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Perspective

Hypoxic regulation of mRNA expression

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Many tumors are hypoxic, and cells that are experimentally rendered hypoxic display a variety of phenotypes which allow them to adapt to the micro-environment. These phenotypes include a shift from aerobic to anaerobic metabolism, a diminution of reactive oxygen species, an arrest of proliferation, apoptosis, and a secretion of pro-angiogenic growth factors. Some of these hypoxic phenotypes are re-capitulated in normoxic tumor cells (e.g., an increase in anaerobic metabolism), and some tumors have undergone mutations that allow them to bypass the cell cycle arrest and apoptosis typically seen in hypoxic cells. Hypoxic regulation of gene expression is responsible for many hypoxia-induced phenotypes, and here we review a variety of mechanisms by which gene expression is altered in hypoxic cells. These include transcription by HIF-1, the hypoxia inducible transcription factor, and other hypoxia-inducible transcription factors, including ones generated by hypoxic activation of the integrated stress response. Recent data from our laboratory demonstrate that nonsense mediated RNA decay is also regulated in hypoxic cells and thus may play an important role in hypoxic gene regulation and hypoxic phenotypes.

The Importance of Cellular Hypoxia in Tumorigenesis

Despite the fact that atmospheric oxygen is approximately 20%, most tissues are physiologically exposed to 4–8% oxygen. Cellular hypoxia (<1–2% oxygen) is a common stress in normal development and numerous pathological conditions, including cancer (reviewed in refs. 1 and 2). By the time a tumor has grown to a detectable size, disordered angiogenesis, leaky vessels and high interstitial tumor pressure all result in significant tumor hypoxia.[3-5] Studies in human tumor xenografts reveal a mean pO2 of <5 mm Hg at a distance of 70–80 μm from a vessel wall.[6] Both non-invasive measurements and direct assessment of tumor oxygenation in patients have demonstrated the presence of profound hypoxia in a diverse group of cancers.[7-10] In one study using oxygen electrodes to assess breast cancer oxygenation in 36 patients and controls, the average pO2 was 30 mm Hg in tumors as compared to 65 mm Hg in normal breast tissue and 67 mm Hg in benign fibrocystic breast disease [11]. Areas of severe hypoxia or anoxia (<5 mm Hg) were noted in >30% of the measurements.

For over 30 years hypoxic tumors have been known to be relatively chemo-resistant and radio-resistant although there is no correlation of tumor hypoxia with tumor size or stage.[12,13] More recently, tumor hypoxia has been correlated with poor clinical response to radiation and/or chemotherapy.[10,14,15] Intriguingly, prospective studies of patients with head and neck or cervical cancer show that tumor hypoxia correlates with local/regional progression, irrespective of whether surgery or radiation is applied as primary treatment.[16] This suggests that while hypoxic tumors are less responsive to cytotoxic therapy, they are also inherently more aggressive than non-hypoxic tumors.

Expression of HIF-1α, a subunit of the hypoxia-inducible transcription transcription factor HIF-1, has also been shown to be an independent indicator of poor prognosis in many cancers, even when HIF-1α is expressed in what appears to be normoxic tumors.[17-19] This observation suggests that many of the aggressive phenotypes observed in hypoxic tumors may be mediated by HIF-1 and/or other hypoxia-induced transcription factors, and serves as a rationale to better understand the regulation of gene expression in hypoxic cells.

Phenotypes of Hypoxic Cells

A brief survey of the variety of phenotypes observed in hypoxic cells provides insight into the aggressiveness of hypoxic tumors, the complexity of the cellular response to hypoxia, and the importance of hypoxic gene regulation in the phenotypic responses to the micro-environment (Fig. 1). As emphasized later, many aspects of gene regulation in hypoxic cells are cell-type specific. Similarly many of the phenotypes of hypoxic cells are also cell-type specific.

Hypoxic metabolism. One of the earliest phenotypes noted in hypoxic cells, as well as one that is relatively consistent amongst disparate cell types, is the shift from aerobic metabolism to less efficient forms of energy production which do not require molecular oxygen. This shift not only provides energy to the hypoxic cell but may also decrease reactive oxygen species (ROS) generation by diminishing flux through mitochondrial electron transport, a vital step in aerobic metabolism. The hypoxic shift from aerobic to anaerobic metabolism is of significant interest to tumor biologists, since many tumors rely on anaerobic metabolism even when normoxic. In fact, the high utilization of glucose for anaerobic energy production in tumors serves as a basis for the clinical use of positron electron emission (PET) imaging with fluorodeoxy glucose.

