes commonly associated with malignancy, including those which increase lactate, and its activation in cancer is highly correlated with poor clinical outcome. HIF-1 activation by hypoxia is well characterized; however, its regulation under normoxia in cancer cells is still uncertain. Here we show that elevated glucose metabolites promote normoxic HIF-1 activation via inhibition of its degradation pathway. Since HIF-1 activation results in elevation of the glucose metabolites that inhibit its degradation, we suggest a novel feed-forward mechanism of malignant progression. HIF-1 activation has also been shown to alter glucose metabolism through inhibition of the Pyruvate Dehydrogenase Complex (PDC). The PDC is inhibited by phosphorylation of its regulatory PDHα subunit, and HIF-1 increases

phosphorylation through expression of Pyruvate Dehydrogenase Kinase-1 (PDK-1). Here we show that increased PDHα phosphorylation and PDK-1 expression are associated with malignancy and normoxic HIF-1 accumulation in cancer cells. Furthermore, we show that inhibition of PDK expression by short hairpin RNA (shRNA) results in reversion from the malignant phenotype and normoxic HIF-1 accumulation in cancer cells both *in vitro* and *in vivo*. The link between HIF-1, glucose metabolism, PDC activity and the malignant phenotype suggests a novel regulatory pathway and further signifies the importance of abnormal glucose metabolism in malignancy.

The Causes and Consequences of Altered Glucose Metabolism in Cancer

By

Thomas McFate

Dissertation submitted to the Faculty of the Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, 2007.

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Abbreviations

2-OG	2-oxoglutarate
Asc	Ascorbate
Asn	Asparagine
ATP	Adenosine Triphosphate
Cys	Cysteine
DAN	2,3-Diaminonaphthalene
DCA	Dichloroacetate
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DETA-NO	Diethylenetetraamine-NONOate
DFO	Desferrioxamine
DMOG	Dimethyloxalylglycine
FIH	Factor Inhibiting HIF
Glc	Glucose
H_2O_2	Hydrogen Peroxide
HIF-1	Hypoxia Inducible Factor-1
His	Histidine
HNSCC	Head and Neck Squamous Cell Carcinoma
HPH (PHD)	HIF Prolyl Hydroxylase
LDH	Lactate Dehydrogenase
NO	Nitric Oxide
Oaa	Oxaloacetate

ODD	Oxygen Dependent Degradation Domain
ODD-GFP	Oxygen Degradation Domain-Green Fluorescent Protein
Pyr	Pyruvate
PDC	Pyruvate Dehydrogenase Complex
PDH (E1)	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PDP	Pyruvate Dehydrogenase Phosphatase
pVHL	von Hippel-Lindau Tumor Suppressor Protein
ROS	Reactive Oxygen Species
shRNA	short hairpin Ribonucleic Acid
Succ	Succinate
VEGF	Vascular Endothelial Growth Factor

Chapter 1 – Introduction

Cancer is one of the worldwide leading causes of death. It has been documented for thousands of years and intensely studied for decades. Research has led to an increased understanding of its pathology and better treatment regimes, but a cure remains elusive. Cancer is a disease of the cells, the building blocks of our body. Normally, cells grow, divide and die as the body needs. However, sometimes this orderly process goes awry and uncontrolled cell growth results in a mass of cells called a tumor. Tumors are either benign or malignant, but only malignant tumors are considered cancerous. Unlike malignant tumors, benign tumors do not invade the surrounding tissue, can often be removed with minimal re-growth and are rarely life-threatening. Thus, cancer is defined by the aggressive characteristics of the uncontrolled dividing cell.

Hanahan and Weinberg (2000) define six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Another commonly observed characteristic of cancer cells, that some consider the seventh hallmark of cancer, is altered glucose metabolism, which is characterized by production of high levels of lactate under normal oxygen conditions when compared to normal tissue (Garber 2006).

Alterations of Glucose Metabolism are Associated with Cancer Progression.

High lactate production in normal oxygen conditions, referred to as aerobic glycolysis, was first observed in cancer in the 1920s by the Nobel laureate Otto Warburg, and has thus come to be known historically as the Warburg effect (Warburg et al. 1924, Warburg 1956). Once considered a leading theory to understanding malignant transformation, the Warburg effect was marginalized with the advent of genetics in the 1950s. However, a growing body of research has led to a reexamination of the importance of aerobic glycolysis in cancer progression. The correlation between high lactate and malignancy has recently been verified using large numbers of tumor samples from head and neck and cervical cancer patients (Walenta and Mueller-Klieser, 2004). In these patient samples high tumor lactate levels have been shown to correlate with higher metastatic rates, cancer recurrence, and poor patient survival (Walenta et al. 2004). Furthermore, evidence has emerged showing that lactate and pyruvate can directly promote angiogenesis (Murray and Wilson 2001; Lee et al. 2001), activate gene transcription (Lu et al. 2002, 2005; Dalgard et al. 2004), stimulate hyaluronan synthesis (Stern et al. 2002) and suppress the immune system (Fischer et al. 2007). Thus, it is now believed that the Warburg effect may directly promote cancer progression.

Aerobic Glycolysis

There are two main pathways in which glucose is metabolized to generate energy in the form of adenosine triphosphate (ATP): glycolysis and oxidative

phosphorylation. Glycolysis, the initial process of glucose metabolism, occurs in the cytosol of every cell type in the body. Glycolysis is an anaerobic process, which means it does not require oxygen. The glycolytic pathway is composed of 10 enzymes that convert the 6-carbon glucose molecule into two 3-carbon pyruvate molecules to produce two ATP molecules. When oxygen is present, pyruvate in most cells is further metabolized in the mitochondria to CO_2 and H_2O via oxidative phosphorylation to produce 36 more ATP molecules. In the absence of oxygen, pyruvate is converted to lactate (Fig. 1A) by the enzyme lactate dehydrogenase (LDH) to regenerate cytosolic nicotinamide adenine dinucleotide (NAD+), a substrate required to maintain glycolysis. Oxidative phosphorylation generates substantially more ATP than glycolysis, and thus the vast majority of cells in our body die when tissue oxygen supply is lost for more than a few minutes. Cancer cells, however, are remarkably tolerant to low oxygen (hypoxia) and exhibit high levels of lactate accumulation, even in the presence of oxygen. This metabolic phenotype is referred to as aerobic glycolysis (Fig 1B).

The origins of the Warburg effect are uncertain, but an emerging premise is that cancer cells upregulate glycolysis as an adaptive response to tumoral hypoxia (Gatenby and Gillies 2004). Solid tumors commonly develop hypoxia as a result of the rapidly dividing cancer cells outgrowing their oxygen supply. This triggers activation of hypoxia induced gene expression, which includes several genes for glycolysis and glucose uptake that promote cell survival in low oxygen

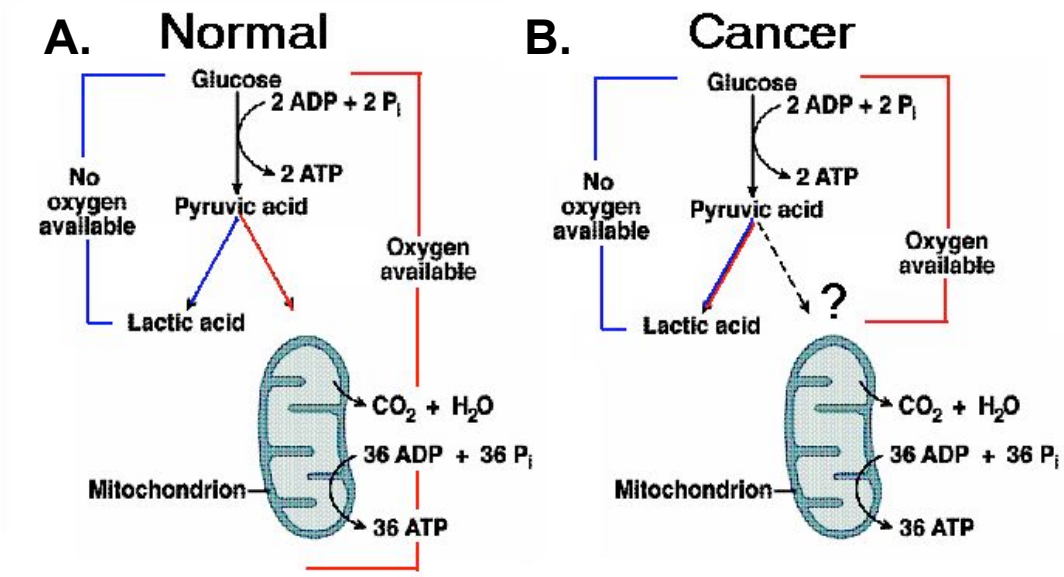

Figure 1. A. Diagram of Normal Glucose Metabolism. Under normal oxygen conditions, glucose is metabolized in the cytosol to pyruvate in a process called glycolysis. It is then further metabolized in the mitochondria to produce the larger percentage of cellular ATP by oxidative phosphorylation (Outlined by the red line to the right of the diagram). When oxygen isn't available, pyruvate is converted to lactate in a process termed anaerobic glycolysis (Outlined by the blue line on the left side of the diagram). **B. Diagram of the Warburg Effect.** Even in the presence of normal oxygen conditions, many cancer cells convert pyruvate to lactate. The mitochondria of these cells often appear to be functional, so it remains uncertain why this altered metabolism occurs.

conditions. Based on our understanding of the hypoxia signaling pathway, normal oxygen conditions should inhibit hypoxic gene expression. For reasons still not fully understood upregulated glycolysis and lactate production persist in cancer cells under normal oxygen conditions.

Leading Theories for the Warburg Effect Point to Normoxic Activation of HIF-1

Research shows that frequent mutations in tumor suppressor and oncogenes, including Ras, PTEN, Myc, Src, HER-2/Neu and p53, can lead to elevated lactate levels in normal oxygen conditions through increased glucose uptake and glycolysis and decreased mitochondrial activity (Bianco et al. 2006; Shim et al. 1998; Bigalow et al. 1997; Dihazi et al. 2003; Matoba et al. 2006). Mitochondrial DNA mutations, which result in decreased oxidative phosphorylation, have also been shown to play a role in the Warburg effect (Zhou et al. 2007). In much of this research, the purported link between mutation and the Warburg effect is that these mutations lead to the constitutive activation of hypoxic gene expression through normoxic stabilization of hypoxia inducible factor-1α (HIF-1α), the regulatory component of the ubiquitous transcription factor HIF-1 (Blum et al. 2005; Karni et al. 2002; Giatromanolaki et al. 2004; Patiar and Harris 2006).

HIF-1, a Master Regulator of Oxygen Homeostasis

In 1991, several groups demonstrated that the hypoxic inducibility of the erythropoietin (EPO) gene was party due to a small hypoxia response element

(HRE) located in its 3' flanking region (Semenza et al. 1991, Imagawa et al 1991). The transcription factor that bound this HRE was designated HIF-1. Subsequent research has revealed that the molecular mechanism behind the regulation of EPO is ubiquitous. HIF was discovered in mammals, and has been shown to be vital for survival because it is required for the establishment of all three components of the circulatory system (blood, heart and vasculature) (Iyer et al. 1998). HIF is also present in metazoan organisms (Bishop et al. 2004), which do not have a specialized system for oxygen delivery, suggesting a more primordial role than the regulation of oxygen transport. To date dozens of direct target genes for HIF have been identified, and its activation is purported to regulate up to 2% of the entire genome, marking it as a master regulator of oxygen homeostasis (Semenza 2007). Most of these HIF-regulated genes promote cell survival, including genes for increased glucose uptake and glycolysis, angiogenesis, and anti-apoptosis. In cancer the expression of the regulatory subunit, HIF-1α, highly correlates with tumorigenesis, malignancy and poor clinical outcome (Semenza 2003).

HIF-1α Protein Stability and Transactivational Activity is Controlled by Protein Hydroxylases

HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits (Wang and Semenza 1995), both of which are constitutively expressed in cells. The regulation of the HIF-1 complex is mainly dependent on the degradation of the HIF-1α subunit. Under normoxic conditions, HIF-1α undergoes ubiquitination and

proteosomal degradation via the E3 ligase component von Hippel-Lindau tumor suppressor protein (pVHL) (Huang et al. 1998; Kallio et al. 1999). As shown in Fig. 2A, this process involves the binding of pVHL to the oxygen dependent degradation domain (ODD) on the HIF-1α protein (Maxwell et al. 1999). In order for pVHL to bind to the HIF-1α ODD, key proline residues must first be hydroxylated (Jaakkola et al. 2001). A family of HIF prolyl hydroxylase (HPH) enzymes (HPH1,2,3) mediates the regulation of this molecular oxygen dependent step. HIF-1α is also regulated by another hydroxylase known as factor inhibiting HIF (FIH)-1. FIH-1 hydroxylates a key asparagine residue on HIF-1α preventing the HIF-1 heterodimer from interacting with the transcriptional co-activator p300, thereby blocking HIF-mediated transcriptional activation (Mahon et al. 2001, Lando et al. 2002, and McNeill et al. 2002). These hydroxylase enzymes require oxygen, iron, and 2-oxoglutarate (2-OG). Ascorbate is also required for optimal activity (Fig. 2B). Limitation of the oxygen substrate during hypoxia halts hydroxylase enzyme activity thus enabling the HIF-1α protein to accumulate, dimerize with HIF-1β to form the HIF-1 complex and transactivate gene expression (Fig. 2B). In addition to hypoxia, HIF hydroxylase activity is also inhibited by the presence of iron-chelators such as desferrioxamine (DFO), iron displacing transition metals such as cobalt, nickel, and manganese, or synthetic 2-OG antagonists such as dimethyloxalylglycine (DMOG) (Zhu and Bunn 1999).

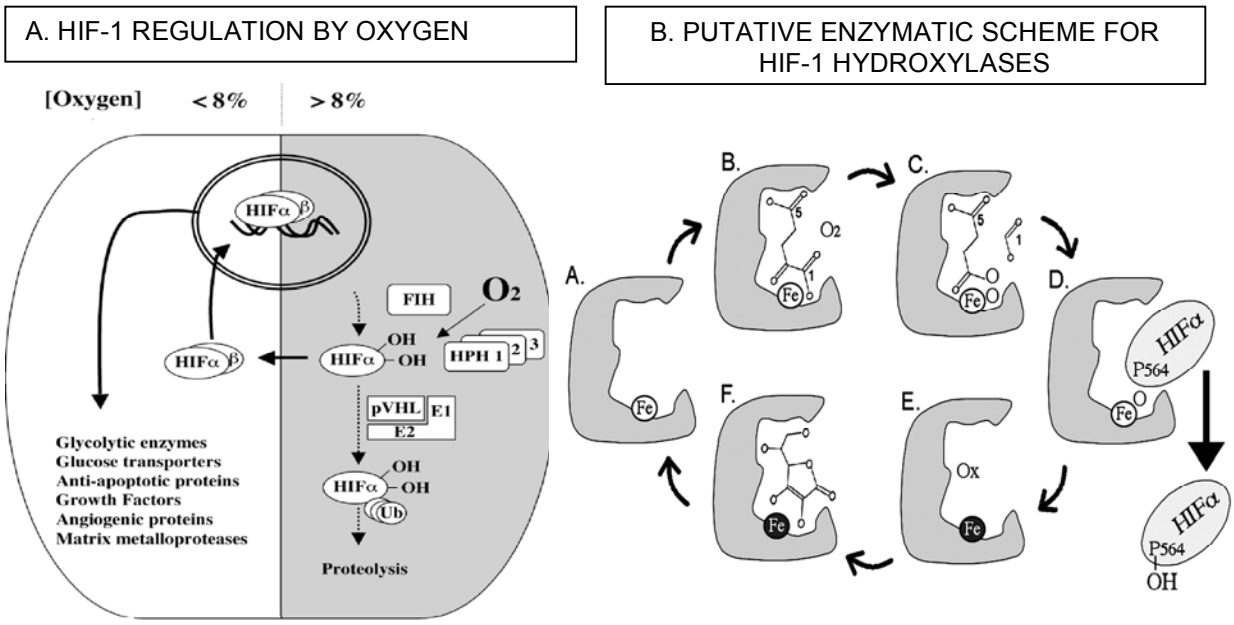

Figure 2 A. The hypoxia signal transduction pathway. The oxygen regulated HIF-1α subunit of HIF-1 is constitutively synthesized in all cells. Under normoxic condition (>8%O2, right side of figure), specific HIF-1α hydroxylases incorporate O2 directly into the HIF-1α protein. Asparaginyl hydroxylation by FIH blocks the transactivating ability of HIF-1α. Proline hydroxylation of HIF-1α by HPH isoforms 1, 2, and 3 generates recognition motifs for binding to the von Hippl Lindau protein (pVHL), which is the E3 component of a ubiqutin ligase complex. Ubiquination and proteolysis of HIF-1α under normoxia assures that HIF-1 responsive genes are not transcribed. Under hypoxia (<8% O2, left side of figure) HIF-1α escapes hydroxylation, dimerizes with HIF-1β, translocates to the nucleus, and activates the transcription of several genes that result in enhanced glycolysis, cell survival and angiogenesis. **B. Putative enzymatic cycle for HIF prolyl hydroxylases.** HPHs and FIH-1 are members of the 2-oxoglutarate dependent dioxygenase enzyme family. These enzymes require iron, 2-oxoglutarate, and oxygen to carry out biological hydroxylations. This figure depicts a putative sequence of events that has been proposed for many members of this enzyme family. A. HPHs (grey C-shaped structure) bind iron (Fe). B. The HPH-iron complex binds 2-oxoglutarate. The 2-oxo group coordinates with iron while the 5-carbon end of the molecule interacts with a different site. C. This complex allows one atom of molecular oxygen to be inserted into the 2-oxoglutarate molecule to yield succinate and carbon dioxide while the other oxygen atom forms a complex with the enzyme-bound iron. D. The iron-complexed oxygen is used to hydroxylate proline 564 within the HIF-1 alpha oxygen dependent degradation domain. HPHs also carry out similar hydroxylation on proline 402 while the FIH-1 enzyme hydroxylates asparagine 803. E. Most enzymes that utilize this mechanism of hydroxylation become syncatalytically inactivated over time. This inactivation may involve redox reactions between oxygen, iron, and the enzyme and can be favored by certain conditions such as the presence of a pseudo-substrate. F. Enzymes inactivated in this way can be re-activated with ascorbate, which appears to bind these enzymes in a manner similar to 2-oxoglutarate (Figure made by Ajay Verma).

