

Regulation of Metabolism by Hypoxia-Inducible Factor 1

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The maintenance of oxygen homeostasis is critical for survival, and the master regulator of this process in metazoan species is hypoxia-inducible factor 1 (HIF-1), which controls both O₂ delivery and utilization. Under conditions of reduced O₂ availability, HIF-1 activates the transcription of genes, whose protein products mediate a switch from oxidative to glycolytic metabolism. HIF-1 is activated in cancer cells as a result of intratumoral hypoxia and/or genetic alterations. In cancer cells, metabolism is reprogrammed to favor glycolysis even under aerobic conditions. Pyruvate kinase M2 (PKM2) has been implicated in cancer growth and metabolism, although the mechanism by which it exerts these effects is unclear. Recent studies indicate that PKM2 interacts with HIF-1 α physically and functionally to stimulate the binding of HIF-1 at target genes, the recruitment of coactivators, histone acetylation, and gene transcription. Interaction with HIF-1 α is facilitated by hydroxylation of PKM2 at proline-403 and -408 by PHD3. Knockdown of PHD3 decreases glucose transporter 1, lactate dehydrogenase A, and pyruvate dehydrogenase kinase 1 expression; decreases glucose uptake and lactate production; and increases O₂ consumption. The effect of PKM2/PHD3 is not limited to genes encoding metabolic enzymes because *VEGF* is similarly regulated. These results provide a mechanism by which PKM2 promotes metabolic reprogramming and suggest that it plays a broader role in cancer progression than has previously been appreciated.

Life on Earth is based on a biological cycle in which plants and blue-green algae capture solar energy and use it to drive the synthesis of glucose from carbon dioxide and water, thereby storing chemical energy in the form of carbon bonds. During this process of photosynthesis, O₂ is formed as a side product. Animals consume the plants and catabolize glucose via glycolysis and the mitochondrial tricarboxylic (TCA) cycle. This oxidative process generates reducing equivalents in the form of NADH and FADH₂, which are delivered to Complex I (NADH:ubiquinone oxidoreductase) and Complex II (succinate:ubiquinone oxidoreductase) of the electron transport chain, respectively, and then transferred to Complex III (ubiquinol:cytochrome *c* oxidoreductase) and, finally, to Complex IV (cytochrome *c* oxidase), where the electrons react with O₂ to form H₂O. The energy derived from electron transfer is used to generate a proton gradient, which in turn is used to drive the synthesis of ATP by Complex V (ATP synthase).

Electron transfer at each step of the respiratory chain is not completely efficient, resulting in the escape of electrons that reduce O₂ to superoxide anion, which is converted to hydrogen peroxide by the action of manganese superoxide dismutase. These reactive oxygen species (ROS) have the potential to damage lipids, proteins, and nucleic acids, leading to cellular dysfunction or death. Electron transport chain activity appears to be optimized for physiological PO₂ and deviations from normal PO₂ (in either direction) are associated with increased ROS production. The utilization of O₂ for energy metabolism is a two-edged sword: the

complete oxidation of glucose to CO₂ and H₂O greatly increases the yield of ATP as compared with the incomplete oxidation of glucose to lactate; however, the transfer of electrons poses an inherent risk of ROS production. As a result, the cellular O₂ concentration must be very tightly regulated through homeostatic mechanisms that control O₂ delivery and O₂ utilization.

HYPOXIA-INDUCIBLE FACTOR 1: MASTER REGULATOR OF O₂ HOMEOSTASIS

All metazoan species express hypoxia-inducible factor 1 (HIF-1), a transcription factor that mediates adaptive responses to reduced O₂ availability (Semenza and Wang 1992). HIF-1 is a heterodimeric protein that is composed of an O₂-regulated HIF-1 α subunit and a constitutively expressed HIF-1 β subunit (Wang and Semenza 1995). Isolation of cDNA sequences (Wang et al. 1995) revealed that both subunits are members of a subfamily of basic helix-loop-helix (HLH) factors containing a PAS domain (first identified in the Per, Arnt, and Sim proteins) that dimerize via HLH and PAS domains and bind to DNA via basic domains located in the amino terminal half of each subunit (Jiang et al. 1996a). The carboxyl terminal half of HIF-1 α contains a transactivation domain (Jiang et al. 1997b; Pugh et al. 1997) that interacts with the coactivator p300 (Arany et al. 1996; Kallio et al. 1998). To activate target gene transcription, HIF-1 binds to hypoxia response elements (HREs) that contain the core HIF-1 binding site sequence 5'-RCGTG-3' (Semenza et al. 1996).