The shift from aerobic to anaerobic metabolism is mediated by the upregulation of several glycolytic enzymes including hexokinase,

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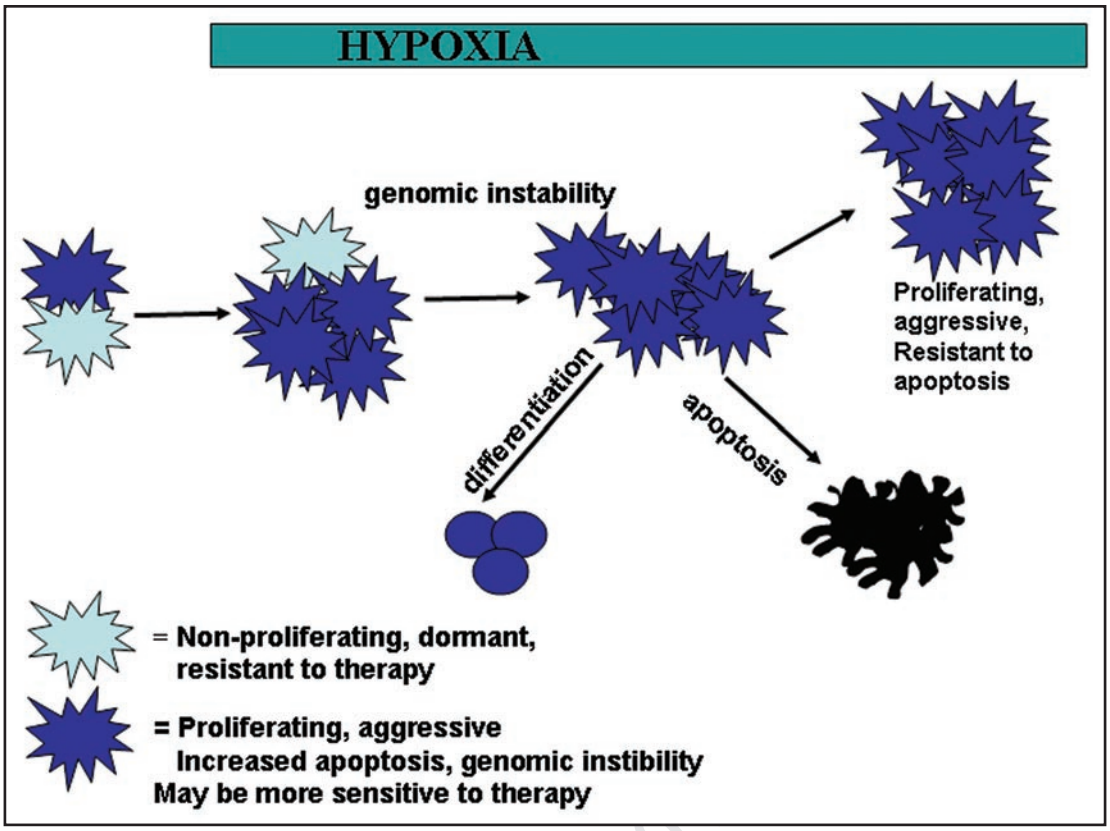

Figure 1. Model of hypoxic tumor growth. After initiation a tumor is heterogeneous, with some cell able to proliferate despite hypoxic conditions (due to mutations in the Rb axis or other mutations). These cells proliferate but then may undergo differentiation and are susceptible to both genomic instability and apoptosis. Those cells which proliferate are able to block apoptosis form an aggressive hypoxic tumor. Cells that do not proliferate when hypoxic may form a dormant tumor which is difficult to eradicate.

enolase and LDH, and over the past decade it has been determined that many of these enzymes are the targets of HIF-1 (reviewed in ref. 20). Normoxic induction of these enzymes in some tumors may be due to normoxic stabilization of HIF-1α, as discussed below. Additionally many of these enzymes are transcriptional targets of the c-myc oncogene which is commonly overexpressed in many malignancies.[21] Recently it has been appreciated that not only is glycolysis increased in hypoxic cells, but its end-product pyruvate is shunted from entering the tricarboxylic acid (TCA) cycle. This shunting is carried out by the enzyme pyruvate dehydrogenase kinase (PDK1) that is also a HIF-1 target.[22,23] By suppressing the TCA cycle, PDK1 not only diminishes the production of intermediate products that require oxygen for further metabolism, but also decrease ROS generation in hypoxic cells. It has also recently been appreciated that ROS generation in hypoxic cells is diminished through HIF-1 regulation of cytochrome c subunits in mitochondria.[24]