Hypoxia-Independent HIF-1α Elevation

Besides inhibitors of the HIF hydroxylases, other hypoxia-independent activators of HIF-1 have been identified. The HIF-1 regulatory protein pVHL is considered a tumor suppressor gene because mutations to it result in normoxic HIF-1 activity and tumorigenesis. Several growth factors, such as insulin-like growth factor-1 (IGF-I) and IGF-II, which act via the phosphoinositide 3-kinase (PI3K) signaling pathway have been shown to elevate HIF-1α protein levels and gene expression in a cell-specific manner in cancer (Semenza 2003). Mutations of many tumor suppressor and oncogenes, such as Ras, PTEN, Myc, Src and HER-2/Neu, result in increased PI3K activity, but it is uncertain if the resultant increased HIF-1α levels involve inhibition the HIF hydroxylases.

Our lab recently identified the glucose metabolites lactate and pyruvate as activators of normoxic HIF-1 (Lu et al., 2002) in cancer cell lines. This finding suggests a feed-forward mechanism whereby increased HIF results in increased lactate and pyruvate, which in turn maintain normoxic HIF expression. Our more recent studies have shown that, in addition to pyruvate and lactate, oxaloacetate and other endogenous 2-oxoacids also activate HIF-1. Interestingly, experiments examining several different cancer cell lines showed that normoxically elevated HIF-1α protein levels and HIF-1-mediated gene expression was specifically reversible by ascorbate (Knowles et al. 2003). Ascorbate was not found to reverse HIF-1α accumulation by hypoxia. The mechanism, as well as the function of any of these hypoxia-independent activators of HIF-1 is unknown.

Putative Mechanism of Hypoxia-Independent HIF-1 Activation.

The discovery of the HIF-1α hydroxylases has greatly advanced our understanding of oxygen regulated signaling. These enzymes belong to the large family of iron and 2-oxoglutarate dependant dioxygenases that follow the general enzymatic cycle shown in figure 2B (Hanauske-Abel and Popowicz 2003). Limitation or competitive antagonism of any of the required substrates halts enzymatic activity as described above. Another means of halting enzymatic activity results from an autocatalytic inactivation. Autocatalytic inactivation refers to an ascorbate reversible loss of enzymatic activity seen after several catalytic cycles. This inactivation is a variable feature for different members of this enzyme family. A well known example of this phenomenon involves the collagen proline-4-hydroxylase enzymes whose inactivation result in scurvy when ascorbate is deficient from the diet. The iron and 2-oxoglutarate dependant dioxygenase family is known to contain several hundred enzymes, but only a few, not including the HIF-1α hydroxylases, have been studied with regards to an autocatalytic inactivation mechanism. Enzymatic pseudo-substrates, such as pyruvate, are known to accelerate inactivation (Hanauske-Abel and Popowicz 2003). Since ascorbate can reverse pyruvate inducible HIF-1 activation in normal oxygen conditions, **we hypothesize that a HIF-1α hydroxylase inactivation mechanism accounts for normoxic HIF-1α accumulation.** The first part of this thesis research will examine this mechanism. The second part of this thesis research explores an important point in the glucose metabolic pathway that may be partly responsible for the maintenance of the aerobic glycolysis phenotype.

Pyruvate Dehydrogenase Complex: Implication in the Warburg Effect

The pyruvate dehydrogenase complex (PDC), a highly regulated enzyme system located at the crux between anaerobic and aerobic glucose metabolism, marks the first step in mitochondrial pyruvate oxidation. The PDC catalyzes the irreversible decarboxylation of pyruvate to acetyl-CoA for use in the Kreb's cycle. Under aerobic conditions, the PDC is generally considered to be primarily responsible for the overall rate of oxidative glucose metabolism (Randle 1986). Decreased activity of the PDC in normal oxygen conditions results in aerobic glycolysis (Stromme et al. 1976), which marks it as a logical point of focus for examining mechanisms underlying the Warburg effect. Furthermore, reduced PDC activity has been observed in several cancer lines with the degree of PDC activity reduction associated with the degree of malignancy (Eboli 1985, Eboli and Pasquini 1994). Despite these observations, decreased PDC activity has been poorly examined as a possible contributing factor to the Warburg Effect and malignant progression of cancer.

The PDC is Regulated by Reversible Phosphorylation

The PDC is composed of three different enzymes, pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). In addition to pyruvate, PDC requires thiamine, coenzyme-A, lipoic acid, and NAD+ to produce acetyl-CoA, NADH and CO_2 (Fig. 3A). The eukaryotic PDC is a highly organized complex with the core

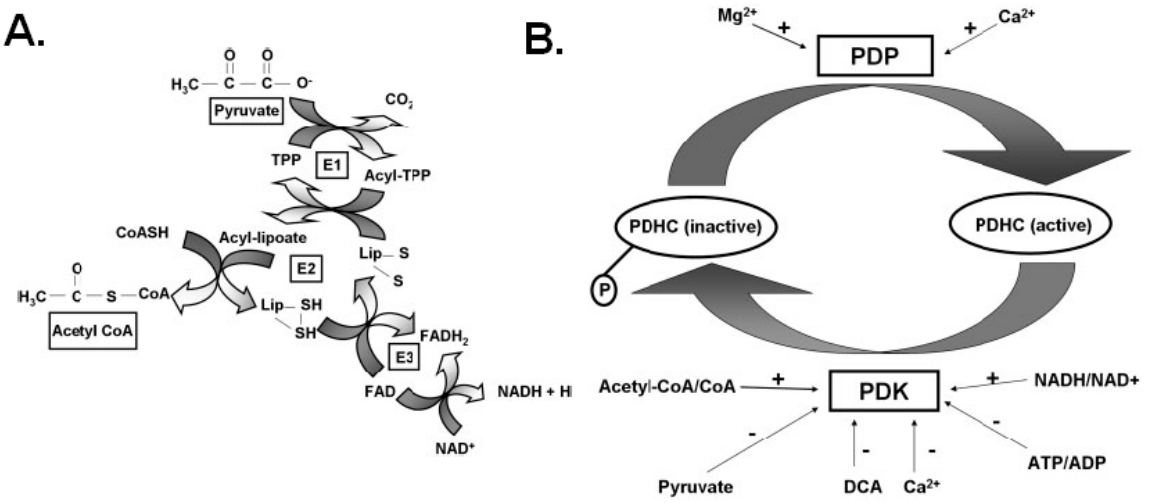

Figure 3. A. Pyruvate Dehydrogenase Complex Activity. The pyruvate dehydrogenase complex catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA. Pyruvate is decarboxylated in the first step by the E1 enzyme pyruvate dehydrogenase. E2, dihydrolipoyl transacetylase uses CoASH to produce Acetyl CoA resulting in a reduced lipoamide. At E3, dihydrolipoyl dehydrogenase, the Dihydrolipoamide is reoxidized to the disulfide, resulting in the reduction of FAD to FADH2 which is reoxidized by electron transfer to NAD+, to yield NADH and H+. **B. Pyruvate Dehydrogenase Kinase and Phosphatase Activity.** The PDC is regulated by phosphorylation of three serine residues on the E1α subunit. The phosphorylated form results in inactivity of the complex. There are several inhibitors and activators of the 4 kinases and 2 phosphatases. The kinases are generally activated by the PDC products and inhibited by the substrates. The pyruvate analog dichloroacetate (DCA) inhibits the kinases. Both phosphatases are activated by Mg^{2+} and PDP1 is activated by Ca^{2+}. (modified from Figures 1 and 2 in Martin et al. 2005)

structure composed of 60 subunits of E2 and 12 monomers of an E3-binding protein to which other components of the complex are bound: 20-30 heterotetramers of E1α and β subunits, 6-12 homodimers of E3, 1-2 homo (or hetero) dimers of pyruvate dehydrogenase kinase (PDK), and 2-3 heterodimers of pyruvate dehydrogenase phosphatase (PDP). Activity of complex is regulated by a reversible phosphorylation cycle of the three serine residues (sites 1-3) located on the E1α component (Patel and Korotchkina 2001). Phosphorylation results in decreased activity with phosphorylation of site 1 conferring maximal inhibition (Sale and Randle 1981).

To date four PDKs (PDK 1-4) and two PDPs (PDP 1 and 2) have been identified. The individual characteristics of these kinases and phosphatases are just beginning to be studied, and initial studies demonstrate differential tissue expression for both (Bowker-Kinley et al. 1998), as well as differential *in vitro* activity levels and serine site preferences for both (Patel and Korotchkina 2006). The kinases are generally activated by the PDC products NADH and acetyl-CoA and inhibited by the substrates pyruvate, NAD+ and CoA (Fig. 3B). Regulation of the PDPs is less understood, but they are known to be activated directly by Mg^{2+} and Ca^{2+} and indirectly by insulin (Macaulay and Jarett 1985). Both increases and decreases in systemic glucose levels due to diabetes and starvation, respectively, have been implicated in the regulation of both the kinases and phosphatases (Roche and Hiromasa 2007). Pharmacologically, the kinases are known to be inhibited by the pyruvate analog dichloroacetate (DCA), which has

been used clinically to treat a variety of disorders connected with decreased PDC activity.

Recent studies with DCA suggest that inhibition of E1α phosphorylation may shift cancer cell metabolism towards aerobic respiration, promote cancer cell apoptosis, and reduce tumor growth (Bonnet et al. 2007). However, DCA also inhibits glutathione S-transferase zeta activity (Tzeng et al. 2000) and cholesterol biosynthesis (Stacpoole and Greene 1992), promotes oxidative stress (Hassoun and Ray 2003), and activates peroxisome proliferator-activated receptors (Laughter et al. 2004). Thus, a role for E1α phosphorylation in regulating cancer biology remains to be established. Interestingly, within the last year, PDK-1 has been identified as a HIF regulated gene (Kim et al. 2006; Papandreou et al. 2006). Considering the connections between HIF-1, aerobic glycolysis and malignancy, **we hypothesize that PDC inactivity promotes aerobic glycolysis and contributes to malignancy in cancer.**

Chapter 2 – Experimental Procedures

Cell Culture and Hypoxia Treatment. Human U251 glioma cells were cultured in Eagle's Modified Eagle's Medium (MEM) (Cellgro, Herdon, VA). The C6 oxygen degradation domain-green fluorescent protein (ODD-GFP) rat glioma cells (D'Angelo et al. 2002), human 22A, 22B, O22 head and neck squamous cell carcinoma cell lines and 22B short hairpin RNA (shRNA) derivatives, human T47d and MDA-MB468 breast cancer cells and E6/E7/hTERT, E6/E7/hTERT/Ras and E6/E7/hTERT/Ras/Akt genetically modified normal human astrocyte cell lines were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA). The C6 ODD-GFP and 22B shRNA cell lines were supplemented with 1.5 and 1mg/ml Geneticin® (Invitrogen, Carlsbad, CA), respectively. The E6/E7 cell lines were supplemented with 1% (v/v) Fungizone ® (Invitrogen, Carlsbad, CA). Human LN18 and LN229 glioma cell lines (ATCC, Manassas, VA) were cultured in RPMI 1640 (Sigma, St. Louis, MO). All media was supplemented with 10% FBS and 1% (v/v) penicillin/ streptomycin (Invitrogen, Carlsbad, CA). Cell lines were maintained in 21% O_2, 5% CO_2 and 74% N_2 in a humidified cell incubator at 37°C until confluent. The E6/E7/hTERT cell lines were kindly provided by Dr. Russell Pieper at the University of California-San Francisco (San Francisco, CA). The 22A and 22B were kindly provided by Dr. Thomas Carey at the University of Michigan (Ann Arbor, MI) and the O22 were kindly provided by Dr. Joseph Califano at Johns Hopkins University (Baltimore, MD). For hypoxia treatments, culture dishes were

sealed in a humidified chamber and exposed to a gas mixture of 1% O_2, 5% CO_2 and 94% N_2 and incubated at 37°C for the indicated times.

Nuclear, Whole Cell and Brain Mitochondria Protein Extraction. Nuclear extracts were prepared according to manufacturers instructions using the Nuclear and Cytoplasmic Extraction Reagents (NE-PER) kit (Pierce, Rockford, IL). Whole cell extracts were prepared by lysing cell pellets in RIPA buffer (0.1% SDS, 1% NP-40, 5 mM EDTA, 0.5% Sodium Deoxycholate, 150 mM NaCl, 50 mM Tris-HCl) freshly supplemented with 2 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche, Indianapolis, IN), 1 mM $NaVO_4$ and 10 mM NaF for 30-60 min on ice. Lysates were centrifuged (4°C) at 16,000 x g for 10 min and supernatant was collected. Brain mitochondria were isolated from adult rats by sucrose centrifugation as described previously (Clark and Niklas 1970). All extracts were assayed for protein concentration using the Bradford method (Bio-Rad, Hercules, CA).

Western Blot Analysis. Western blots were performed with 50-75 µg of nuclear, 35-50 µg of whole cell or 10 µg of mitochondrial protein extracts in equal amounts of Laemmli Sample Buffer (BioRad, Hercules, CA) supplemented with 200mM DTT. Extracts in sample buffer were heated at 90°C for 5 minutes, then ran on 4-12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes by standard procedures. Membranes were blocked in Tris Buffered Saline (TBS) (bio-WORLD, Dublin, OH) with 0.1%

Tween 20 (Sigma, St. Louis, MO) containing 5% nonfat milk for one hour and then either incubated overnight at 4° C or one hour at room temperature with the respective primary antibody. Immunoreactive bands were visualized using horseradish peroxidase (hrp) conjugated anti-mouse or anti-rabbit secondary antibodies, 1:10,000 with enhanced chemiluminescence reagent (Pierce, Rockford, IL). Densitometry was performed using Image J software (NIH, Bethesda, MD) where bands on digitally scanned film were analyzed for size and shade. Western blot analysis of recombinant PDH peptides (Korotchkina and Patel 2001) performed in Dr. Mulchand Patel's lab.

Antibodies and Chemical Reagents. Mouse monoclonal anti-HIF-1α, 1:400, was purchased from Becton Dickinson Transduction Laboratories (San Jose, CA). Mouse monoclonal anti-GFP antibody, 1:1000, was purchased from Roche (Indianapolis, IN). Mouse monoclonal anti-β-actin antibody, 1:10,000, was purchased from Abcam (Cambridge, MA). Mouse monoclonal HIF-1β, 1:1000, was purchased from Novus Biologicals (Littleton, CO). Mouse monoclonal PDH E1α (PDHα), 1:5000, was purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-PDH E1α Phospho-Serine293 (pPDHα), 1:5000, was developed by Ajay Verma in coordination with Novus Biologicals (Littleton, CO) (see below for details). Rabbit polyclonal anti-PDK-1, 1:2500 was purchased from Assay Designs (Ann Arbor. MI). Rabbit polyclonal anti-PDK-3, 1:1000, was purchased from Abgent (San Diego, CA). Rabbit polyclonal anti-PDK-2, 1:1000, and PDK-4, 1:1000, were kindly provided by Dr. Robert Harris (Indiana University School of

Medicine, Indianapolis, IN). The proteosome inhibitor MG-132 was purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Cellular Reactive Oxygen Species and Nitrite Measurements. (performed with Sasha Tait) Reactive oxygen species were measured using the cell permeable 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Molecular Probes, Inc., Eugene, OR). CM-H2DCFDA fluoresces due to intracellular oxidation. Cells (80% confluent) were loaded with CM-H2DCFDA (5 μM) in Krebs buffer for 1 h and then treated with 2 mM pyruvate or oxaloacetate for 2 h. Positive controls were treated with 40 μM H_2O_2 for 2 h. Cells were then collected, washed twice with phosphate-buffered saline, and resuspended in phosphate-buffered saline plus 0.1% (w/v) bovine serum albumin before measuring fluorescence with excitation at 488 nm and emission read at 520 nm using a Coulter flow cytometer (BeckmanCoulter, Fullerton, CA). Nitrite concentration in culture medium was measured as described in Misko et al. 1993. In brief, 100μl media from cultured cells (80% confluent) treated with the nitrite producing chemical diethylenetetraamine-NONOate (DETA-NO) (100μM and 300μM), pyruvate (2 mM) or oxaloacetate (2 mM) for 2h was mixed with 10μl of freshly prepared 2,3-Diaminonaphthalene (DAN) (0.05 mg/ml in 0.62 *M* HCL). DAN reacts with nitrite under acidic conditions to form the fluorescent product 1-(*H*)-naphthotriazole. Fluorescence was measured with excitation at 365 nm and emission read at 450

nm using the FLUOstar Optima fluorescent plate reader (BMG Labtech, Durham, NC).

Calcein Fluorescence Assay for Cellular Iron. The ability of 2-oxoacids to chelate the labile intracellular iron pool was measured using the calcein (CA) fluorescence assay as described (Epsztejn et al. 1997). The iron chelators desferrioxamine and bipyridyl were used as positive controls. Cultured cells (80% confluent) were incubated with 0.25μM calcein-acetoxymethyl ester (CA-AM) (Molecular Probes, Inc., Eugene, OR) in for 3-5 min at 37° C in bicarbonate-free and serum free growth medium containing 1mg/ml BSA, 20mM Hepes, pH 7.3, washed of excess CA-AM, resuspended in loading medium without CA-AM to 5 x 10^5 cells/ml, and maintained at room temperature until used. Just prior to measurements, 1ml of the CA-loaded cell suspension was centrifuged in a microcentrifuge; the cells were resuspended in 2ml of prewarmed Hepes buffered saline (HBS) buffer (20mM Hepes, 150mM NaCl, pH 7.3). The cell suspension was transferred to a stirred thermostated cuvette and fluorescence was monitored at an excitation of 488 nm and emission of 520 nm using the Perkin Elmer Luminescence Spectrometer LS 50B (Perkin Elmer, Waltham, MA).