The levels of HIF-1 α protein increase exponentially as O₂ concentration declines over the entire physiological range of PO₂ (Jiang et al. 1996b). Changes in HIF-1 α protein levels are the result of O₂-dependent ubiquitination and proteasomal degradation of HIF-1 α (Salceda and Caro 1997). In the presence of O₂, HIF-1 α is hydroxylated on two proline residues by a family of prolyl-4-hydroxylase domain (PHD) proteins (Bruick and McKnight 2001; Epstein et al. 2001). These dioxygenases utilize O₂ and the TCA cycle intermediate α -ketoglutarate as substrates, with one oxygen atom inserted into the prolyl residue and the other oxygen atom used to split α -ketoglutarate into succinate and CO₂. Prolyl hydroxylation increases the binding to HIF-1 α of the von Hippel–Lindau protein (VHL), which is the substrate recognition subunit of an E3 ubiquitin–protein ligase that targets HIF-1 α for degradation (Kaelin and Ratcliffe 2008).

HIF-1 α transactivation domain function is also negatively regulated by O₂ (Jiang et al. 1997b; Pugh et al. 1997), through the activity of factor inhibiting HIF-1 (FIH-1) (Mahon et al. 2001), which uses O₂ and α -ketoglutarate to hydroxylate an asparaginyl residue in the transactivation domain, thereby blocking the binding of p300 (Lando et al. 2002a,b). Thus, both the half-life and transcriptional activity of HIF-1 α are regulated by O₂-dependent hydroxylation reactions that provide a direct mechanism to transduce changes in O₂ availability to the nucleus as changes in transcription mediated by HIF-1, which is now known to regulate the expression of hundreds of genes (Manalo et al. 2005; Mole et al. 2009; Xia et al. 2009; Schödel et al. 2011).

HIF-1 was discovered based on its regulation of the human *EPO* gene, which encodes erythropoietin, the hormone that controls red cell production and, thus, blood O₂-carrying capacity (Semenza and Wang, 1992). HIF-1 was subsequently shown to regulate the expression of *VEGF*, which encodes vascular endothelial growth factor, the principal protein for stimulating the formation of new blood vessels (Forsythe et al. 1996). EPO and VEGF illustrate the role of HIF-1 in increasing O₂ delivery in response to systemic or local hypoxia, respectively. Mouse embryos lacking HIF-1 α die at midgestation with defects in the heart and vasculature (Iyer et al. 1998; Ryan et al. 1998; Compornolle et al. 2003) and reduced blood cell production (Yoon et al. 2006), indicating that all three components of the circulatory system are dependent upon HIF-1 for normal development.

HIF-1 also increases the ability to survive O₂ deprivation by switching cells from oxidative to glycolytic metabolism by coordinately regulating the genes encoding glycolytic enzymes (Semenza et al. 1996; Iyer et al. 1998; Seagroves et al. 2001) and pyruvate dehydrogenase (PDH) kinase 1 (PDK1) (Kim et al. 2006; Papandreou et al. 2006). The key decision point for glucose metabolism is the fate of pyruvate, which is a substrate for either PDH, which converts it to acetyl coenzyme A for entry into the mitochondrial TCA cycle, or lactate dehydrogenase A (LDHA), which converts it to lactate for export from the cell. Under hypoxic conditions, HIF-1 activates

expression of both LDHA and PDK1, thereby shunting pyruvate away from mitochondrial oxidation. HIF-1 also activates the expression of BNIP3 and BNIP3L, two proteins that trigger mitochondrial-selective autophagy, which is a strategy that eliminates oxidative metabolism of both glucose and fatty acids by eliminating the mitochondria in which these reactions occur (Zhang et al. 2008; Bellot et al. 2009). HIF-1 also activates transcription of a microRNA, miR-210, which inhibits the expression of a protein required for formation of iron-sulfur clusters that are components of the TCA cycle enzyme aconitase and electron transport chain Complex I (Chan et al. 2009; Chen et al. 2010; Favaro et al. 2010).