Hypoxic cell cycle effects. When mammalian cells are rendered hypoxic in vitro, they undergo a cell cycle arrest (reviewed in ref. 25). This also occurs in vivo, as evidenced in animal models where proliferation ceases in tissue regions that are hypoxic.[5] Under moderate hypoxic conditions (~1% oxygen) a G_1 arrest associated with a decrease in CDK2 activity and a hypo-phosphorylation of the retinoblastoma (Rb) protein has been commonly observed. This arrest is p53 independent, but does appear dependent on Rb. Conflicting studies, potentially complicated by differences in cell lines, culture conditions and degree of hypoxia, have alternatively implicated p27 and p21 in this cell cycle arrest.[26-28] HIF-1 independent upregulation of p27,[26,27,29] and HIF-1 dependent mechanisms for this arrest have been implicated[28] including, for example, direct interactions between HIF subunits (HIF-1α and HIF-2α) and other transcription factors involved in regulating the cell cycle, such as Myc. In the case of Myc, for example, HIF-1α interacts with Myc, leading to a de-repression of p21 transactivation and cell cycle arrest.[28] In contrast, HIF-2α also interacts with Myc and has been reported to *increase* proliferation in some hypoxic cells.[30]

Under more severe hypoxic conditions (~0.1–0.5% oxygen) a proliferation arrest occurs which has been characterized as an inhibition of the initiation of DNA replication. This S phase arrest is p53, p27 and p21 independent, but can be bypassed by overexpression of the E1a oncoprotein.[31,32] Thus it is readily apparent how some tumor cells with either defects in the Rb axis or other oncogenic alterations may proliferate despite being hypoxic. The proliferation of some hypoxic cells may render them more sensitive to chemotherapy and radiation, but may also make them more susceptible to apoptosis and genomic instability and thus select for cells that have additional mutations which may render them inherently more aggressive than other tumors (Fig. 1).

Hypoxic apoptosis and geonomic instability. Studies have noted that apoptosis of hypoxic cells is increased in the presence of wild-type p53,[33] in acidic conditions[34] and in more severely hypoxic cells.[35] Several pro-apoptotic proteins, including the HIF-1 target BNIP3, have been implicated in hypoxia-induced apoptosis.[36] On

the other hand, several transcription factors generated via activation of the integrated stress response as detailed below, including XBP-1 and ATF-4, protect cells from death associated with hypoxic stress.[37,38] Hypoxia-induced apoptosis may also be blocked by BCl-2 family members[31] and in vivo studies have demonstrated that p53$^{-/-}$ cells are selected for in growing hypoxic tumors.[33] These studies suggest that hypoxia may select for cells with increased resistance to apoptosis. Since proliferating cells may be more susceptible to apoptosis than dormant cells,[39] the selection of cells resistant for apoptosis may be even more rigorous in hypoxic proliferating cells.

In addition to the hypoxic selection for of apoptotic-defective clones, several studies have indicated that hypoxic cells undergo mutations at a higher rate than non-hypoxic cells. The basis for this is not entirely clear, but several mismatch repair enzymes are downregulated in hypoxic cells, perhaps in a HIF-1α dependent mechanism.[40-42] Collectively, the genetic changes associated with hypoxia may promote the development of tumors that can proliferate in hypoxic conditions, withstand hypoxia-induced apoptosis, and thus form clinically aggressive tumors.

Hypoxic metastases. Hypoxia promotes metastases, in part due to the upregulation of genes involved in invasion and angiogenesis, such as matrix metalloproteinase II, VEGF and other pro-angiogenic growth factors.[43] The best known regulator of VEGF is HIF-1, but additional mechanisms contribute to the higher expression of VEGF mRNA and other pro-angiogenic growth factors in hypoxic cells, including activation of the integrated stress response and hypoxic stabilization of mRNAs (see below). An additional mechanism theorized to be important in the spread of tumors is epithelial to mesenchymal transformation (EMT). EMT, first noted in embryogenesis, describes the ability of epithelial cells to transform to a mesenchymal phenotype as demonstrated by the upregulation of markers such as smooth muscle actin and vimentin, the downregulation of e-Cadherin and consequent dissolution of tight junctions, and an increase in migrational capacity. Many of these features, including e-Cadherin downregulation and an increase in cell migration, have also been noted in hypoxic cells and may, in part, be due to the hypoxic upregulation of Twist by HIF-1.[44,45]