EF-5 Assay for Cellular Hypoxia. Cellular hypoxia was monitored via immunofluorescence detection of 2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)-acetamide] (EF-5) cellular adducts as described (Koch et al. 1995). In brief, cultured cells were grown to confluency, rinsed with glucose-free

Kreb's buffer, then incubated in glucose-free Kreb's buffer with EF-5 (500μM) in 1% O_2, 21% O_2, or 21% O_2 plus pyruvate (3 mM) for 4 h followed by fixation with 4% paraformaldehyde in PBS for 1h at 4° C. The cells were then rinsed twice in PBS. Non-specific binding was blocked by the addition of PBS containing 0.3% Tween-20 (Sigma, St. Louis, MO), 1.5% BSA, 20 % non-fat milk and 5% mouse serum (Jackson Laboratories, Bar Harbor, ME) (4° C, overnight). The blocking solution was then removed, the cells rinsed with PBS and EF-5-Cy3 antibody was added at a concentration of 75 μg/ml for 6h at 4° C. Extensive rinsing (three changes of PBS with 0.3% Tween 20 for 40 min each) was followed by storage of cells in PBS with 1% paraformaldehyde. Immunofluorescence was imaged using a fluorescent microscope with a Texas Red emission wavelength filter.

Generation of Site 1 (S293) Phospho-Specific PDHα Antibody. A phospho-specific antibody was generated to pS293 of the PDH E1α protein (of the immature human and rodent protein) by Novus Biologicals. New Zealand white rabbits were immunized with the peptide YRYHGH(pS)MSDPG conjugated to the adjuvant Keyhole Limpet Hemocyanin (KHL) at 21 day intervals until peak antibody titer were reached. The resulting serum was then affinity purified against the immunizing peptide unphosphorlyated at S293. To determine the phospho-serine293 specificity of the antisera, human recombinant PDHα either phosphorlyated or unphosphorlyated at serine 293, were ran on 4-12% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted (as described above) with pPDHα affinity-purified antibody. Dr. Mulchand Patel

generated recombinant PDHα specifically phosphorylated at site 1 by treating recombinant PDHα mutated at sites 2 and 3 (S2A,S3A) with PDK1 in the presence of 500 μM ATP (Korotchkina and Patel 2001). Following immunoblotting with pPDHα affinity-purified antibody, membranes were stripped, using stripping buffer (Pierce, Rockford, IL) for 30 min at 37° C, and reprobed with anti-PDHα as described above.

^{32}P-γATP and ATP In Vitro Phosphorylation Assays. To evaluate the specificity of the anti-pPDHα antibody *in vitro*, manipulation of brain mitochondria (1μg/ml) phosphorylation was accomplished by incubation with 6 μCi/mmol ^{32}P-γATP either alone or with the addition of 10 mM $MgSO_4$ (to maximize dephosphorylation) or 1 mM EDTA (to inhibit dephosphorylation) and by incubation with 1mM ATP alone or with the addition of 5 or 20 mM of the PDK inhibitor dichloroacetate (DCA) for 30 min at 37°C. Both reactions were terminated by the addition of Laemmli Sample Buffer supplemented with 200mM DTT. The proteins were ran on 4-12% SDS-polyacrylamide gels, transferred to nitrocellulose membrane and evaluated for anti-pPDHα and anti-PDHα expression using Western blot procedures described above and ^{32}P-γATP phosphorylation by exposure to film for 24hrs.

Two Dimensional Gel Electrophresis. (performed by Nader Halim) 2D-electrophresis was performed on whole cell lysate from rat brain using the Zoom® bench top proteomics system according to the manufactures protocol

(Invitrogen, Carlsbad, CA). Briefly, homogenates were loaded onto a pH 3-10 IPG strip according to the manufacturer's instructions. Proteins on the IPG strip were separated by isoelectric point using a step-voltage protocol (175 V, 15 min; 175-2000 V, 45 min; 2000 V, 45 min). The IPG strip was then removed, treated with NuPAGE® LDS Sample Buffer with NuPAGE® Sample Reducing Agent, then NuPAGE® LDS Sample Buffer with 125 mM iodoacetamide prior to separation in the second dimension. To separate using SDS-PAGE, the treated IPG strip was placed into a NuPAGE® Novex 4-12% Bis-Tris Zoom® Gel specifically designed for running proteins separated on IPG strips. Immunoblotting were preformed as described above.

Cell Number and Viability Determination. (performed with Ahmed Mohyeldin) Cells were plated in 6-well plates at 0.1-0.25 x 10^6 cells per well. At various times points, the medium was collected and cells were trypsinized, counted and recombined with medium. The total cell number and viability (trypsinized cells and floating cells in the medium) was performed using the Vi-CELL Series Cell Viability Analyzer (BeckmanCoulter, Fullerton, CA), which determines viability based on the trypan blue dye exclusion method.

Colony Formation. (performed by Shaoyu Zhou) Cells (10^4) from each cell line were placed in 1 ml of DMEM with 0.3% low-melting agarose (soft agar) and 10% FBS, and overlaid onto 1 ml/well of DMEM with 0.5% agarose and 10% FBS.

After 18 days, colonies were counted directly under microscope using the Nikon SMZ1500 microscope and photographed with the Nikon DXM camera.

Lactate Measurements. (performed with Ahmed Mohyeldin) At various time points, 50 µl of media was taken from cells grown in 2 ml of media in 6-well cell culture plates, and frozen until the time of measurement. Measurements were performed using the CMA 600 Analyzer (CMA-Microdialysis, Acton, MA). This instrument performs an automated enzymatic conversion of the metabolite to H_2O_2. Peroxidase catalyses a reaction between H_2O_2 and other substrates to form the red violet colored quinonediimine. The rate of formation of quinonediimine per minute and absorption is measured at 546 nm. The signal is quantified based on intensity of color, which is proportional to lactate concentration.

Cell Transfections and Selection for Stable Lines. (performed with Huasheng Lu) Validated plasmids encoding PDK-1 shRNA (TCTCGcagatgcagttatctacatta-CTTCCTGTCAtaatgtagataactgcatctgCT) and PDK-2 shRNA (TCTCgctcctgtgt-gacaagtattaCTTCCTGTCAtaatacttgtcacacaggagcCT) and negative control shRNA (TCTCggaatctcattcgatgcatacCTTCCTGTCAgtatgcatcgaatgagattccCT) (SuperArray, Frederick, MD) were purified for transfection into 22B cells using the QIAfilter Maxi Plasmid Kit (Qiagen Sciences, Germantown, MD) and introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according manufacturer instructions for cells plate in 6-well dishes. Cells were

90-95% confluent at the time of transfection and switched to medium without antibiotics. For each transfection sample 4.0µg of DNA was diluted in 250µl of OptiPro™ SFM (Invitrogen, Carlsbad, CA) and mixed gently. Gently mixed Lipofectamine™ 2000 (10µl) was diluted in 250µl of OptiPro™ SFM and incubated for 5 minutes at room temperature. After a 5 minute incubation, the diluted DNA was combined with diluted Lipofectamine™ 2000 (total volume = 500 µl), mixed gently and incubated for 20 minutes at room temperature. The 500 µl mixture of complexes was then added to the 6-well dishes containing cells and 1.5ml of medium followed by gentle mixing by rocking the plate back and forth. Cells were then incubated at 37°C in an incubator for 24hr. After 24hr, the cells were passaged at 1:10 into fresh growth medium and stable cell lines were selected for the following day using 1.5 mg/ml Geneticin® (Invitrogen, Carlsbad, CA) over a period of 1-2 weeks.

PDC Activity Assay. (performed by Nam Ho Jeoung) PDH activity was assayed according to previously published methods with minor modifications for use with culture cells (Jeoung et al. 2006). Cells were grown to confluency in 10 cm dishes and rinsed with PBS (without Ca^{2+}/Mg^{2+}). Extraction buffer (400 µl @ 4°) was added directly to the plates and the cells were scraped and pelleted. Samples were homogenized with a motor driven homogenizer, and then centrifuged at 13,000 rpm for 10 min with tabletop microcentrifuge. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 µM final concentration) was added to the PDC extraction and re-suspension buffer just before use.

Glucose Oxidation. (performed with Jay Thakar) Glucose oxidation was assayed via $^{14}CO_2$ capture using uniformly labeled glucose (D-[U-^{14}C] glucose) according to previously published methods (Itoh et al. 2003) with slight modifications. Cells were grown in 25 cm^2 flasks. Immediately before starting the assay, cultures were washed twice with 1 ml of PBS at room temperature. Kreb's buffer prewarmed to 37°C containing 2.5 mM D-[U-14C] glucose was then added to the culture flasks. The culture flasks were then capped with rubber stoppers fitted with hanging wells containing accordion-folded, 1.5 x 2.5 cm pieces of filter paper that had been wetted with 100 µl of 1.0 M NaOH to trap $^{14}CO_2$ produced. The culture flasks were incubated for 60 min in a 37°C water bath with gentle shaking. The reactions were terminated by injection of 250 µl of 60% (w/v) perchloric acid through the rubber stopper, and the flasks were kept at 4°C overnight to trap the $^{14}CO_2$. The hanging wells including the filter paper were then transferred to 20-ml glass scintillation vials, and 250 µl of water and 10 ml of scintillation fluid were added. ^{14}C in the vials was assayed by liquid scintillation counting using the Wallac 1450 Microbeta trilux liquid scintillation and luminescence counter (Perkin Elmer, Waltham, MA). Cell carpets left in the incubation flasks after removal of the reaction mixtures were digested with 1 ml of 1 M NaOH, and their protein contents were determined using the Bradford assay.

VEGF Enzyme-linked Immunosorbent Assay. (performed with Ahmed Mohyeldin and Jeremy Henriques) Culture medium VEGF was measured via an enzyme-linked immunosorbent assay according to manufacturer's instructions (R & D Systems, Minneapolis, MN). Cells were plated in 6-well dishes and media was replaced at the start of the 24 hr experiment. At end point, media samples were taken and frozen to assay VEGF. Cells were trypsinized and counted for normalization to secretion of VEGF per cell. Absorbance was measured at 450 nm with the correction wavelength set at 570 nm using the Multiskan Ascent plate reader (Thermo Fisher Scientific, Inc., Waltham, MA). Undiluted samples were in the linear range when compared to the standard curve using recombinant VEGF provided in the enzyme-linked immunosorbent assay kit.

Cell Invasion and Migration Assay. (performed with Ahmed Mohyeldin) Cell invasion experiments were performed using 24-well Biocoat Matrigel™ Invasion Chambers with an 8-μm pore polycarbonate filter according to manufacturer's instructions (Beckton Dickinson Labware, Bedford, MA). Regular growth factor Matrigel invasion chambers were used (BD Biosciences, San Jose, CA). Prior to experimentation all invasion chamber inserts were hydrated according to manufacturer's protocol. Briefly, cells in the growing phase were trypsinized and suspended at a concentration of 4×10^5 cells/ml. The lower compartment of the plates received 750 μl of serum free media and/or the treatment condition designated in each experiment. 2×10^5 cells (for serum free media experiments)

and 2.5 x 10^4 (for 10% serum media experiments) were plated in each insert and allowed to invade for 48 hrs at 37°C in a humidified incubator with 21% O_2.

Xenograft of Human Tumors. (performed with Ahmed Mohyeldin and Jake Friedman) BALB/C male nude mice were purchased from National Cancer Institute (Frederick, MD) and housed in a pathogen-free room controlled for temperature and humidity. All animal protocols and handling were performed under approved USUHS IACUC guidelines. Twenty (22B control shRNA *n=10*, 22B PDK1 shRNA *n=10*) 6-8 weeks old mice were injected subcutaneously in the scruff with 1x10^7 cells in 100 µl of PBS. Tumor bearing mice (9 from each group) were used for the experiment. Tumor volume was assessed bi-weekly 1 week after injection for 2 months and calculated using the following formula for a rotational ellipsoid: $V = (a \times b^2)/2$, where *a* is the widest diameter of the tumor and *b* is the diameter perpendicular to *a*. The results from individual mice were graphed as average tumor volume over 60 days.

Statistical Analysis. Results are expressed as means plus or minus standard deviation. Data were analyzed using Student's t test or ANOVA. If the f value, which is a probability function of an F distribution, was significant at $\leq$0.05, Tukey pairwise multiple comparison procedure was used to compare means.

Chapter 3

Examination of the Mechanism Underlying HIF-1α Regulation by Glycolytic Metabolites in Normoxia

Summary

The regulatory alpha subunit of the HIF-1 transcription factor is constitutively expressed and degraded in normal oxygen conditions. Degradation of HIF-1α prevents HIF-1 transcriptional activation, and thus the expression of many genes important for hypoxic survival and associated with malignant progression, including those for anaerobic metabolism, angiogenesis, cell survival and intercellular matrix modification. HIF-1α is marked for degradation via prolyl-hydroxylation by the oxygen, 2-oxoglutarate, iron and ascorbate-dependent HIF hydroxylases (HPHs). Hypoxia prevents hydroxylation, which results in HIF-1α protein stabilization and HIF-1 activation. In normoxia, glucose metabolites can also stabilize HIF-1α and activate HIF-1. But unlike HIF-1 activation by hypoxia, the mechanism of normoxic activation by glucose metabolites has not been elucidated, yet. Using cells which express the HIF-1 oxygen degradation domain fused to the reporter protein GFP, we show that the glucose metabolites pyruvate and oxaloacetate inhibit normoxic HPH activity in a reversible manner by ascorbate, cysteine and histidine. We also demonstrate that pyruvate treatment in hypoxia delays the rate of HIF-1α degradation upon re-oxygenation, which suggests an inhibition of the degradation pathway. Furthermore, we show that pyruvate does not promote HIF-1α stabilization through local hypoxia, reactive oxygen species production or iron chelation. Reversible HPH inhibition in normoxia by glucose metabolites signifies a novel mechanism for HIF-1 regulation. Enhancement of HIF-1 by glucose metabolites may constitute a novel feed-forward signaling mechanism that promotes

malignant progression, since HIF-1 itself promotes glycolytic metabolism and malignancy.

Introduction

The heterodimeric transcription factor HIF-1 directly regulates the expression of over 60 genes related to cell survival in hypoxia. The expression of many of these genes can also promote carcinogenesis by inducing angiogenesis, tumorigenesis, treatment resistance and invasiveness in cancer cells and tumors (Semenza 2003). HIF-1 transcriptional activity requires dimerization of its constitutively expressed HIF-1α and HIF-1β subunits. Dimerization is normally suppressed in oxygen tensions above ~5% because oxygen promotes the rapid decay of the HIF-1α regulatory subunit. In the presence of oxygen, proline residues (Pro402 and Pro564) within the HIF-1α oxygen-dependent degradation (ODD) domain are hydroxylated (Ivan et al. 2001; Jaakkola et al. 2001). Hydroxylated HIF-1α is recognized by the E3 ubiquitin ligase von Hippel-Lindau tumor suppressor gene product pVHL and degraded via an ubiquitin-proteasome degradation pathway (Salceda et al. 1997; Huang et al. 1998; Maxwell et al. 1999). Oxygen also prevents HIF-1 transcriptional activity by a separately controlled, O2-dependent hydroxylation of asparagine 803 in the HIF-1α C-terminal transactivation domain, which inhibits HIF-1 interaction with the p300/CBP coactivator (Lando et al. 2002; Hewitson et al. 2002). To date, four O2-dependent HIF-1α hydroxylases have been identified, which include three HIF-1α prolyl hydroxylases (HPH1to 3; also referred to as PHD3 to 1, respectively) and one HIF-1α asparaginyl hydroxylase (factor inhibiting HIF, or FIH) (Bruick et al. 2001; Epstein et al. 2001; Mahon et al. 2001). These enzymes belong to a family of dioxygenases which, in addition to oxygen, require ferrous

iron and 2-oxoglutarate (2-OG) for their activity. Many of these dioxygenase enzymes also require ascorbate (Schofield and Ratcliffe 2004; Hanauske-Abel and Popowicz 2003). The dependence on Fe(II) and 2-OG explains why treatment with iron chelators or the 2-OG analogs *N*-oxalylglycine and its cell permeable precursor dimethyloxalylglycine (DMOG) can prevent HIF-1α proteolysis and activate HIF-mediated gene expression in normal oxygen (normoxic) conditions. Normoxic or basal HIF-1α expression has been observed in cancer, but it's uncertain if the mechanism involves HIF-1α hydroxylases inhibition.

Although not as well understood as the hypoxia-dependent mechanisms of HIF-1 activation, normoxic HIF-1α expression is highly relevant to the progression of human cancers because HIF-1α protein levels are not only observed in hypoxic tumor zones, but also in well oxygenated tumor areas and metastatic nodules (Semenza 2003). Also, many cancer cell lines cultured in room air (21% O_2) demonstrate high HIF-1α protein and HIF-1 regulated gene expression, as well as biological activities controlled by HIF-1 (Zhong et al. 2001). The mechanisms underlying normoxic HIF-1α expression in cancer are just beginning to be examined. Growth factors (*e.g.* insulin, insulin-like growth factor-1 (IGF-1), endothelial growth factor (EGF), and heregulin) that stimulate the phosphatidylinositol 3-kinase-Akt mTOR pathways have been shown to activate HIF-1 in the presence of O_2 through increased HIF-1α translation (Semenza 2003). Reactive oxygen species (ROS) (Gerald et al. 2004) and nitric oxide (NO) may promote HIF-1 activation by direct actions on HPHs (Metzen et

al. 2003). We recently discovered that accumulation of the glycolytic metabolites lactate and pyruvate can also promote normoxic HIF-1 activation (Lu et al. 2002). Knowles et al. (2003) showed that ascorbate selectively reverses normoxic but not hypoxia-induced HIF-1α accumulation, which suggests a distinct oxygen-independent mechanism for HIF-1 regulation and implicates HPH inhibition in that ascorbate is required to maintain HPH in the active state. Despite these clues, the main factors underlying high normoxic HIF-1 levels in cancer cells remain unclear. We therefore set out to identify the major determinants of hypoxia independent HIF-1 regulation in cancer cell lines. The data presented in this chapter, which were published in Lu et al. (2005), summarize my contribution to the understanding that HIF-1 activation by glucose metabolites in normoxia involves a reversible HPH inhibition mechanism.