According to conventional wisdom, the driving force behind the switch from oxidative to glycolytic metabolism is the maintenance of ATP production. However, mouse embryo fibroblasts (MEFs) that are homozygous for a null (knockout) allele at the locus encoding HIF-1 α die when exposed to 0.5%–1% O₂ for >48 h, despite the fact that these cells have higher ATP levels than their wild-type counterparts (Zhang et al. 2008). In the absence of HIF-1 activity, the MEFs do not die from ATP depletion but instead are killed by toxic levels of ROS because of the failure to switch from oxidative to glycolytic metabolism (Kim et al. 2006; Zhang et al. 2008).

HIF-1 is expressed in *Caenorhabditis elegans* (Epstein et al. 2001), a nematode that consists of more than 1000 cells and has no specialized systems for O₂ delivery. However, these worms must adapt their metabolism when they burrow into the soil for food. Thus, a major (and probably primordial) function of HIF-1 is to balance the risks and benefits of O₂ utilization over the entire physiological range of PO₂. This point is underscored by the finding that HIF-1 also regulates respiration by directing a subunit switch in cytochrome *c* oxidase that appears to increase the efficiency of electron transfer at Complex IV under hypoxic conditions (Fukuda et al. 2007). Remarkably, a homologous subunit switch in Complex IV occurs when *Saccharomyces cerevisiae* is subjected to hypoxia (Burke and Poyton 1998), although by a different mechanism because HIF-1 is only present in the metazoan lineage, which suggests that metabolic adaptation to hypoxia is critical for survival of all eukaryotic cells.

ACTIVATION OF HIF-1 IN CANCER

HIF-1 α protein levels are increased in the majority of human cancers and their metastases (Zhong et al. 1999), and high levels of HIF-1 α in the primary tumor biopsy are associated with increased risk of mortality in many cancer types (Semenza 2010). There are three general mechanisms by which HIF-1 α levels are increased in human cancer. First, intratumoral hypoxia is common and often severe. In breast cancer, the mean PO₂ is 10 mmHg, compared with 65 mmHg in normal breast tissue, and patients with intratumoral PO₂ < 10 mmHg are at increased risk of mortality, independent of tumor size, stage, histology, grade, or lymph node status (Vaupel

et al. 2004). Second, other alterations of the tumor microenvironment also increase HIF-1 α levels, such as the presence of nitric oxide (Quintero et al. 2006) or ROS (Gao et al. 2007).

Third, cancer-specific genetic alterations lead to dysregulation of HIF-1 α levels in an O₂-independent manner. The most dramatic effect is observed with loss of function of the VHL tumor suppressor protein in the clear cell type of renal cell carcinoma and in cerebellar hemangioblastoma (Maxwell et al. 1999; Kaelin 2007). Increased HIF-1 α levels are also associated with loss of function of p53 (Ravi et al. 2000) and PTEN (Zhong et al. 2000; Zundel et al. 2000), which are the two most commonly mutated tumor suppressors. Other tumor suppressors whose inactivation is associated with increased HIF-1 activity include p14^{ARF} (Fatyol and Szalay 2001), TSC2 (Brugarolas et al. 2003), PML (Bernardi et al. 2006), and LKB1 (Shackelford et al. 2009). The *SDHB*, *SDHC*, *SDHD*, and *FH* genes encode the TCA cycle enzymes succinate dehydrogenase and fumarate hydratase, which also function as tumor suppressors that, when inactivated, result in increased HIF-1 α levels (Isaacs et al. 2005; Selak et al. 2005). Activated oncogenes such as HER2^{neu} in breast cancer (Laughner et al. 2001), EGFR in prostate (Zhong et al. 2000) and lung (Phillips et al. 2005) cancer, and BCR-ABL in chronic myelogenous leukemia (Mayerhofer et al. 2002) increase HIF-1 α levels through the phosphatidylinositol-3-kinase/AKT/mTOR signaling pathway. HIF-1 activity is also increased by transforming proteins of many oncogenic viruses, including Rous sarcoma virus (Jiang et al. 1997a), Kaposi sarcoma herpesvirus (Sodhi et al. 2000), hepatitis B virus (Yoo et al. 2004), Epstein–Barr virus (Wakisaka et al. 2004), and human papillomavirus (Nakamura et al. 2009).

Taken together, the data suggest that HIF-1 activation represents a final common pathway in most advanced human cancers. The selection for cells with HIF-1 activation reflects the many HIF-1 target genes that play important roles in critical aspects of cancer biology, including angiogenesis (Liao and Johnson 2007); epithelial–mesenchymal transition, invasion, and metastasis (Chan and Giaccia 2007; Sullivan and Graham 2007); genetic instability (Bindra et al. 2007); immortalization and stem cell maintenance (Barnhart and Simon 2007); immune evasion (Lukashev et al. 2007); metabolism (Kim et al. 2007a); pH regulation (Swietach et al. 2007); and resistance to radiation therapy (Moeller et al. 2007).