Transcriptional Regulation of Hypoxic Genes by HIF-1 and Others

While there are several potential mechanisms for the biological behavior of hypoxic cells described above, it is likely that these and other phenotypes are in large part determined by the hypoxic induction and repression of specific genes. It is estimated that ~1 to 1.5% of the human genome is transcriptionally responsive to hypoxia.[46] Over the past decades our understanding of the mechanisms by which genes are regulated in hypoxic cells has greatly increased, and as a result the complexity of hypoxic gene regulation is beginning to be appreciated. The best explored transcription factor induced in hypoxic cells is HIF-1, which controls a transcriptional network of more than 100 downstream genes involved in diverse cellular processes including angiogenesis, cell cycle control, proliferation and energy metabolism[2,47] (Fig. 2). However, other transcription factors have been increasingly thought to play important roles in the hypoxic response, as will be discussed below.

HIF-1 regulation in normoxic and hypoxic cells. HIF-1 and HIF-2 are heterodimeric transcription factors composed of one of two HIFα proteins (HIF-1α, or HIF-2α respectively) and HIF-1β (also called the aryl hydrocarbon receptor, ARNT). HIFα proteins and HIF-1β belong to the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors and possess conserved domains including a bHLH region for DNA binding and two PAS domains for dimerization and target gene specificity.[48] HIF-1α and HIF-2α share 48% overall amino acid identity, with significant homology in their bHLH (83%) and PAS (70%) domains.[49,50] Although evidence suggests that HIF-2α may not be regulated or active in some hypoxic cell types,[51] most studies indicate that HIF-2α may play an important role in hypoxic gene regulation; in fact some mouse models of cancer suggest HIF-2α, and not HIF-1α, can promote tumorigenesis.[52-55] These observations argue that despite their sequence homology HIF-1α and HIF-2α are not functionally redundant. In addition, HIF-1α$^{-/-}$ as well as HIF-2α$^{-/-}$ mice both exhibit embryonic lethality with blood vessel defects, demonstrating that one isoform does not "rescue" the other during development.[49,56-59] Furthermore, while HIF-1α is ubiquitously expressed, HIF-2α expression is restricted to specific cell types, including vascular endothelium, kidney and heart.[49,50,60] Finally, gene expression analyses in mammalian cell lines have shown that HIF-1α and HIF-2α regulate both shared and unique target genes. For example, HIF-1α uniquely activates glycolytic gene expression, while HIF-2α preferentially activates at least 15 others, including VEGF, cyclin D1, Oct4 and Gadd45B.[55,61,62]

In normal tissues, while HIF-1β is constitutively expressed, HIFα levels are tightly regulated in response to changes in oxygen tension. In normoxic cells, prolyl residues located within oxygen-dependent degradation domains (ODDs) of HIF-α are hydroxylated by a family of prolyl-4-hydroxylases.[63-65] Hydroxylation of HIF-α subunits generates a binding site for the von Hippel Lindau protein (pVHL).[66,67] pVHL exists within a multi-subunit E3 ligase that contains elongin B, elongin C, Cul2 and Rbx1 (also called ROC1 or Hrt1)[68-70] and functions as the substrate recognition motif that targets HIF-α subunits for degradation by the ubiquitin-proteasome system.[71-73] In hypoxic cells prolyl hydroxylation of HIF-α is suppressed and pVHL no longer binds to HIF-α subunits, which leads to their accumulation. Accumulation of HIFα subunits then complex with HIF-1β, translocate to the nucleus, and bind to consensus hypoxia-response element (HRE) in HIF responsive genes to activate transcription. In addition to oxygen, prolyl hydroxylase activity requires the cofactors ascorbate and iron and the co-substrates 2-oxoglutarate. Iron chelation and cobalt have also been noted to increase HIF-α and may explain the clinical observation that cobalt ingestion increases erythropoiesis[74] (presumably via increased expression of the HIF target erythropoietin).