Results

To study HPH mediated oxygen-dependent HIF-1α degradation, we used a live cell assay with C6 rat glioma cells that had been stably transfected with a vector encoding the HIF-1α ODD region fused to GFP (ODD-GFP). The ODD-GFP protein is hydroxylated and degraded in an oxygen-dependent manner similar to HIF-1α. As with HIF-1α, a normoxic as well as a hypoxia-inducible accumulation of ODD-GFP has been reported (D'Angelo et al. 2002). We found that normoxic ODD-GFP accumulation in C6 cells cultured in DMEM for 24 h was selectively inhibited by ascorbate, whereas the hypoxia-inducible ODD-GFP expression was not (Fig. 4A). This was expected since ascorbate can not activate HPH when the substrate oxygen is limited. In Krebs buffer, normoxic ODD-GFP accumulation was also stimulated by glucose, pyruvate and oxaloacetate, but not by succinate. The ineffectiveness of succinate *versus* pyruvate or oxaloacetate in these cells was not due to a relative difference in cell permeability since the same pattern was seen in digitonin-permeabilized C6 cells (Lu et al. 2005). Moreover, histidine and cysteine also reduced ODD-GFP induction by pyruvate and oxaloacetate (Fig. 4B). These results showed that 2-oxoacids could selectively block O_2-dependent HPH-mediated protein degradation.

Changes in mitochondrial electron transport chain activity have been shown to alter local oxygen concentrations in cell culture (Doege et al. 2005). To confirm that the metabolite effects on HIF-1 were indeed oxygen-independent, we sought to rule out the possibility that 2-oxoacids such as pyruvate might

induce local hypoxia in cell culture through enhancement of cell respiration. To determine if pyruvate could induce local hypoxia, we used the hypoxia-localizing nitroimidazole compound EF-5, which forms cellular adducts only upon reductive metabolism to reactive intermediates in anaerobic environments (Koch et al. 1995). Whereas EF-5 adducts were clearly detected by immunofluorescence in hypoxic cells, no adducts were found under normoxia, with or without added pyruvate, despite the accumulation of HIF-1α by pyruvate (Fig. 5A). Reactive oxygen species have been shown to increase HIF-1α levels in normoxia. Since ascorbate, cysteine, histidine and glutathione all have antioxidant and/or iron-reducing properties and can reverse normoxic HIF-1α (Lu et al. 2005), we also wanted to rule out enhanced production of oxidants such as H_2O_2 or NO by pyruvate or oxaloacetate. Despite confirming the ability of H_2O_2 and NO to induce HIF-1α accumulation in U251 cells, using assays described in Chapter 2, we did not detect an increase in H_2O_2 or NO levels upon treatment of U251 cells with pyruvate or oxaloacetate (Fig. 5B).

Ascorbate is required for preventing the syncatalytic inactivation of 2-OG-dependent dioxygenases. The ascorbate reversible induction of HIF-1α accumulation by pyruvate and oxaloacetate thus suggested that these 2-oxoacids could somehow inactivate cellular HPHs. To test this notion, we treated U251 cells in glucose-free Krebs buffer to either hypoxia (1% O_2) or 2mM pyruvate for 4 h. Nuclear HIF-1α protein accumulation as observed by immunocytochemistry was induced by both treatments (Fig. 6A). We then returned the hypoxia treated cells to 21% O_2 and removed the pyruvate by

extensively washing the cells with glucose-free Krebs buffer. HIF-1α immunoreactivity disappeared rapidly upon reoxygenation (return to 21% O_2) of hypoxic cells, as would be expected with the resumption of oxygen-dependent HIF-1α hydroxylation and proteolysis. However, an entirely different pattern was seen in pyruvate treated cells. Even after extensive washing out of pyruvate, nuclear HIF-1α protein levels did not decay promptly, which suggested that pyruvate inactivated the HPH enzymes. Similar results, which support the immunocytochemistry results, were observed using Western blot analysis (Lu et al. 2005)

Ferrous iron is required for HPH activity and therefore the decay of HIF-1α. Iron chelators, such as DFO, increase HIF-1α by inhibiting HPH activity. To rule out the possibility that pyruvate chelated intracellular iron, we used a live cell fluorescence-based assay for labile iron. Interaction of calcein trapped inside cells with iron specifically lowers its fluorescence. Iron chelators in turn increase fluorescence (Misko et al. 1993). Calcein-loaded U251 cells monitored for fluorescence in real-time displayed an immediate increase in fluorescence when treated with the iron chelators DFO (150 μM) or bipyridyl (100 μM) and a immediate decrease in fluorescence when 10 μM Fe(II) was added (Fig. 7A). Treatment with pyruvate (1 mM), however, showed no detectable effect on fluorescence level, which suggests that pyruvate does not inactivate HPH enzymes via iron chelation. Figure 7A demonstrates representative images of experiments performed in duplicate.

Discussion

The HIF-1α hydroxylases require Fe(II), 2-OG, molecular oxygen (O2) and ascorbate. It is thought that activity of the enzymes (Fig. 8, A–C) initially starts with bidentate ligation of the 1-carboxylate and 2-oxoacid functional groups of 2-OG to the Fe(II) bound enzyme. The functional enzyme complex, which provides access of molecular oxygen to the ferrous iron site, may also require binding of the 5-carboxylate of 2-OG to a distinct subsite, as well as HIF-1α binding. Proper assembly of these components allows for one oxygen atom of O_2 to be inserted into 2-OG to form succinate and CO_2, and the other oxygen atom likely to form a ferryl intermediate, which subsequently produces HIF-1α hydroxylation (reviewed in Schofield and Ratcliffe 2004; Hanauske-Abel and Popowicz 2003). These enzymes are inhibited by reduced availability of Fe(II), 2-OG and oxygen by chelators, 2-OG analogues and hypoxia, respectively. Syncatalytic inactivation, due to oxidization of the Fe(II) to Fe(III) or at critical amino acid residues, can also inhibit these enzymes (Fig. 8D). The reducing capacity of ascorbate is thought to block or reverse this oxidation induced syncatalytic inactivation (Fig. 8E; reviewed in Hanauske-Abel and Popowicz 2003). The fact that ascorbate and Fe(II) can readily reverse normoxic HIF-1α induction in cancer cells suggests that the HPHs are inactivated, and furthermore, that HPH inactivation may be a prominent feature of malignancy or a result of cell culture conditions and tumor microenvironment.

ROS and NO have been shown to increase normoxic HIF-1α levels under physiological conditions and in cancer (Chandel et al. 1998). HPH inactivation via

oxidation of Fe(II) to Fe(III) may be the underlying mechanism. Interestingly, the accumulation of HIF-1α by ROS and NO is reversed by ascorbate, cysteine and histidine (Gerald et al. 2004; Lu et al. 2005). Cysteine and histidine, like ascorbate, are known to modulate iron redox status (Gerald et al. 2004; Myllyla et al. 1978; Winkler et al. 1984). The antioxidant glutathione has also been shown to reduce normoxic HIF-1α (Lu et al. 2005). In cancer, increased oxidation, due free radical producing radiation therapy, mutations in signaling pathways involved in cell growth and division and mitochondrial mutations of genes encoding electron transport chain proteins (Gerald et al. 2004; BelAiba et al. 2004; Moeller et al. 2004; Zhou et al. 2007) or deficiencies in iron reducing capacity (Shoichet et al. 2002), may result in normoxic HPH inactivation and concomitant nuclear and cytosolic HIF-1α accumulation. Although, we could not detect significant ROS or NO production upon treatment of cells with pyruvate or oxaloacetate, we did find that ascorbate, cysteine, histidine and Fe(II) quickly reversed (~30min) HIF-1α accumulation by these 2-oxoacid stimuli. Since we show that 2-oxoacids do not chelate or oxidize free iron, and to the contrary of the latter are well known as potent antioxidants, it remains uncertain how the HPHs are inhibited by 2-oxoacids (Andrae et al. 1985, O'Donnell-Tormey et al. 1987). It is possible that the absence of a C5 carboxyl group allows pyruvate and oxaloacetate to interfere with allosteric enzyme mechanisms for the scheduled sequential binding of other reactants, which results in oxidation of HPH-bound iron, but prevention of HIF-1α hydroxylation. Fumarate and succinate have been shown to inhibit HPHs in a 2-OG reversible manner (Selak et al. 2005). Selective

reversal of the actions of pyruvate and oxaloacetate by ascorbate, but not 2-OG, supports the possibility of unique recognition sites for ascorbate and 2-OG within the HIF-1α hydroxylases (Majamaa et al. 1985). The lowering of normoxic HIF-1α in cancer cells by ascorbate supports previous observations of the anti-cancer effects of this vitamin (Cameron and Pauling 1976). Complete reversal of normoxic HIF-1α by ascorbate *in vitro* has been observed in the 25 – 100 μM range (Knowles et al. 2003; Lu et al. 2005). Clinical data with ascorbate administration have shown that human plasma levels are tightly controlled at ~100 μM following oral dosing, while intravenous ascorbate administration can easily achieve millimolar levels (Padayatty et al. 2004). The combined use of intravenous ascorbate along with histidine and cysteine may therefore have significant therapeutic value in cancer.

Aerobic glycolysis is a hallmark of malignancy indicated by lactate accumulation despite adequate O_2 availability (Warburg et al. 1924). This association with malignancy has been demonstrated by extensive quantitative analysis of lactate concentrations in frozen biopsy sections from various primary carcinomas, which show that tumor lactate levels can reach as high as 40 mM with levels above 8 mM being highly correlated with malignant progression, metastasis and poor clinical outcome (Walenta et al. 2004; Walenta and Mueller-Klieser 2004). Elevated lactate levels arise through the buffering action of lactate dehydrogenase which converts pyruvate to lactate. We demonstrate the ability of pyruvate to increase normoxic HIF-1α. Although similar quantitative screening of tumoral pyruvate levels has not yet been performed, intracellular lactate and

pyruvate concentrations in normal tissue have been found to be between 4 - 5 mM and 200 - 500 μM, respectively. These physiological concentrations of lactate and pyruvate are within the range that we found to activate HIF-1 in cultured cells, suggesting that HIF-1 is activated in normoxia by 2-oxoacids *in vivo* (Lu et al. 2005). Furthermore, tissue lactate/pyruvate ratios are generally between 6 and 15, which suggest a much higher pyruvate level in tumors (Ashcroft and Christie 1979; Constantin-Teodosiu et al. 1999; Walenta et al. 2004, Walenta and Mueller-Klieser 2004). Intracellular pyruvate levels are regulated by the relative rates of glycolysis, lactate dehydrogenase and pyruvate dehydrogenase. Marked differences in these metabolic activities may contribute to normoxic HIF-1 activation. The following parts of this dissertation explore this possibility by examining the role of pyruvate dehydrogenase inhibition as a causal factor in the maintenance of a malignant phenotype associated with the Warburg effect.

Figures

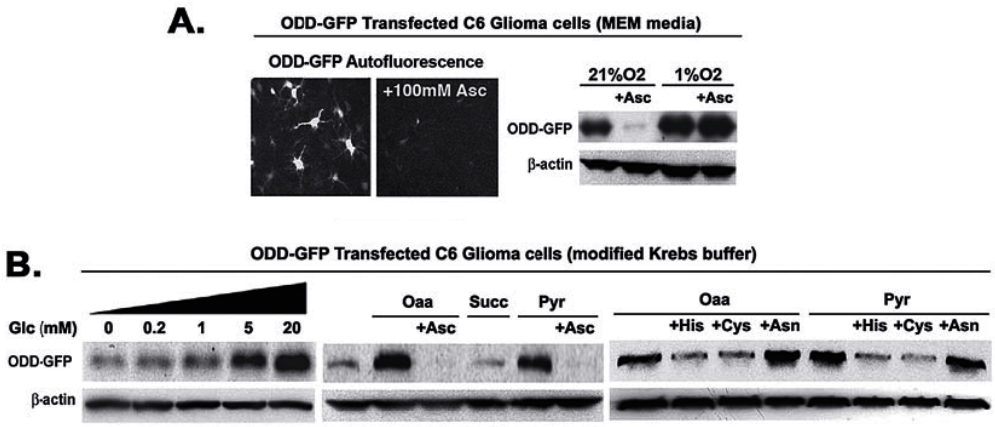

Figure 4 A. GFP expression in ODD-GFP-transfected C6 glioma cells was measured by fluorescence microscopy or Western blotting. **B.** ODD-GFP transfected C6 glioma cells were grown for 24 h in Krebs buffer modified to contain the indicated amounts of glucose (Glc) or 2 mM oxaloacetate (Oaa), succinate (Succ), or pyruvate (Pyr). Ascorbate (Asc) (100 μM) and the amino acids histidine (His), cysteine (Cys), and (Asn) asparagine (0.5 mM each) were added where indicated. (Modified from Figure 3 published in Lu et al. 2005)

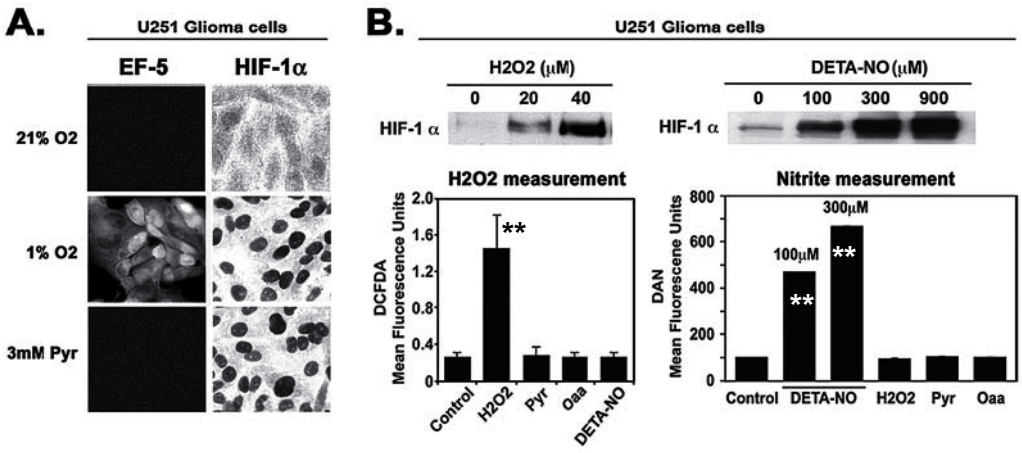

Figure 5 A. EF-5 staining in U251 glioma cells demonstrates that pyr treatment does not induce HIF-1α through hypoxia. EF-5 (500μM) was added to the culture medium, and cells were then incubated under the indicated conditions for 4 h followed by fixation and immunofluorescence staining for EF-5 adducts. **B**. **Top.** Nuclear HIF-1α in U251 cells treated with the indicated doses of H_2O_2 or the NO producing chemical diethylenetetraamine-NONOate (DETA-NO) for 4 h. **Bottom.** ROS and nitrite in cells were determined after 4 h of culture in glucose-free Krebs (control) supplemented with 40μM H_2O_2, 3 mM pyr, 3mM oaa, or 300 μM DETA-NO. **B bottom left**. 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) treatment to measure H_2O_2. H_2O_2 treated cells demonstrated significantly greater fluorescence when compared to control, pyr, oaa and DETA-NO treated cells (**p<0.001; n=3; ANOVA), while control did not significantly differ from pyr, oaa and DETA-NO treated cells. **B bottom right.** 2,3-Diaminonaphthalene (DAN) treatment to measure nitrite. DETA-NO treated cells demonstrated significantly greater fluorescence when compared to control, H_2O_2, pyr and oaa treated cells (**p<0.001; n=3; ANOVA), while control did not significantly differ from H_2O_2, pyr and oaa treated cells. (modified from Figure 3 published in Lu et al. 2005).