REPROGRAMMING OF METABOLISM IN CANCER CELLS BY HIF-1

Compared with normal cells, cancer cells have long been known to exhibit dramatically increased glucose uptake and lactate production, even under conditions in which O₂ is not limiting for respiration (Warburg et al. 1927). Infection of normal cells with Rous sarcoma virus was shown to increase aerobic glycolysis and this effect was temporally and genetically associated with

V-SRC-mediated transformation (Steck et al. 1968; Singh et al. 1974; Carroll et al. 1978), which provided the first link between an oncogene and metabolism. Oncogenic forms of SRC increase HIF-1 α protein levels and HIF-1-dependent activation of glycolytic gene expression, providing the first molecular mechanism by which an oncogene promotes metabolic reprogramming (Jiang et al. 1997a; Karni et al. 2002). Oncogenic forms of MYC cooperate with HIF-1 to activate glycolytic gene transcription (Kim et al. 2007b). In renal carcinoma cells, loss of VHL tumor suppressor function leads to a switch from oxidative to glycolytic metabolism that can be dramatically reversed by expression of wild-type VHL or inhibition of HIF-1 activity (Zhang et al. 2007).

The mechanisms and consequences of alterations of metabolism in cancer cells are the focus of considerable research effort (McKnight 2010; Dang et al. 2011; Frezza et al. 2011; Maddocks and Vousden 2011; Semenza, 2011; Shanware et al. 2011; Yecies and Manning 2011). One area of particular recent interest is the role of the pyruvate kinase M2 (PKM2) isoform in cancer metabolism. PKM2 is normally expressed in the embryo, whereas PKM1 is expressed in adult cells. These two isoforms arise from alternative splicing of the primary RNA transcript: PKM1 mRNA contains sequences encoded by exon 9, whereas the PKM2 mRNA contains sequences encoded by exon 10 (Noguchi et al. 1986; Mazurek et al. 2005). Many cancer cells re-express PKM2 (Mazurek et al. 2005) as a result of MYC-dependent expression of heterogeneous nuclear ribonucleoprotein 1, A1, and A2, which bind to RNA sequences encoded by exon 9 and promote the splicing out of the PKM1-specific sequences (Clower et al. 2010; David et al. 2010). PKM2, but not PKM1, is subject to allosteric regulation that is modulated by tyrosine kinase signaling (Christofk et al. 2008b; Hitosugi et al. 2009).

Knockdown of PKM2 in cancer cells and replacement with PKM1 expression resulted in decreased lactate production and increased O₂ consumption as well as decreased tumor xenograft growth, indicating that PKM2 plays important roles in tumor growth and metabolic reprogramming (Christofk et al. 2008a). However, the mechanism by which PKM2 altered the balance between oxidative and glycolytic metabolism was unclear because PKM2 catalyzes the conversion of phosphoenolpyruvate to pyruvate—upstream of the decision point, which is the conversion of pyruvate to either acetyl CoA or lactate that is regulated by HIF-1 as described above.

We performed a proteomics-based screen to identify proteins that interact with the carboxyl terminus (amino acid residues 531–826) of HIF-1 α and identified PKM2, which was subsequently shown to co-immunoprecipitate with HIF-1 α in nuclear extracts of hypoxic HeLa cells (Luo et al. 2011). HIF-1 α bound to the PKM2-specific domain encoded by exon 10 but not to the PKM1-specific domain encoded by exon 9. We hypothesized that PKM2 contributes to metabolic reprogramming of cancer cells by stimulating HIF-1 activity. To test this hypothesis, we co-transfected HeLa

cells with an HIF-1-dependent luciferase reporter plasmid and an expression vector encoding PKM1 or PKM2. PKM2 vector increased hypoxia-induced luciferase activity, whereas PKM1 did not. PKM2 did not affect the levels of HIF-1 α or HIF-1 β protein. However, PKM2 stimulated luciferase activity in cells co-transfected with an expression vector encoding the DNA binding domain from GAL4 fused to the HIF-1 α transactivation domain and a luciferase reporter plasmid containing five GAL4 binding sites (Luo et al. 2011).