In cancer tissues HIFα is frequently overexpressed not only because hypoxia is a characteristic feature of tumor microenvironments[75] but because alterations in key tumor suppressor and/or oncogenic proteins lead to constitutive activation of HIF-1 and/or HIF-2 in an oxygen-*independent* manner in a variety of different tumor types. In the case of kidney cancer, for example, mutations in the pVHL tumor suppressor lead to constitutive upregulation of HIF-1 activity in normoxia.[76] In other cancer types, HIF-1α is also stabilized by ras, AKT, Her-2 overexpression and other oncogenic alterations.[77-80] Mutations in succinate dehyrogenase and fumarate hyroxylase, which lead to increases in intermediaries that act as

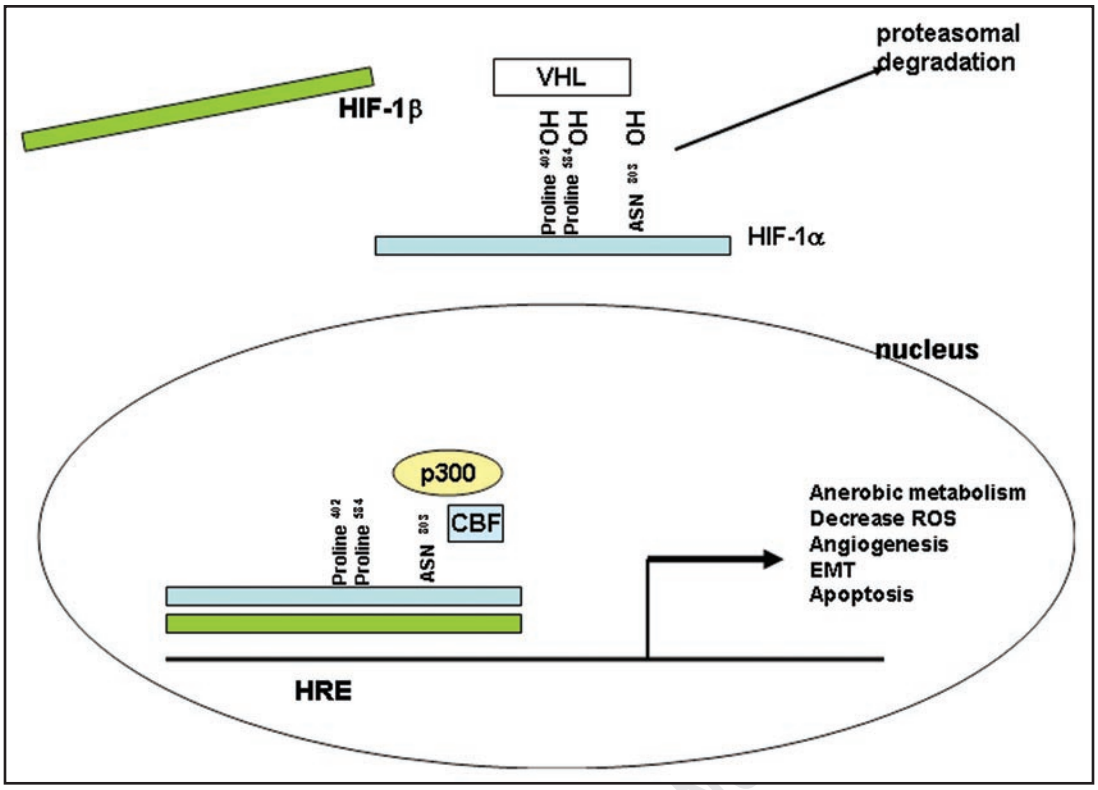

Figure 2. Model of transcription by HIF-1. Under hypoxic conditions HIF-1α is not prolyl hydroxylated, and does not serve as a target for pVHL and degradation. HIF-1α may also be stabilized through a variety of oncogenic alterations, and thus may be expressed in normoxic cells. HIF-1α dimerizes with HIF-1b, translocates to the nucleus, and forms the active transcription factor HIF-1. HIF-1 is able to transactivate genes important for metabolism, angiogenesis and other features which may promote hypoxic survival and tumor growth.

competitive inhibitors of prolyl hydroxylase, lead to increase HIF-1 activity and hereditary cancer syndromes[81]

The fact that erythropoietin (a HIF target) and erythropoiesis are increased in patients with stabilizing mutations in HIF-2α[82] and pVHL[83] and that other HIF targets are also upregulated in pVHL null renal cancers suggest that HIF-1 expression is sufficient to transactivate target genes even in normoxic cells. An unresolved question is whether genes regulated by HIF aberrantly activated in normoxic cells are the same as those regulated by HIF induced in hypoxic cells. Evidence to address this (albeit indirectly) comes from gene expression analyses comparing normoxic pVHL mutant kidney cancer cell lines to wild-type pVHL revertant cell lines rendered hypoxic.[84,85] These studies have generally showed concordance between "pVHL-responsive" genes (i.e., those detected in pVHL mutant cell lines grown in normoxia) and hypoxia-responsive genes in pVHL wild-type revertants. Despite this fact there are exceptions, with some genes regulated by VHL but not hypoxia, suggesting additional transcription pathways in kidney cancer cells that are pVHL-dependent, HIF-independent.