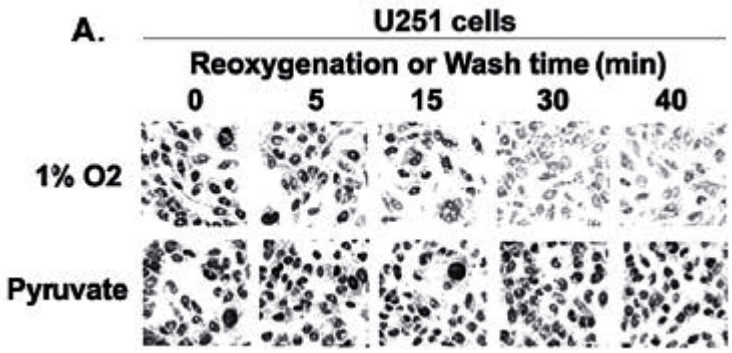

Figure 6 A. U251 cells cultured for 4 h in glucose-free Krebs either in 1% O_2 or with 3mM pyruvate in 21% O_2 were washed (three times) and maintained in oxygenated glucose-free Krebs buffer until formalin fixation and staining for HIF-1α immunoreactivity at the indicated times. (modified from Figure 5 published in Lu et al. 2005)

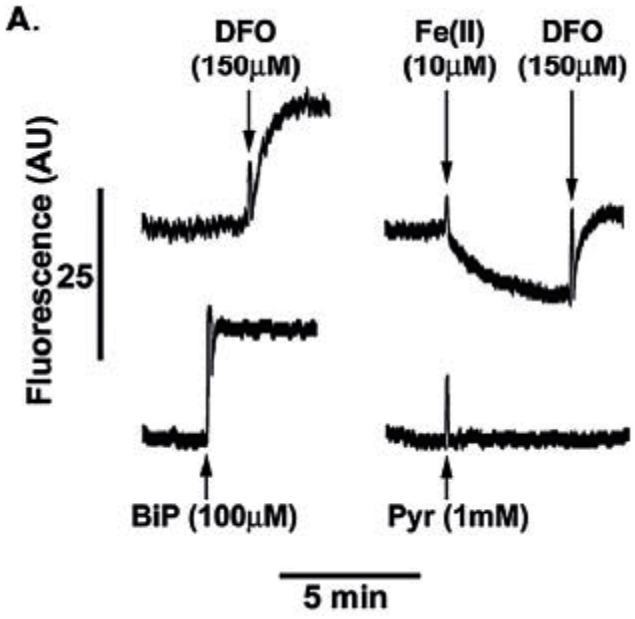

Figure 7 A. Intracellular iron levels were measured via fluorescence in calcein-loaded U251 cells exposed to the indicated agents. (modified from Figure 5 published in Lu et al. 2005)

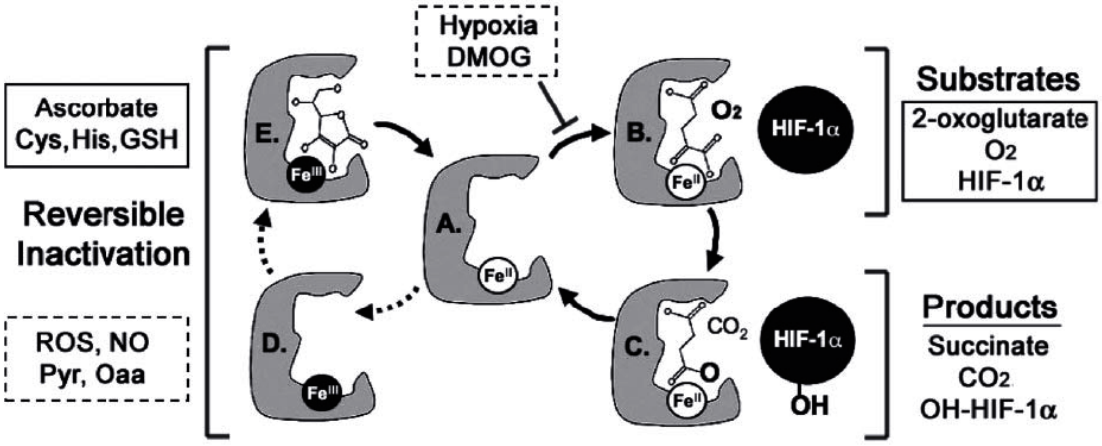

Figure 8. Model for regulation of HIF-prolyl hydroxylases by oxygen availability (A–C) or by reversible inactivation (D–E). (A–C) Demonstrates HIF-1α hydroxylation in normoxia. The Fe(II) dependent HIF-prolyl hydroxylases use the substrates HIF-1α, O_2 and 2-oxoglutarate (2-OG) to produce hydroxylated-HIF-1α, CO_2 and succinate. The process is inhibited by hypoxia and the 2-OG analogue DMOG. **(D–E)** Demonstrates proposed reversible inactivation mechanism of HIF-prolyl hydroxylases. Prolyl hydroxylases are known to be syncatalytically inactivated in normoxia by oxidization of the Fe(II) to Fe(III) or at critical amino acid residues, which is reversed by ascorbate, cysteine and histidine. Ascorbate, cysteine and histidine reverse HIF-prolyl hydroxylase inactivation by ROS, NO, pyruvate and oxaloacetate, so it is proposed that these inactivating factors may be causing oxidation of oxidization of the Fe(II) to Fe(III) or critical amino acid residues (Figure published in Lu et al. 2005).

Chapter 4

Development of an Assay for Determining PDHα Phosphorylation Status

Summary

Decreased Pyruvate Dehydrogenase Complex (PDC) activity in normal oxygen conditions, via phosphorylation of its pyruvate dehydrogenase (PDH or E1) α subunit, results in aerobic glycolysis, i.e. lactate production in aerobic conditions. Aerobic glycolysis is a classic marker for malignant transformation. We developed an antibody to detect phosphorylated PDHα levels in human cancer cells. Here we demonstrate specificity of the antibody using Western blot analysis of recombinant phosphorylated and unphosphorylated PDHα proteins, autoradiography and Western blot analysis of isolated mitochondria incubated with ^{32}P-γATP treated to increase or decrease phosphorylation, Western blot analysis of isolated mitochondria treated with kinase inhibitors to decrease phosphorylation and 2-dimensional gel analysis of whole cell lysate.

Introduction

The Pyruvate Dehydrogenase Complex (PDC) is a key regulator of glucose metabolism in that it catalyzes the irreversible conversion of the glycolytic end-product pyruvate to acetyl-CoA for use in the Krebs cycle. If PDC activity is inhibited, pyruvate and lactate levels accumulate (Stromme et al. 1976). Altered glucose metabolism or aerobic glycolysis, indicated by high lactate levels in normal oxygen conditions, has long been associated with malignant progression in cancer (Warburg et al. 1924). However, PDC inhibition in cancer has been poorly examined. The PDC is composed of three different enzymes, pyruvate dehydrogenase (PDH or E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). PDC activity is regulated by reversible phosphorylation of its PDH/E1α subunits with phosphorylation resulting in decreased activity. Most studies examining E1α phosphorylation have utilized ^{32}P incorporation approaches which are sensitive to many experimental variables and do not reflect the true endogenous phosphorylation status. PDC activity is regulated by reversible phosphorylation of three serine residues on its E1α subunit where phosphorylation of E1α results in decreased activity, and phosphorylation of site 1 (S293, in the immature rodent and human peptide) results in near maximal inhibition (~97%)(Sale and Randle 1981). The reversible phosphorylation is carried out by PDC specific kinases and phosphatases. To date four pyruvate dehydrogenase kinases (PDK1-4) and two pyruvate dehydrogenase phosphatases (PDP1-2) have been identified (Patel and

Korotchkina 2006). The kinases are known to be inhibited by the pyruvate analog dichloroacetate (DCA) and the phosphatases are known to be activated by magnesium (Mg^{2+}) (Patel and Korotchkina 2001). In order to trap and directly assess the phosphorylation status of the E1α protein, we generated a phospho-antibody that specifically recognizes phosphorylation of site 1 (Halim et al. in preparation).

Results

Using a recombinant human PDHα total peptide, the antibody was found to recognize PDHα phosphorylated at serine 293 (site 1), but had an undetectable level of cross-reactivity with the recombinant unphosphorlyated PDHα (Fig 9A). To demonstrate the specificity of this antibody in tissue extracts, we performed an *in vitro* PDHα phosphorylation/dephosphorylation assay in isolated rat whole brain mitochondria using ^{32}P-γATP (6 μCi/mmol). The phosphorylation of PDHα can be easily manipulated in isolated mitochondria, because the PDC specific kinases and phosphatases are contained inside isolated intact mitochondria (Hopper et al. 2006). Autoradiographic analysis of SDS-PAGE separated proteins transferred to nitrocellulose membranes revealed the most prominent ^{32}P-labeled band to be at approximately 42 kDa. Treatment with Mg^{2+}, which is required for PDP activity, greatly reduced labeling of this band (Fig. 9B, top panel). Subsequent immunoblotting of the same membrane with anti-PDHα ^{pS293} or anti-PDHα demonstrated Mg^{2+} induced dephosphorylation of PDHα when the PDHα ^{pS293} targeted antisera was employed. This reduction in immunoreactivity was not seen with PDHα targeted antisera (Fig. 9B).

Previous reports demonstrated cross-reactivity of a phospho-CREB antibody with phospho-PDHα (Platenik et al. 2006). Both proteins are ~40kDa (Browning et al. 1981) and this cross-reactivity was only determined using two-dimensional electrophoresis, which separates proteins by both molecular weight and isoelectric point. Therefore, to confirm that our antibody was specific for PDHα ^{pS293} we performed two-dimensional electrophoresis on rat brain protein

extracts. Subsequent immunoblotting of separated proteins produced a train of spots, characteristic of phospho-proteins, at the molecular weight (42 kDa) and isoelectric point (pI) observed for PDHα (Hopper et al. 2006) (Fig. 9C). To determine the spotting differential of the PDHα ^{pS293} antibody to the total PDHα antibody we stripped the membrane and then re-probed it using a mouse monoclonal anti-PDHα antibody. Immunoblotting with anti-PDHα produced a similar train of spots at a similar MW and pI as the PDHα ^{pS293} antibody, although one more spot was observed (Fig. 9C, see arrow). The extra spot may indicate the ability of the anti-PDHα antibody to recognize total protein, where as anti-PDHα ^{pS293} only recognizes PDHα phosphorylated at site 1. To further demonstrate the specificity of the antibody we used isolated whole brain mitochondria treated with the PDK inhibitor DCA. We observed a DCA dose dependent decrease in the anti-phospho-PDHα antibody immunoreactivity while the anti-PDHα immunoreactivity remained unchanged (Fig. 9D).

Discussion

Using a variety of methods, we demonstrate the specificity of our PDH E1α phospho-serine 293 antibody for Western blot analysis. By separating proteins on a gel using SDS-PAGE, transferring them to nitrocellulose membrane and detecting their immunoreactivity by Western blot analysis, we have determined that this antibody specifically recognizes recombinant phospho-PDHα, while it does not recognize recombinant nonphospho-PDHα. We used isolated mitochondrial extracts incubated with ^{32}P-γATP and either a phosphatase activator to decrease phosphorylation or a phosphatase inhibitor or increase phosphorylation, then separated these radiolabeled protein extracts using SDS-PAGE and imaged them using autoradiography. We used the nitrocellulose membrane from the autoradiography experiment for Western blot analysis and showed that the antibody matched the unphosphorylated-phosphorylated pattern. Also by Western blot analysis, we show that the antibody specifically detects a dose dependent decrease in immunoreactivity in protein extracts treated with the PDK inhibitor DCA. A limitation of Western blotting is that cross-reactivity with a protein of a similar molecular weight may go unnoticed. Based on findings in the literature showing that the antibody to phospho-CREB, which has a similar molecular weight to PDHα, cross-reacted with PDHα, we used 2-dimensional gel analysis to not only separate proteins by molecular weight, but also by isoelectric point. Although CREB and PDHα have similar molecular weights, they have very different isoelectric points. Two-dimensional gel analysis results demonstrating a single immunoreactive pattern

expected for PDHα show that there is no cross-reactivity between the phospho-PDHα antibody and the CREB protein. It remains to be determined if this antibody can be effectively used for other immunoreactivity assays beside Western blot, such as immunocytochemistry and immunohistochemistry.

Although phosphorylation of serine 293 has been shown to maximally inhibit PDC activity, phosphorylation of serine at sites 2 and 3 can also inhibit PDC activity. Since this antibody is specific for site 1 phosphorylation, it's conceivable that site 1 could be dephosphorylated, yet the PDC would remain inactive due to site 2 and 3 phosphorylation. However, this is unlikely given that the dephosphorylation rate of site 1 has been shown to occur at a slower rate when compared to the dephosphorylation rates of sites 2 and 3 (Patel and Korotchkina 2001). The effectiveness of this tool as a measure of PDC activity could be more fully established by developing antibodies specific for sites 2 and 3 and comparing the immunoreactivity of all three to the relative PDC activities.

This antibody will be used to determine phospho-PDHα levels in cancer in the following chapter. However, this tool is not limited to the exploration of cancerous metabolic patterns. The manuscript entitled "Phosphorylation Status of Pyruvate Dehydrogenase Distinguishes Metabolic Phenotypes of Rat Cerebral Cortical Astrocytes and Neurons", in which the antibody data will be published (Halim et al. in preparation), explores the role of PDHα phosphorylation in the astrocyte/neuron lactate shuttle hypothesis. The hypothesis proposes that despite having similar concentration of mitochondria per cell, astrocytes primarily derive energy from glycolysis and produce lactate for neurons that primarily use

oxidative phosphorylation for energy production (Pellerin et al. 2007). Using the antibody, it appears that *in vitro* PDHα is indeed phosphorylated to a greater degree in astrocytes compared to neurons. Furthermore, the follow-on reduction of PDC activity observed in astrocytes is reversible by dephosphorylation to levels comparable to neurons. Moreover, decreased mitochondrial activity has been implicated in many disease states, which may or may not be due to decreased PDC activity as a mechanism of their pathology, including diabetes, heart disease (Roche and Hiromasa 2007) and degenerative neurological disorders, such as Parkinson's syndrome, Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease (Hoshi et al. 1996, Petrozzi et al. 2007). The antibody may prove to be an important tool in determining if decreased PDC activity due to increased phosphorylation is involved in the pathology of these diseases.

Figures

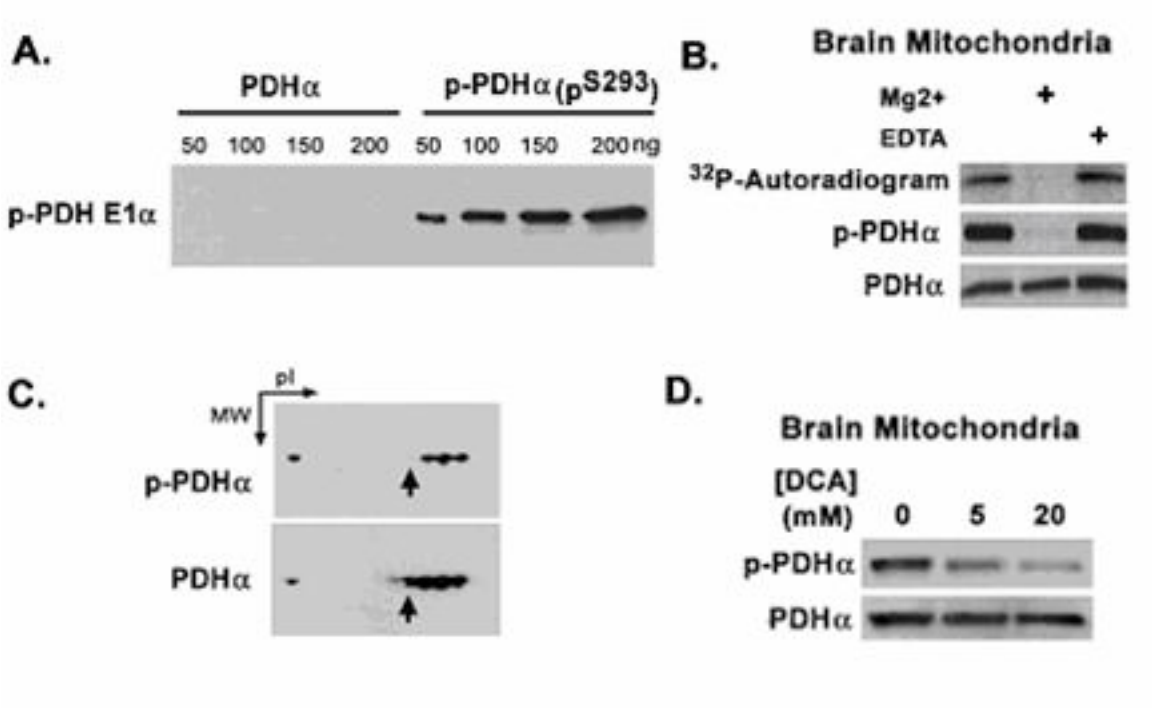

Figure 9. A. Generation of phospho-specific antibody to serine293 (site 1) of the E1α subunit of pyruvate dehydrogenase (PDH) was confirmed using phosphorlyated and unphosphorlyated recombinant peptides, an *in vitro* kinase assay, and two-dimensional electrophoresis. The antiserum is specific to phosphorlyated S293 and has no cross-reactivity to the unphosphorlyated peptide **B**. *In vitro* kinase assay and subsequent immunoblotting demonstrated a Mg^{2+} dependant dephosphorylation of isolated rat brain PDH detected with antibodies to PDHα and p-PDHα **C.** Dose dependant dichloroacetate (DCA) induced dephosphorylation of rat brain mitochondria demonstrated by immunoblotting with p-PDH and PDH antibodies. (Figures to be published in Halim et al. *in preparation*)

Chapter 5

Determination if Decreased PDC Activity via Phosphorylation of its Regulatory PDHα Subunit Contributes to Malignancy in Cancer

Summary

Malignant progression has long been associated with altered glucose metabolism characterized by increased lactate production despite adequate oxygen, but the causes and functional roles of this link still remain unclear. Here we show that phosphorylation dependent inhibition of pyruvate dehydrogenase complex (PDC) activity contributes to this metabolic pattern in human cancer cells. Expression of pyruvate dehydrogenase kinase-1 (PDK-1) and inhibitory phosphorylation of the pyruvate dehydrogenase α (PDHα) subunit is associated with enhanced lactate production and normoxic accumulation of hypoxia-inducible factor-1α (HIF-1α). Knockdown of PDK-1 via shRNA reverses these phenotypes and also lowers hypoxic survival, invasiveness, and tumor formation by cancer cells. Oncogenic transformation of cells also promotes PDK-1 expression and PDHα phosphorylation. Since PDK-1 is itself a HIF-1 regulated gene, our findings suggest that PDC inhibition sustains a feed-forward loop for malignant progression.

Introduction

Altered glucose metabolism reflected by lactate accumulation despite adequate oxygen availability is commonly associated with malignant transformation. This metabolic phenotype, termed aerobic glycolysis and historically known as the Warburg effect, is characterized by high glycolytic rates and reduced mitochondrial oxidation (Warburg et al. 1924; Semenza et al. 2001), features that may favor survival in hypoxic microenvironments found in tumors. Hypoxia may in fact select for this phenotype by activating hypoxia-inducible transcription factor-1 (HIF-1), which induces transcription of glucose transporters, glycolytic enzymes and also many other genes associated with hypoxic survival, angiogenesis, and tissue invasion (Semenza et al. 2001). Hypoxia, HIF-1 activation and high lactate levels in tumors are independently correlated with poor outcome for many human cancers (Harris 2002; Walenta et al. 2004; Mabjeesh and Amir 2007). Recently, several intermediary glucose metabolites have been shown to promote HIF-1 activation independently of hypoxia by interacting with the HIF prolyl hydroxylases (HPH 1-3). These enzymes promote degradation of the HIF-1α subunit (Jaakkola et al. 2001) and require oxygen, iron, ascorbate and 2-oxoglutarate (2-OG) for their activity. Fumarate and succinate inhibit HPHs by competing with 2-OG (Isaacs et al. 2005; Selak et al. 2005) while lactate, pyruvate and oxaloacetate inactivate PHDs in an ascorbate reversible manner (Lu et al. 2005). Alterations of glucose routing may thus directly impact malignant progression through novel signaling actions of metabolites.