The amino acid sequence encoded by exon 10 was remarkable for the presence of the hexapeptide ³⁹⁸LRRLAP⁴⁰³, which fits the consensus for HIF-1 α prolyl hydroxylation sites in all vertebrate species (LXXLAP). Mass spectrometry revealed that both P⁴⁰³ and P⁴⁰⁸ were hydroxylated. Mutation of both prolines (P403A/P408A) resulted in decreased physical and functional interaction of PKM2 with HIF-1 α (Luo et al. 2011). Similar effects were observed when cells were treated with the α -ketoglutarate analog dimethylxalylglycine (DMOG), which inhibits hydroxylase activity. The prolyl hydroxylase PHD3 was found to bind to PKM2 and stimulate its hydroxylation and interaction with HIF-1 α . Surprisingly, PKM2 was hydroxylated at similar levels in HeLa cells incubated at 20% or 1% O₂, despite the known loss of hydroxylase activity at lower O₂ concentrations (Epstein et al. 2001; Kaelin and Ratcliffe 2008). Analysis of PHD3 protein levels revealed a marked increase under hypoxic conditions, consistent with previous studies, which reported that PHD3 is encoded by an HIF-1 target gene (Aprelikova et al. 2004; Marxsen et al. 2004; Pescador et al. 2005). Thus, the increased expression of PHD3 compensates for the loss of enzymatic activity under conditions of chronic hypoxia to maintain PKM2 hydroxylation, as has previously been reported for HIF-1 α hydroxylation (Stiehl et al. 2006).

Chromatin immunoprecipitation (ChIP) assays revealed that knockdown of PKM2 reduced the hypoxia-induced occupancy by HIF-1 α , HIF-1 β , and p300 of HREs in the *LDHA* and *PDK1* genes in HeLa cells (Luo et al. 2011). Recruitment of both PKM2 and PHD3 to these HREs in hypoxic cells was also demonstrated. Knockdown of PKM2 reduced the levels of histone H3 acetylated at lysine 9 (H3K9ac) at HREs as determined by ChIP, whereas there was no effect on total H3 binding to the HRE or on total cellular levels of H3 or H3K9ac. These data indicate that PKM2 specifically enhances the formation of an active HIF-1 complex at HREs and promotes H3K9 acetylation, which is an epigenetic modification that is associated with transcriptional activation. PKM2 binding to HIF-1 α may provide a mechanism to overcome the repressive effect of asparaginyl hydroxylation by FIH-1, thereby increasing transactivation function under aerobic conditions in cancer cells in which HIF-1 α protein expression has been dysregulated by genetic alterations as described above.

Knockdown of PKM2 in HeLa cells significantly reduced the levels of GLUT1, LDHA, and PDK1 mRNA and protein under hypoxic conditions (Luo

et al. 2011). Knockdown of PHD3 in RCC4 renal carcinoma cells, in which HIF-1 α is constitutively expressed because of VHL loss of function, significantly reduced GLUT1, LDHA, and PDK1 mRNA and protein levels, leading to significantly decreased glucose uptake and lactate production and increased O₂ consumption under non-hypoxic conditions.

Analysis of PKM1 and PKM2 expression in immortalized mouse embryo fibroblasts revealed that both mRNAs were induced by hypoxia in an HIF-1-dependent manner (Luo et al. 2011). ChIP assays identified HIF-1 binding sites within intron 1 of the human *PKM2* gene and a 55-bp sequence encompassing these sites functioned as an HRE in reporter gene assays. Thus, the genes encoding PKM2 and PHD3 are both direct HIF-1 targets, thereby establishing a feed-forward mechanism for amplifying HIF-1-dependent transcriptional activation in cancer cells. PKM2 coactivator function, by increasing the expression of *LDHA*, *PDK1*, and other HIF-1 target genes that mediate the metabolic switch from oxidative to glycolytic metabolism, provides a mechanism underlying the contribution of PKM2 to metabolic reprogramming of cancer cells. However, the effect of PKM2 coactivator function was not restricted to genes encoding metabolic enzymes, as recruitment of HIF-1 α and HIF-1 β to the *VEGF* gene was also impaired by PKM2 knockdown. These data imply that PKM2 may play a far broader role in cancer progression than has been appreciated heretofore. Finally, these data suggest that therapeutic strategies based on the inhibition of PKM2 catalytic activity in cancer cells may have limited efficacy, because mutation of a key catalytic residue in PKM2 that eliminated its enzymatic activity as a glycolytic enzyme had no effect on its HIF-1 coactivator function (Luo et al. 2011).

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