Functional role of HIF-1. The functional role of HIF-1 activity in both normal and cancer cells is complex. A dominant hypothesis is that HIF-1 activation results in expression of downstream target genes that promote cellular survival. In support of this, HIF-1 is necessary for growth in a hypoxic environment in a mouse model of astrocytomas, and HIF-1 activity in endothelial cells also seems necessary for solid tumor growth.[86,87] In contrast, however, expression of HIF-1 is correlated with slower growth of embryonic stem cells and transformed astrocytes in vitro, and increased HIF-1 leads to death in thymocytes.[86,88,89] HIF-1 activity is necessary for the growth of some human colon cancer cells as explants in nude mice, but not for others, and is also required for the normoxic growth of some human colon cancer cell lines.[90] Thus, the net effect of HIF-1 on cell growth (growth promoting versus growth inhibiting) depends on both the cell-type and physiologic context.

Illustrating these concepts, HIF-1 target genes differ dramatically in various cell types despite similar expression of HIF-1α.[46,61,77,91] These observations suggest that the HRE sequence is necessary but not sufficient for HIF-1 transactivation, and that there may be cell specific factors that modulate HIF-1 transcriptional activity. It is known that the co-activators p300 and CBP are required for HIF-1 transactivation of target genes. Oxygen dependent hydroxylation of HIF-1α by factor inhibiting HIF-1 (FIH-1) blocks the interaction of HIF-1α with the co-activators p300 and CBP.[92] However, to date cell or tissue specific alterations in co-activator levels or FIH activity have not been described.

An additional potential mechanism to modulate HIF-1 activity is through the differential expression of other transcription factors. These transcription factors may be able to suppress, or synergistically activate HIF-1 targets. In addition, the fact that HIF-1α and HIF-2α interact with other transcription factors (such as p53 and Myc) raises the possibility that the activity of HIF may be modulated by these factors. Conversely, HIF can alter the activity of other transcription factors. For example, it has been suggested that a direct HIF1α-Myc interaction de-represses Myc inhibition of the

p21 promoter and stimulate p21 activity and cell cycle arrest,[28] and contrastingly HIF-2α may collaborate with Myc by binding and stabilizing Myc-Max heterodimers.[30] Finally, an additional Max partner that can inhibit the formation of Myc-Max heterodimers, Mxi-1, is a direct target of HIF-1.[93] These few examples demonstrate the complexity of HIF transactivation and the many mechanisms by which the activity of HIF-1, as well as other transcription factors including Myc, may be modulated n different types of hypoxic cells.

An additional factor complicating the delineation of HIF-1 target genes is that most studies have not differentiated between direct and indirect HIF-1 targets. Indirect HIF-1 targets are genes that are upregulated by a transcription factor that is upregulated by HIF-1 or by conditions that occur in the hypoxic cell in the setting of HIF-1 transcription. For example, the enzyme GAPDH is a typical HIF-1 target in many cells but also serves as a co-activator to upregulate genes that promote S phase.[94] As another example, HIF-1 has been shown to diminish ROS, which in turn may regulate transcription factors and other cellular processes. In addition to HIF-1 and HIF-1 target genes, other transcription factors appear to be directly upregulated in hypoxic cells, including AP-1, NFκB and Egr1.[95,96] The mechanisms for the upregulation of these factors are not clear, although reactive oxygen species and activation of p38 may play a role. The induction of these "stress-responsive" factors, however, does not correlate with oxygen tension as sensitively or specifically as it does for HIF-1. Thus, their contribution to the hypoxia "transcriptome" appears to be relatively small when compared to HIF-1.[46] Finally, the hypoxic upregulation of a transcription factor does not necessarily mean that it is active in hypoxic cells. For example, while p53 is both upregulated in hypoxic cells (due to an inhibition of MDM2) and hypoxia-induced p53 promotes apoptosis and activates reporter constructs with p53 responsive elements in their promoters,[97] many endogenous targets of p53 are *not* induced in hypoxic cells and thus p53 is not thought to be particularly transcriptionally active in this setting.[98]