The pyruvate dehydrogenase complex (PDC) controls pyruvate entry into the tricarboxylic acid (TCA) cycle. Differentially expressed pyruvate dehydrogenase kinases (PDK-1-4) and pyruvate dehydrogenase phosphatases (PDP-1, 2) tightly regulate PDC activity via reversible inhibitory phosphorylation of the PDH E1α (PDHα) subunit (Roche and Hiromasa 2007). PDK-1 was recently identified as a HIF-1 regulated gene, thus implicating PDHα phosphorylation in the hypoxic induction of aerobic glycolysis in cancer cells (Kim et al. 2006; Papandreou et al. 2006). We hypothesized that by allowing accumulation of glycolytic end-products, PDHα phosphorylation may also potentially establish a feed-forward stimulatory loop between glucose metabolites and HIF-1, an action that may accelerate malignant progression.

Results

To elucidate a possible role for PDHα phosphorylation in the malignant phenotype of cancer cells, we utilized the human head and neck squamous cell carcinoma (HNSCC) cells lines UM-22A, UM-22B, and JHUO22. Two of these were generated from a primary (UM-22A) and metastatic lesion (UM-22B) in the same patient (Zou et al. 2001). Although these cancer cell lines showed comparable anchorage-dependent growth properties (Fig. 10A), the 22B cells displayed much greater anchorage-independent growth in soft agar colony formation assays (Fig. 10B) and twice the lactate accumulation seen with the other cell lines (Fig. 10C). Also, the 22B cells alone accumulated observable nuclear HIF-1α protein following 12h in culture under normal oxygen conditions (Fig. 10D). Normoxic HIF-1α expression has been linked to aerobic glycolysis and is additive with hypoxia-induced HIF-1α accumulation (Lu et al. 2005). In keeping with this, 22B cells also displayed the highest induction of HIF-1α following 4h exposure to hypoxia (1%O_2) (Fig. 10D). To determine the respective PDHα phosphorylation status of the HNSCC cell lines, we used a PDHα phosphoserine 293 (site 1) specific antisera since phosphorylation of this site produces nearly complete inhibition of PDC activity (Sale and Randle 1981). A monoclonal antibody was used to measure total PDHα expression. The highest ratio of phospho-PDHα to total PDHα was observed in the 22B cells (Fig. 10E). Although differential expression of PDK isoforms 1-4 was seen among the head and neck cancer cell lines, we observed the highest expression of PDK-1 in 22B cells (Fig. 10E). We also found prominent PDHα phosphorylation and PDK-1

expression in malignant breast cancer and glioma cells, as well as astrocytes transformed with Ras alone or Ras plus Akt (Figs. 13 A-C) with greater HIF-1α expression corresponding to higher malignancy and PDHα and PDK-1 expression in the glioma lines (Figs. 13 D,E). HIF-1α accumulation in these cells was only seen after 3 days in culture, while HIF-1α in 22B cells was observed in 12h. In addition to greater normoxic HIF-1α expression, preliminary results characterizing a variety of cancer cell lines for their malignant phenotypes by measuring growth rate, lactate production, invasiveness, VEGF production, and colony formation, suggested to us that 22B cells were our most malignant cell line. We therefore used 22B cells to determine the effect of decreasing PDHα phosphorylation on the malignant phenotype.

Recent studies with the weak PDK inhibitor dichloroacetate (DCA) suggest that inhibition of PDHα phosphorylation may shift cancer cell metabolism towards aerobic respiration, promote cancer cell apoptosis and reduce tumor growth (Kim et al. 2006; Papandreou et al. 2006; Bonnet et al. 2007; Cairns et al. 2007). We found that DCA decreased PDHα phosphorylation dose-dependently in 22A cells, but was surprisingly without significant effect in 22B cells, even at concentrations up to 20 mM (Fig. 10, F and G). Also, the time and dose dependent inhibition of lactate release by DCA observed in 22A cells was absent in 22B cells (Fig. 14A,B). Moreover, at the high doses required for its effects, DCA produced toxicity in the 22B cells, but not the 22A cells (Fig. 14C,D). In addition to weakly inhibiting PDK, DCA blocks glutathione S-transferase zeta (Tzeng et al. 2000), inhibits cholesterol biosynthesis (Stacpoole and Greene 1992), promotes

oxidative stress (Hassoun and Ray 2003) and activates peroxisome proliferator-activated receptors (Laughter et al. 2004). This along with our observations that DCA treatment causes cell death in malignant 22B cells independent of PDHα dephosphorylation persuaded us to use more specific strategies for implicating a role for PDHα phosphorylation in cancer biology.

We therefore utilized short hairpin RNA (shRNA) to knockdown PDK-1 expression in 22B cells. Cells were transfected with a vector under control of the U1 promoter containing a neomycin resistant gene and stably expressing cell lines were selected by antibiotic treatment. Western blot analysis showed the effective reduction of PDK-1 expression in the stably transfected PDK-1 shRNA (shPDK-1) cells when compared to cells transfected with control shRNA (shCt) (Fig. 11A). Western blotting confirmed reduction of PDHα phosphorylation but not PDHα expression following PDK-1 knockdown. Measurement of PDC activity in cell lysates showed that shCt cell extracts had 60% lower PDC activity than the shPDK-1 cells (Fig. 11B). However, after addition of recombinant PDP-1 to maximally dephosphorylate PDHα, and thus fully activate PDC, the shCt cell extracts showed greater total activity than the shPDK-1 cell extracts, confirming strong phosphorylation dependent inhibition. The ratio of active to total PDC was 4 times higher in the 22B shPDK-1 cells compared to the 22B shCt cells (Fig. 11B; $p<0.001$). Consistent with this, the shPDK-1 cells were found to oxidize uniformly labeled ^{14}C glucose to $^{14}CO_2$ at twice the rate of shCt cells (Fig. 11C; $p<0.01$). Furthermore, lactate production by the shPDK-1 cells was decreased

(Fig. 11D; $p<0.001$) to levels similar to those observed in 22A and O22 cell lines from Fig. 10 C.

Removal of media glucose or its substitution with non-glycolytic fuel sources has been shown to diminish normoxic HIF-1α accumulation and also to lower HIF-1α stabilization in hypoxia (Lu et al. 2002; Lu et al. 2005), suggesting a key role for glycolytic metabolism in regulating normoxic and hypoxic HIF-1 activation. To examine whether altering the ratio of glycolysis to glucose oxidation via PDC activation had an impact on HIF-1α stabilization, we measured nuclear HIF-1α accumulation in cells following 16h culture with or without exposure to hypoxia (1%O_2) for the final 4h. As shown in Figure 11E, the normoxic and hypoxia dependent HIF-1α accumulation seen in the parent 22B cells was retained in the shCt cells. However, PDK-1 knockdown eliminated normoxic and reduced hypoxic accumulation of HIF-1α (Fig. 11E). A similar effect was seen in 22B cells stably transfected with shRNA targeting PDK-2, which has been proposed to form functional heterodimers with PDK-1 (Boulatnikov and Popov 2003). HIF-1α accumulation following treatment with the proteasome inhibitor MG-132, which blocks the final step of HIF-1α degradation, was similar in all transfectants, which suggests that HIF-1α reduction by PDK knockdown was not due to decreased protein production (Fig. 11F). The regulation of HIF-1 by glucose metabolism is most readily and specifically demonstrated using short term culture of cells in glucose containing Krebs buffer rather than complete medium (Lu et al. 2002; Lu et al. 2005). A robust glucose-dependent HIF-1α accumulation was seen under these conditions within 4h in the shCT cells.

However, this effect was diminished in shPDK cells (Fig. 11G). Pyruvate can substitute for glucose in promoting normoxic HIF-1α accumulation in many cell lines (Lu et al. 2002; Lu et al. 2005), and the response to pyruvate was also diminished in shPDK cells (Fig. 11H). Glucose-dependent and normoxic HIF-1α accumulation is known to be selectively blunted by ascorbate (Lu et al. 2002; Knowles et al. 2003; Lu et al. 2005; Zhou et al. 2007). A prominent accumulation of ascorbate-sensitive HIF-1α was seen over 24h in the parent and shCt 22B cells, but not in the shPDK cells (Fig. 11I). Moreover, ascorbate did not affect the phosphorylation status of PDHα, suggesting that the reduction of normoxic HIF-1α accumulation by ascorbate occurs independently from PDC activity. These observations suggested that reduction of PDC activity by PDK mediated phosphorylation sensitizes cells to HIF-1α stabilization by glucose or glycolytic metabolites.

We next examined several biochemical and phenotypic markers relevant to the malignant phenotype to determine if they were altered by differential PDC activity in 22B cells. No difference was observed in the anchorage dependent growth of 22B cells following knockdown of PDK-1 (Fig. 12A). However, the shPDK1 cells showed significantly higher cell death as measure by trypan blue exclusion under prolonged hypoxia (Fig. 12B; $p<0.01$; $n=3$), as well as a profound reduction in the ability to form colonies in soft agar (Fig. 12C; $p<0.001$; $n=3$). Moreover, the shPDK-1 cells also displayed a 50% decrease in vascular endothelial growth factor (VEGF) secretion as assessed by VEGF ELISA analysis (Fig. 12D; $p<0.001$; $n=6$) and showed a loss of invasiveness and

migration in Matrigel™ coated Boyden chambers (Fig. 12E; $p<0.001$; n=6). Decreased PDHα phosphorylation and VEGF production was also observed in shPDK-2 cells (Fig. 15 A-B). These *in vitro* results suggested that PDC activity may be an important determinant of aggressive tumor growth. To examine the impact of PDK knockdown on in vivo tumor growth, we performed xenograft studies using nude mice. We injected 10×10^6 cells into the posterior neck scruff of 10 mice for each group. Palpable tumors were seen at one week and subsequently measured bi-weekly for 2 months. At end-point, 9 out of 10 mice formed tumors in each group. Figure 12F shows that PDK-1 knockdown led to a dramatic reduction of tumor growth ($p<0.05$; n=9). Three examples of representative tumors show the marked differences seen in tumor morphology. The shPDK1 tumors remained white with the larger tumors developing what appear to be central necrotic areas. The much larger shCt tumors exhibited no externally visible necrotic area and appeared to be well vascularized based on their dark color (Fig. 12G). Western blot analysis of tumor tissue from 3 separate tumors for each group showed that the differential PDHα phosphorylation state and HIF-1α (Fig. 12H) accumulation observed *in vitro* was also observed *in vivo*.

Discussion

Despite being recognized for nearly 80 years, the causes and consequences of aerobic glycolysis in cancer cells have only recently begun to emerge. Several common oncogenes including Myc, Ras and Akt can upregulate glycolysis (Shim et al. 1997; Blum et al. 2005; Elstrom et al. 2004). Decreased oxidation can result from cancer related mitochondrial DNA mutations (Zhou et al. 2007), p53 mutations (Zhou et al. 2003) and rare familial mutations in the TCA cycle enzymes succinate dehydrogenase and fumarate hydratase (Gottlieb and Tomlinson 2005). Together these genetic alterations in cancer cells favor the accumulation of glycolytic metabolites such as pyruvate, lactate and TCA cycle intermediates. All of these mutations have been also linked to the regulation of HIF-1α expression. In the present study, we have demonstrated that PDHα is basally phosphorylated in several human cancer cells and that this contributes to the Warburg phenotype and associated hypoxia-independent HIF-1 activation. We have shown that stable knockdown of PDK-1 and 2 diminishes HIF-1α accumulation and reduces malignant features. Our study thus supports a direct role for biological actions of glucose metabolites in influencing cancer progression. This is consistent with newly recognized signaling roles for lactate and pyruvate in angiogenesis (Lee et al. 2001; Hunt et al. 2007), metastasis (Walenta et al. 2004) and immune evasion by cancer cells (Fischer et al. 2007). Our study provides support for PDK isoforms as potential targets for killing cancer cells. Although DCA has shown some potential to be used as a drug to kill cancer cells, the toxicity, low potency and lack of specificity warrant the clinical

development of new PDK inhibitors. Recent advances in the ability to quantitatively image tumor lactate and pyruvate in biopsies and *in vivo* (Golman et al. 2006) offer an ideal opportunity to test therapeutic aimed at reverting the Warburg effect in cancer.

Figures

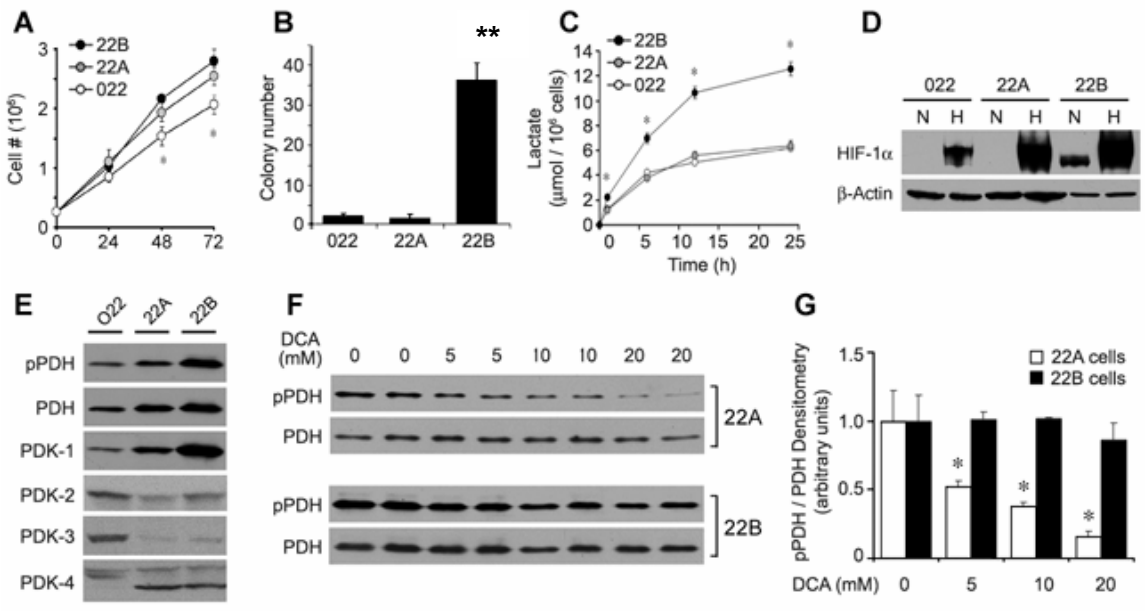

Figure 10. Growth, metabolic phenotype, and PDH phosphorylation in HNSCC cells. **A.** 22B, 22B, and O22 cells demonstrate similar anchorage-dependent growth (*p<0.01(22B compared to O22 at 48 and 72hr); n=3; ANOVA). **B.** Only the 22B cells develop significant anchorage-independent colonies (**p<0.001; n=3; ANOVA). **C.** 22B cells demonstrate higher lactate production than O22 and 22A cells (*p<0.01(22B compared to O22 and 22A at 6, 12 and 24hr; n=3; ANOVA). **D**. Western blot analysis of nuclear extracts shows 22B cells have greater normoxic and hypoxic HIF-1α protein expression than O22 and 22A cells. **E.** Western blot analysis of whole cell lysate for pPDHα, PDHα and PDK1-4 protein expression shows 22B cells to have the highest pPDHα and PDK-1 expression. **F.** Western blot analysis of pPDHα and PDHα protein expression in whole cell lysates from 22A and 22B cells treated with 0, 5, 10 and 20mM DCA for 24hr shows dose dependent decrease of PDHα phosphorylation in 22A, but not 22B cells. **G.** Densitometric ratio of immunoreactve pPDHα/PDHα from Fig. 10F (*p<0.01(22A at 5, 10 and 20mM DCA compared to 0mM DCA; n=3; ANOVA).

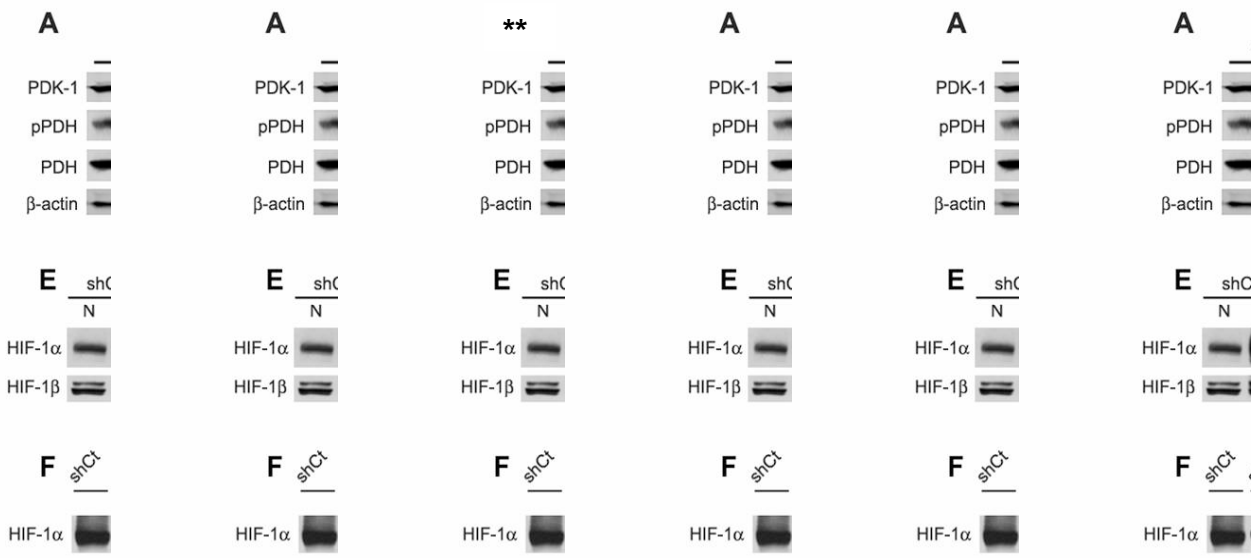

Figure 11. Dependence of Warburg phenotype and normoxic HIF-1 accumulation on PDK expression. **A.** Western blot of 22B cells stably transfected with shPDK1 show reduced PDK-1 and pPDHα expression when compared to shCt cells. **B.** Active:total PDC activity is higher in shPDK1 compared to shCt cells. Total activity was determined as activity following PDP-1 treatment (**p<0.001; n=3; Student's t test). **C.** $^{14}CO_2$ captured from uniformly labeled glucose is higher in shPDK1 compared to shCt cells (*p<0.01; n=4; Student's t test). **D.** Lactate production is decreased in shPDK1 cells compared to shCt. (*p<0.01 at 12, 24 and 48hr; n=3; ANOVA). **E.** Western blot of nuclear extracts shows decreased normoxic and hypoxic HIF-1α expression in shPDK1 and shPDK2 cells compared to shCt. **F.** Western blot of normoxic HIF-1α expression following treatment with the 26S proteosome inhibitor MG132 (15 μM) shows equivalent HIF-1α expression. **G.** Western blot shows higher normoxic HIF-1α expression in shCt cells following incubation (4hr) in Krebs buffer compared to shPDK1 cells. **H.** Western blot of normoxic HIF-1α expression is higher in shCt cells following incubation (4hr) in glucose-free Krebs buffer with pyruvate compared to shPDK1 cells. **I.** Ascorbate treatment (100μM for 24hr) decreases normoxic HIF-1α expression independently of PDH phosphorylation demonstrated by Western blot of nuclear (HIF-1α and β-actin) and cytoplasmic (pPDHα and PDHα) cell extracts.

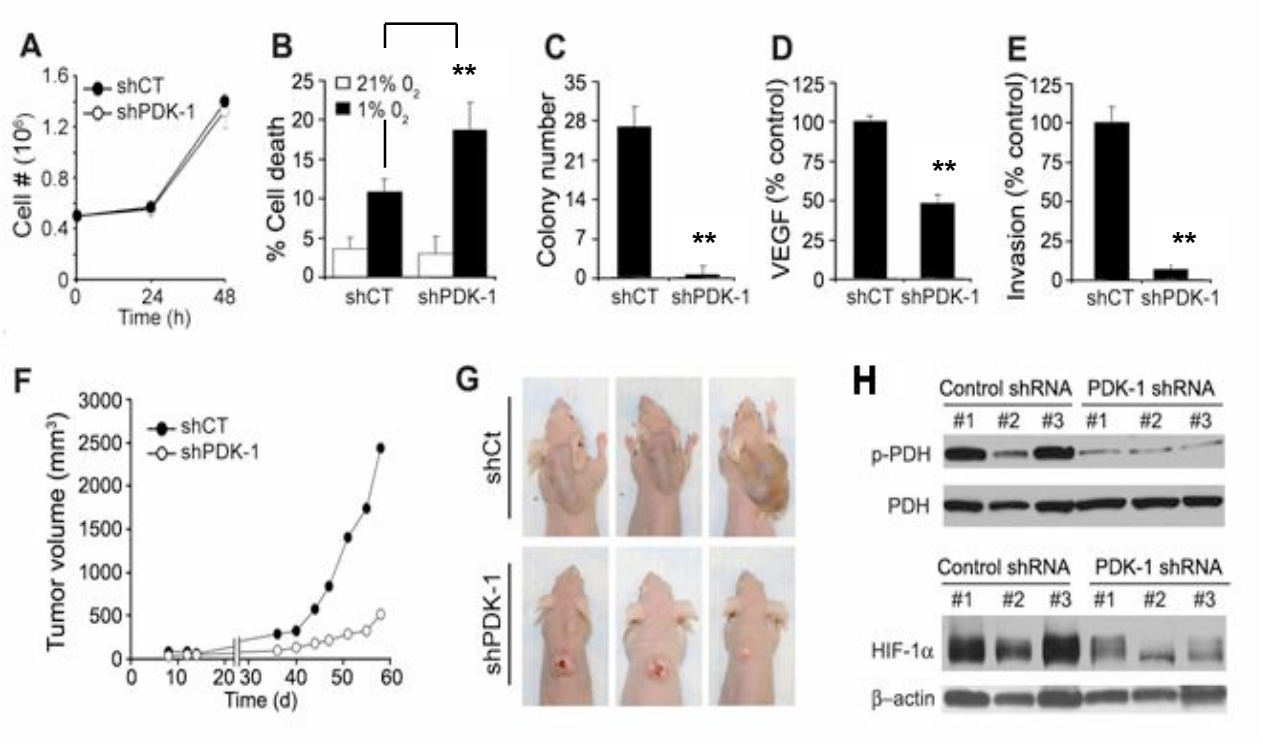

Figure 12. *In vitro* and *In vivo* reversal of malignant phenotype by PDK inhibition in 22B cells **A.** shPDK1 and shCt cells demonstrate no significant difference in anchorage-dependent growth rates. **B.** Hypoxia treatment increases shPDK1 cell death 2-fold greater than 22B shCt (**p<0.001(1% O_2 shPDK1 compared to 1% O_2 shCT); n=3; ANOVA). Comparison of survival in 21% showed no significant difference between cell lines. **C.** shPDK1 cells demonstrate decreased capacity to form colonies in soft agar compared to shCt (**p<0.001; n=3; Student's t test). **D.** VEGF production, assayed by ELISA, is reduced in shPDK1 cells compared to shCt cells (**p<0.001; n=6; Student's t test). **E.** Invasiveness and migration through Matrigel™ coated Boyden chambers is decreased in shPDK1 cells compared to 22B shCt cells (**p<0.001; n=6; Student's t test). **F.** Xenograft tumor growth in nude mice is reduced in shPDK1 cells compared to shCt cells (p<0.05; n=9; ANOVA). **G.** Three representative images of shCt and shPDK1 tumors. **H.** Western blot analysis of cytoplasmic (pPDHα and PDHα) and nuclear (HIF-1α and β-actin) cell extracts from 3 separate xenograft tumors from each group shows that the decreased pPDHα and HIF-1α expression in shPDK1 compared to shCt cells observed *in vitro* is maintained *in vivo*.

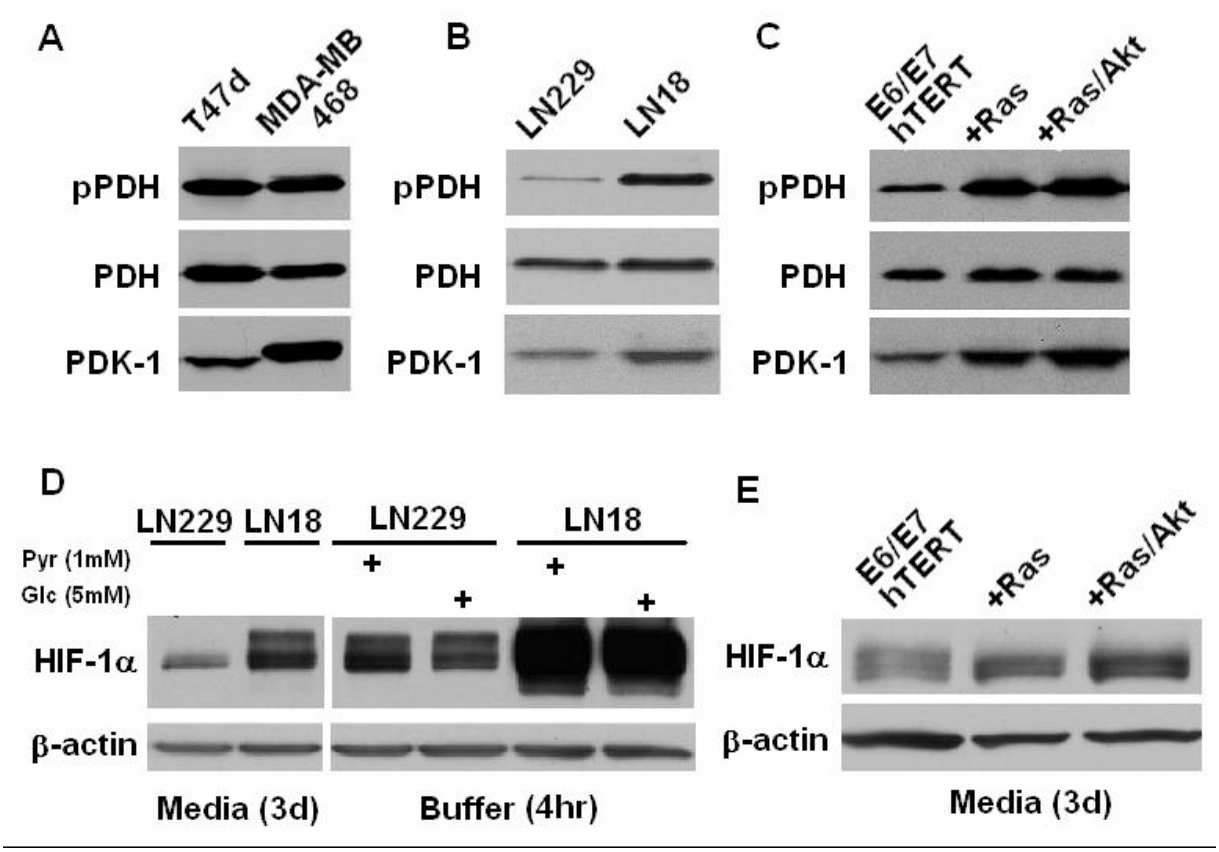

Figure 13 Phosphorylated PDHα and PDK1 expression is indicative of normoxic HIF-1α expression in malignant cell lines. Western blot analysis of whole cell lysates from malignant breast cell lines T47d and MDA-MB468 **A.**, glioma cell lines LN229 and LN18 **B.**, and from genetically modified human astrocyte cell lines shows that man diverse human cancer cells maintain a significant levels of PDH in the phosphorylated state and that this generally mirrors PDK-1 expression status. The LN18 cells have previously been shown to have much higher rates of glycolysis as well as high levels of phospho-akt, features lacking from the LN18 cells (Elstrom et al. 2004). The immortalized human astrocytes, generated via stable E6/E7 and hTERT expression, do not form tumors in rodent brain. When also made to express constitutively active Ras, these cells form tumor resembling grade 3 anaplastic astrocytoma. With further forced expression of constitutively active akt, these cells develop grade 4 glioblastoma multiforme like tumors in rodent brain (Sonoda et al. 2001). **D.** Western blot analysis of nuclear extract from glioma cell lines demonstrates greater basal (normoxic) HIF-1α expression in the LN18 cell line both in media and glucose-free Krebs buffer supplemented with pyruvate or glucose. **E.** Western blot analysis of nuclear extract from genetically modified human astrocyte cell lines demonstrates greatest HIF-1α expression in the most transformed E6/E7/hTERT/Ras/Akt cell line.

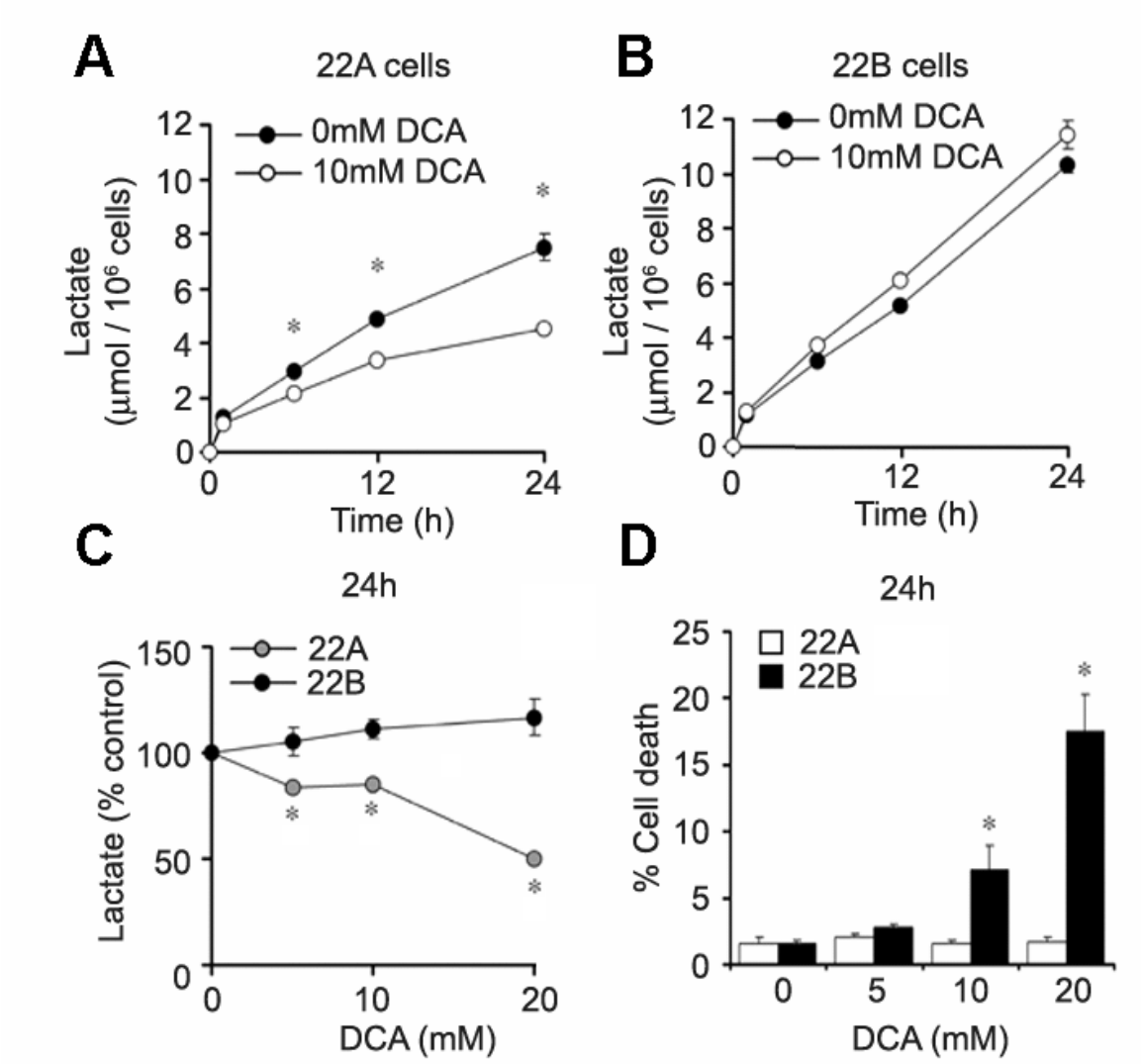

Fig. 14 DCA treatment increases cell death in 22B cells independent of lactate reduction. **A.** Lactate production is reduced in 22A cells treated with 10mM DCA (*p<0.01 at 6, 12 and 24hr; n=3; ANOVA). **B.** Although there is a statistical difference between the DCA treated and untreated 22B cells, lactate production does not significantly change over time (*p<0.01(22B compared to 22B 10mM DCA); n=3; ANOVA). **C.** DCA dose dependently reduces lactate in 22A, but not 22B cells (*p<0.01(22A at 5, 10 and 20mM DCA compared to 0mM DCA; n=3; ANOVA). **D.** DCA dose dependently increases cell death as measured by trypan blue exclusion in 22B, but not 22A cells (*p<0.01(22B at 10 and 20mM DCA compared to 22A; n=3; ANOVA).

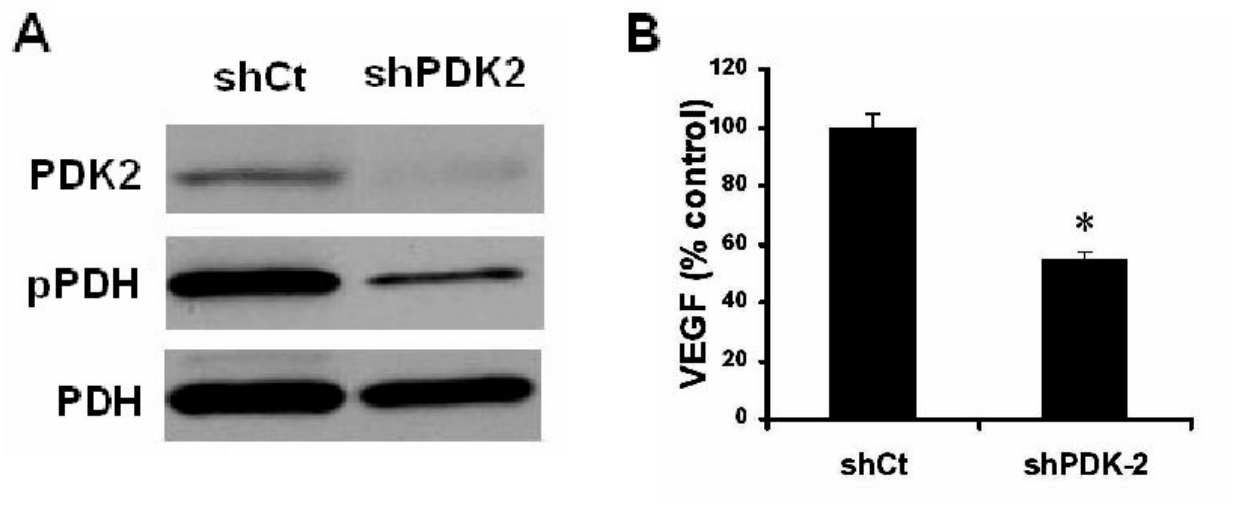

Fig. 15 PDK2 knockdown inhibits PDH phosphorylation and malignant phenotype. **A.** Western blot of 22B cells stably transfected with shPDK2 show reduced PDK2 and pPDH expression when compared to shCt cells. **B.** VEGF production, assayed by ELISA, is reduced in shPDK2 cells compared to shCt cells. ($p<0.01$, n=6, Student's t test).