While the induction of genes in hypoxia has received much attention, the repression of genes in hypoxia also occurs. Although, as discussed below, many genes are repressed at the translational level in hypoxic cells, expression arrays demonstrate that many genes are repressed at the transcriptional level {Denko, 2003 #41; Denko, 2003 #34;Manalo, 2005 #332}. Genes repressed in hypoxic cells include those important in DNA replication, RNA metabolism and transcriptional regulation.[47] The possible mechanisms by which genes may be repressed in hypoxic cells are not clear, but may include hypoxia-induced expression of transcriptional repressors including the HIF-1 target Dec1, and global histone modifications.[99,100] In addition, HIF-1 has recently been shown to regulate microRNAs, which silence gene expression of target genes by either mRNA degradation and/or translational repression.[101,102]

ATF-4 (and Others): Hypoxia-Induced Transcription Factors Generated from Activation of the Integrated Stress Response

As detailed above, many of the genes activated in hypoxic cells promote energy conservation as part of the cellular adaptation to a hostile environment. An additional approach by which cells limit energy expenditure and maintain homeostasis under hypoxic conditions is through the suppression of mRNA translation. It has long been appreciated that protein synthesis is repressed in hypoxic cells, but only recently have the mechanisms and consequences of the hypoxic inhibition of protein translation started to be delineated.

The inhibition of protein translation in hypoxic cells. Protein translation is suppressed in hypoxic cells through several mechanisms. Cap-dependent initiation of protein translation is inhibited in hypoxic cells when eIF4E, a vital component of the cap-dependent initiation complex, is sequestered by the 4E binding protein 4E-BP1 when 4E-BP1 is hypo-phosphorylated. 4E-BP1 is normally phosphorylated by the mTOR kinase, and mTOR activity is suppressed in hypoxic cells via several mechanisms including the activation of the AMPK/TSC2 pathway by low adenosine triphosphate (ATP) stores and by REDD1, a HIF target[103,104]. Although cap dependent translation is limited in hypoxic cells through mTOR inhibition, cap *independent* translation, through internal ribosomal entry sites (IRESs) appears to occur robustly in hypoxic cells, thus promoting the translation of the transcription factors HIF-1α and Myc, as well as BCl-2 and a variety of other mRNAs.[103,105,106]

Global protein translation may also be inhibited by the phosphorylation of the initiation factor eIF2α, which then binds to eIF2B preventing it from exchanging GDP for GTP which is required for translation. eIF2α phosphorylation has been noted to rapidly occur in severely hypoxic cells (environmental oxygen concentrations <0.5%) and with slower kinetics in more moderately hypoxic conditions (oxygen concentration 1–1.5%).[38] Although eIF2α can be phosphorylated by a variety of stress activated kinases, the eIF2α kinase most active in hypoxic cells is the endoplasmic reticulum (ER) kinase PERK. PERK is activated when unfolded proteins in the ER sequester chaperones that normally inactivate PERK. An additional marker of ER stress, the splicing of XBP-1 mRNA also occurs in hypoxic cells supporting the conclusion that ER folding is disturbed;[107] however the formal demonstration of the accumulation of unfolded proteins in the ER and/or the mechanism for improper protein folding in hypoxic cells have not yet been demonstrated. During prolonged hypoxia eIF2α phosphorylation appears to play a diminished role in regulating protein translation and hypo-phosphorylation of 4E-BP1 may play the predominant role.[108] Although at least one study suggests that regulation of the cap binding complex is predominant in regulating protein translation in hypoxic cells,[109] the majority of studies indicate that phosphorylation of eIF2α is an important component. In particular it has been found that protein translation occurs robustly in hypoxic cells carrying genetic manipulations that reduce or eliminate phosphorylation eIF2α cells.[110,111]

Activation of the integrated stress response as a mechanism for upregulating transcription factors. Not only does the cessation of global protein synthesis serve as a protective mechanism for the cell, but activation of the integrated stress response leads to an *upregulation* of multiple mRNAs which also serve adaptive roles in the hostile hypoxic environment. The mechanisms by which activation of the integrated stress response upregulates mRNAs are unique. For example, in the setting of increased ER stress the ER endoribonuclease IRE1 is activated and splices XBP-1 mRNA producing an altered reading frame that encodes the active transcription factor. XBP-1 plays an important role in lymphocyte differentiation, and potentially regulates a host of genes.[112,113] Although xbp-1 promotes survival of hypoxic cells[37] the mechanism is not entirely clear and the full range of target genes and complete functional role of

xbp-1 in hypoxic cells has not been well studied.