Discussion

Elevated lactate levels despite adequate oxygen supply, referred to as aerobic glycolysis or the Warburg effect, has long been associated with malignant progression in cancer (Warburg 1924). This thesis research examines causes and functions of this metabolic phenomenon. The first part of this research is my contribution to a continuation of a discovery by our lab that the glycolytic end-products pyruvate and lactate can activate the transcription factor Hypoxia Inducible Factor-1 (HIF-1) in normal oxygen conditions through accumulation of its HIF-1α regulatory subunit (Lu et al. 2002). HIF-1 is a ubiquitous transcription factor that controls the expression of many genes important for cell survival, and its role in malignant progression has been well documented (Semenza 2003). Since HIF-1 activation leads to increased glycolysis and decreased oxidative phosphorylation, which results in elevated pyruvate and lactate, HIF-1 is often implicated as a major culprit for aerobic glycolysis (Robey et al. 2005). Normoxic accumulation of HIF-1α by pyruvate and lactate suggests a novel feed-forward mechanism for malignant progression. A thorough understanding of the mechanism by which pyruvate and lactate result in HIF-1α accumulation may provide insight into mechanisms of malignant progression and lead to new treatment regimes. Understanding what promotes aerobic glycolysis in cancer and determining if decreasing aerobic glycolysis can decrease malignancy may also lead to new treatment regimes.

In the second part of my dissertation I focus on what promotes aerobic glycolysis in cancer and determining if decreasing aerobic glycolysis can decrease malignancy. To do this I examined the possible role of Pyruvate Dehydrogenase Complex (PDC) inhibition in the maintenance of aerobic glycolysis in cancer and in turn, if PDC activation can decrease aerobic glycolysis, and therefore the malignant phenotype. PDC inhibition results in aerobic glycolysis and is indicated by phosphorylation of its PDHα. If PDC activity is involved in malignancy, it offers an attractive therapeutic target in that its activity is specifically regulated by a set of 4 enzyme bound kinases and 2 phosphatases.

Therefore, this thesis aimed to address two simple questions:

1. What is the mechanism by which glucose metabolites stabilize HIF-1α in normal oxygen conditions?
2. Does decreased PDC activity via phosphorylation of its regulatory PDHα subunit contribute to malignancy in cancer?

Chapter 3, **Examination of the Mechanism Underlying HIF-1α Regulation by Glycolytic Metabolites in Normoxia,** addresses the first question. This chapter presents data published in Lu et al. 2005. Briefly, this paper shows that pyruvate and other 2-oxoacids can inhibit the activity of the HIF Prolyl Hydroxylases (HPH) in normoxia in an ascorbate, ferrous iron, cysteine and histidine reversible manner. The HPHs promote HIF-1α degradation by hydroxylation of proline residues. Once hydroxylated HIF-1α is targeted by pVHL,

the E3 recognition subunit of an ubiquitin ligase, and targeted for degradation. We showed that 2-oxoacids inhibit HPH binding to HIF-1α, HIF-1α hydroxylation and pVHL binding. Other researchers have found that a build-up the Krebs cycle intermediates fumarate and succinate, due to either germline mutations of fumarate hydratase (FH) and succinate dehydrogenase (SDH), respectively, or direct treatment of cells with cell permeating fumarate and succinate derivatives, can also stabilize HIF-1α in normoxic conditions (Selak et al. 2005). We did not see this effect in our observations (Lu et al. 2005), which may be due to methodological differences in that we used supplemented glucose-free Krebs to isolate the effect of each metabolite. In a paper by Isaacs et al. (2005), FH inhibition was shown to correlate with increased fumarate and HIF-1α accumulation. Interestingly, but not well emphasized in the paper, FH inhibition also resulted in a large increase in lactate. This large increase in lactate may contribute to the observed HIF-1α based on our observations that glucose-free Krebs supplemented with lactate results in HIF-1α accumulation.

Using electrospray ionization mass spectrometry (ESI-MS), the Krebs cycle intermediates 2-oxoglutarate, fumarate, succinate, isocitrate and malate were shown to form a complex with recombinant HPH2 enzyme (Hewitson et al. 2007). Interestingly, pyruvate and the Krebs cycle intermediate oxaloacetate, two molecules in which we observed a robust ability to promote normoxic HIF-1α stabilization, did not form complexes. Furthermore, in the same publication the fumarate, succinate, isocitrate and oxaloacetate were shown to inhibit HPH2 activity, while pyruvate did not. No Western blot evidence was presented to show

that any of these inhibitors led to HIF-1α stabilization in cell culture. In findings from Koivunen et al. (2007), using recombinant HPH1-3 enzymes, oxaloacetate, fumarate and succinate were to found to inhibit enzyme activity, but isocitrate and pyruvate showed no enzyme activity inhibition. Interestingly, although succinate was shown to inhibit HPH activity, it was not found to stabilize HIF-1α in normoxia; a finding that supports our findings, but contradicts the findings of Selak et al. (2005). It's uncertain if the inconsistencies among these findings are attributable to methodological differences, but taken as a whole, they offer further support for the regulation of HIF-1 in normoxia by a mechanism whereby glucose metabolites inhibit HPH enzyme activity.

From the field of wound healing, elevated lactate has been linked to aerobic angiogenesis via normoxic HIF-1α accumulation (Hunt et al. 2007). It is noted that collagen deposition is important for angiogenesis and aerobic conditions are needed for collagen deposition. The enzymes responsible for collagen deposition, like the HPH enzymes, are oxygen-dependent dioxygenases that also require ascorbate for proper functioning. Ascorbate deficiency leads to the inactivation of these enzymes, resulting in scurvy. This example in wound healing of a physiological role for HIF-1 activation by aerobic glycolysis suggests that other physiological conditions where aerobic glycolysis is observed, such as prolonged muscle activity, may also result in HIF-1 activation. If there is a general role for HIF-1 activation by aerobic glycolysis in normal physiological conditions, further understanding of this benign normoxic regulation of HIF-1 may provide insight into why normoxic HIF-1 activation promotes malignancy in cancer.

It appears that glucose metabolites are not the only metabolites that affect HIF-1α regulation. Some of the 2-oxoacids observed to stabilize normoxic HIF-1α derive from branched-chain amino acid catabolism (Lu et al. 2005). This observation may have important consequences in the metabolic disease Maple Syrup Urine Disease (MSUD) where a build-up of branched-chain amino acids correlates with cerebral edema (Levin et al. 1993) that may be caused by HIF-1 induced vascular endothelial growth factor (VEGF). Our lab is currently exploring this and other pathological implications of deviant branched-chain amino acid metabolism. Whether the branched-chain amino acids cause normoxic HIF-1α accumulation via HPH inhibition has yet to been examined.

First discovered as the regulator of hypoxia inducible EPO, a hormone which promotes red blood cell production, and thus oxygen carrying capacity, HIF-1 is generally considered a regulator of the hypoxic survival response. However, the growing body of research showing that HIF-1 can be activated independently of hypoxia by various metabolites shows the importance of metabolism in HIF-1 regulation and marks HIF-1 as a regulator of gene expression broader than that only required for survival in hypoxia. Recent findings identifying PDK-1, a PDC inhibitory enzyme, as a HIF-1 regulated gene shows that HIF-1 activation can not only increase glycolysis, but also decrease oxidative phosphorylation demonstrating the importance HIF-1 regulation for metabolism.

Chapter 4 and 5 address the second question, “Could decreased PDC activity via phosphorylation of its regulatory PDHα subunit contribute to

malignancy in cancer?" To explore this question we developed a novel antibody specific for phosphorylated PDHα. Chapter 4, **"Development an Assay for Determining PDH E1α Phosphorylation Status"**, presents data demonstrating that our antibody is specific for phosphorylated PDHα. We determined this by Western blot analysis of recombinant phosphorylated and unphosphorylated PDHα, isolated mitochondria treated to increase or decrease phosphorylation and whole brain lysate separated by electrophoresis and isoelectric point.

For Chapter 5, **"Determination if Decreased PDC Activity via Phosphorylation of its Regulatory PDHα Subunit Contributes to Malignancy in Cancer"** we used the phospho-PDHα (pPDHα) antibody to determine phosphorylation in a variety of human cancer cell lines. We found that elevated pPDHα, as well as PDK-1, was associated with degree of malignancy based on our malignant phenotype characterizations and previous research characterizing the cell lines we tested (Elstrom et al. 2004, Sonoda et al. 2001). We also found that increased pPDHα and PDK-1 expression was comparable with increased HIF-1α levels in the head and neck (O22, 22A, 22B) and glial (LN229, LN18, E6/E7/hTERT, +RAS, +RAS/AKT) cancer lines we tested. It's predictable that HIF-1α expression would correspond with pPDHα and PDK-1 expression, considering PDK-1 is a HIF-1 regulated gene (Kim et al. 2006; Papandreou et al. 2006). The findings of this thesis research are the first to demonstrate this link in cancer.

Preliminary results in which we characterized a variety of cancer cell lines for their malignant phenotypes by measuring growth rate, lactate production,

invasiveness, VEGF production, colony formation and normoxic HIF-1α expression led us to choose the 22B cell line to inhibit PDK expression using shRNA. The 22B demonstrated the most robust normoxic HIF-1α expression of all the cell lines we tested. When compared to other head and neck squamous cell carcinoma (HNSCC) lines, except for growth rate, the 22B line was invariably the most malignant (Figs. 10 A-D). The 22B line when compared to other HNSCC lines also had the highest pPDHα and PDK-1 expression (Fig. 10 E). As expected, transfection with PDK-1 shRNA inhibited PDK-1 expression, decreased pPDHα levels, increased PDC activity and glucose oxidation (Figs. 11 A-C). Interestingly, this also resulted in a dramatic reduction in the *in vitro* and *in vivo* malignant phenotype as measured by lactate production, normoxic HIF-1α expression, colony formation, VEGF production, invasiveness and migration, and xenograft tumor growth rate (Figs. 11 D,E; Fig. 12).

HIF-1 regulates PDK-1 expression; our data demonstrates a novel finding that PDK expression can in turn regulate HIF-1. Based on our observation that the glucose metabolites lactate and pyruvate can stabilize HIF-1α (Lu et al. 2002), and inhibition of PDK reduces lactate, we reason that the reduction of HIF-1α expression is due to reduced lactate (Fig. 16). The PDK enzymes are thought to only be expressed in the mitochondrial matrix (Harris et al. 1995), and are not known to have any function outside of regulating PDC activity. Furthermore, evidence suggests that binding to the E2 subunit of the PDC is important for their activity (Tuganova et al. 2005). However, it is possible that PDK may have direct or indirect effects on the regulation of HIF-1 independent of

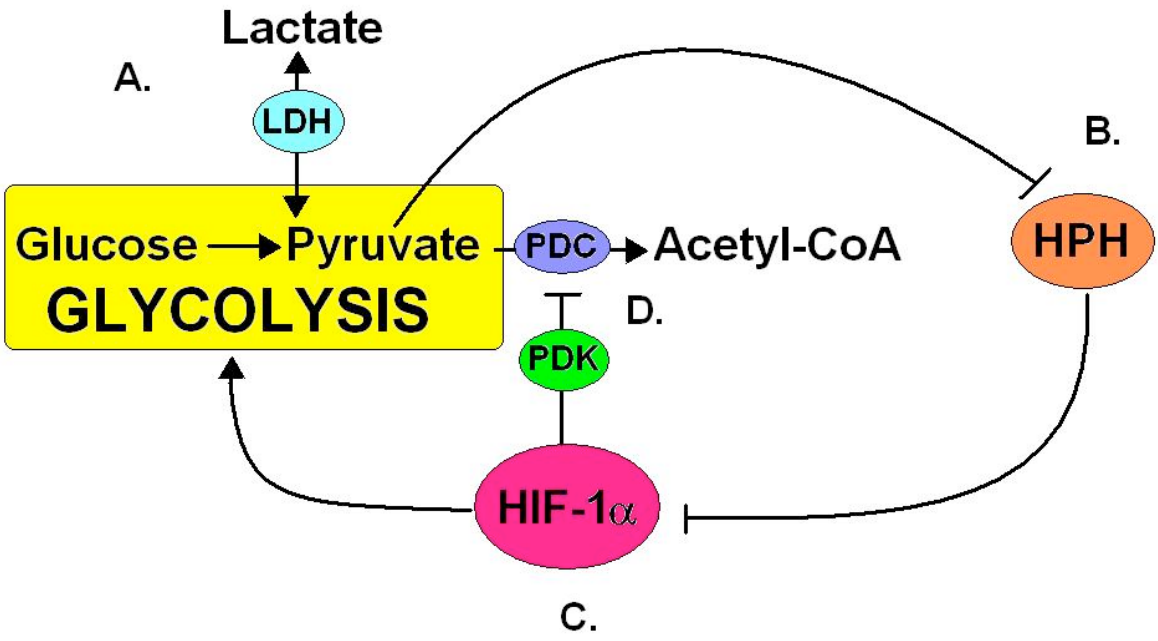

Figure 16. Hypothetical Mechanism of How Aerobic Glycolysis Promotes Malignancy. A. Aerobic glycolysis, i.e., lactate in aerobic conditions has long been associated with malignant progression, and is historically referred to as the Warburg effect. **B.** Evidence suggests that aerobic glycolysis promotes malignancy through inhibition of HIF-1 prolyl hydroxylase (HPH). Active HPH targets the alpha subunit of the Hypoxia Inducible Factor-1 (HIF-1α) transcription factor for degradation in aerobic conditions by hydroxylating proline residues. **C.** Aerobic inhibition of HPH results in HIF-1α accumulation and activation of genes that code for proteins that promote aerobic glycolysis, including Pyruvate Dehydrogenase Kinase-1 (PDK-1) and glycolytic enzymes, as well as genes that promote malignancy through angiogenesis, tumorigenesis, invasiveness and increased cell survival. **D.** The pyruvate dehydrogenase complex (PDC) catalyzes the irreversible conversion of pyruvate to acetyl-CoA. PDC phosphorylation by PDK inhibits PDC activity and results in increased lactate. Suppression of PDK by shRNA results in decreased lactate, HIF-1α accumulation and the malignant phenotype.

PDH phosphorylation. Increased HPH expression, which would result in the increased rate of HIF-1α degradation, is an alternative explanation for the observed decrease in HIF-1α. Our data which shows a differential HIF-1α induction between the shPDK and shCT cells treated with equal amounts of glucose or pyruvate in buffer (Figs. 11G,H), along with the observation from Figure 11F, showing equal HIF-1α protein expression when degradation is blocked suggests differential degradation. A more detailed measurement of intercellular pyruvate and lactate concentrations, LDH activity and metabolite transporters may provide further insight into the mechanism by which decreased PDK expression results in decreased HIF-1α levels.

Our findings of differential pPDHα and PDK1 expression in association with normoxic HIF-1α accumulation in the LN glioma and the immortalized E6/E7/hTERT human astrocytes cell lines supports our findings that pPDHα and PDK1 expression associates with malignancy and implicates a possible role for activation of the Ras and Akt signaling pathways in PDC inhibition. Comparing the two LN glioma cell lines, LN229 and LN18, we observed greater pPDHα and PDK1 expression in association with normoxic HIF-1α accumulation in the LN 18 cells (Figs. 13 B, D). LN 18 cells are known to have greater Akt activity and greater lactate production compared to the LN229 cells (Elstrom et al. 2004). Comparing the three immortalized human astrocytes cell lines, E6/E7/hTERT, E6/E7/hTERT/Ras and E6/E7/hTERT/Ras/Akt, we observed greater pPDHα and PDK1 expression in association with normoxic HIF-1α accumulation in the more transformed E6/E7/hTERT/Ras and E6/E7/hTERT/Ras/Akt cell lines (Figs. 13

C,E). The more transformed E6/E7/hTERT/Ras and E6/E7/hTERT/Ras/Akt cell lines have been shown to exhibit a greater capacity for colony formation when compared to the E6/E7/hTERT cell line, and to demonstrate characteristics consistent with higher tumor grade when grown *in vivo* (Sonoda et al. 2001). Activation of the Ras and Akt pathways are known to correlate with malignancy and have been implicated in the maintenance of aerobic glycolysis and HIF-1 activation in cancer (Shim et al. 1998). Our observation of the association of increased Ras and Akt pathway activity with increased pPDHα and PDK1 expression may be a direct effect or indirect effect via HIF-1 activation. Further studies examining the effect of manipulating the activity of these pathways on pPDHα, PDK1 and HIF-1α expression may provide valuable insight into how activation of the Ras and Akt signaling pathways promote malignancy in cancer.

The evidence showing that lactate and pyruvate can directly promote angiogenesis (Murray and Wilson 2001; Lee et al. 2001), activate gene transcription (Lu et al. 2002, 2005; Dalgard et al. 2004), stimulate hyaluronan synthesis (Stern et al. 2002) and suppress the immune system (Fischer et al. 2007) suggests that altering metabolic pathways may ameliorate these malignant symptoms. Indeed, our promising results show that inhibition of PDK, which results in decreased lactate, results in the reduction of several malignancy markers. The PDC is a major regulatory gateway between glycolysis and oxidative phosphorylation controlled by specific kinases and phosphatases, which makes it an attractive therapeutic target for altering glucose metabolism. Results using the PDK inhibitor DCA are encouraging in that malignant cell lines

were selectively killed by DCA treatment when compared to normal cell lines (Bonnet et al. 2007). Our results showing DCA to be selectively toxic towards the more malignant metastatic 22B cell line when compared to the 22A primary tumor cell line also support potential therapeutic benefits of pharmacologic inhibition of PDK. However, given the high doses required for DCA treatment, potential toxic side effects and evidence that DCA effects may be independent of PDHα phosphorylation (Fig. 14), there is a need for the development of more specific PDK inhibitors or PDP activators.

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