Another mechanism by which ER stress induces mRNAs is via eIF2α phosphorylation, the same modification that inhibits the general synthesis of proteins. Translation of a small subset of mRNAs actually increases in the setting of eIF2α phosphorylation; thus these mRNAs have increased poly-ribosomes associated with them in hypoxic cells.[111] While the translation of several mRNAs are increased in hypoxic cells, including those encoding adhesion molecules and proteins important for angiogenesis,[111,114] the best example of the increased translation of a mRNA in hypoxic cells due to eIF2α phosphorylation is the transcription factor ATF-4 (Fig. 3). ATF-4 translation is induced in hypoxic cells when eIF2α is phosphorylated because of unique features in its upstream open reading frames.[115] Hypoxia-induced ATF-4 then acts as a transcription factor to upregulates a variety of proteins including gadd34 (an eIF2α phosphatase), CHOP (which has been reported to be pro-apoptotic) and in part ATF-3 (which is responsible for part of the hypoxic downregulation of Id-1.[107,111] The full range of ATF-4 targets in hypoxic cells is not known and probably varies in distinct organs and cell types. Experimentally cells which cannot phosphorylate eIF2α, via mutations in eIF2α or through expression of a dominant negative PERK, do not survive in hypoxic conditions and do not grow as transformed cells in mouse models.[38] It is unclear if the poor survival, which is more severe than seen in normoxic cells, is due to an inability to inhibit protein translation or an inability to upregulate ATF-4, ATF-4 targets, and other mRNAs that are induced in hypoxic cells in an eIF2α dependent manner (see below).

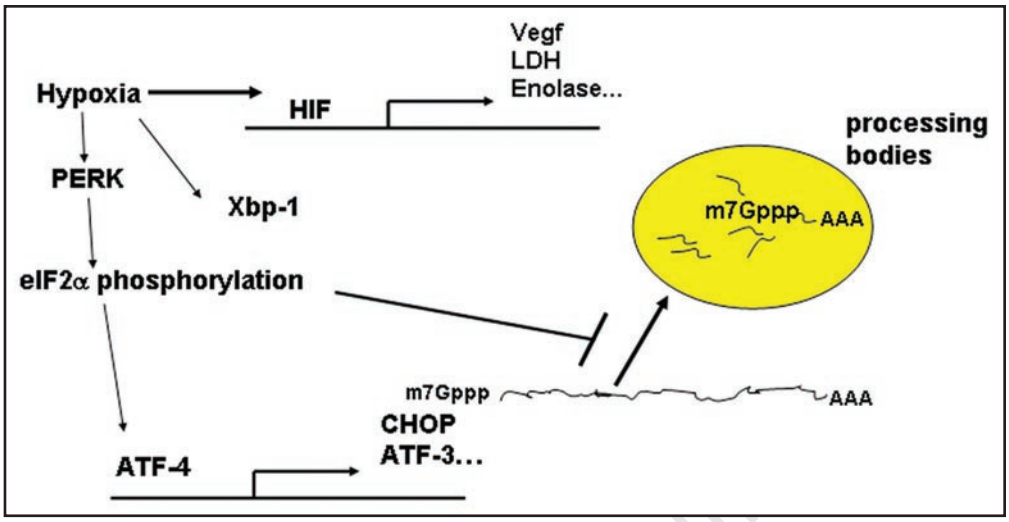

Figure 3. Hypoxic regulation of gene expression by activation of the integrated stress response and by inhibition of NMD. In hypoxic cells, unfolded proteins in the ER lead to activation of the PERK kinase. PERK induced phosphorylation of eIF2α: (1) Suppresses global translation, (2) Increase the translation of the transcription factor ATF-4, which then upregulates other genes like ATF-3 and CHOP (3) Suppresses NMD, and thus enhancing the stability of a variety of mRNAs including ATF-4, ATF-3 and CHOP.

Hypoxic Regulation of mRNA Degradation

Historically, the role of HIF-1 in altering hypoxic gene expression has been emphasized as the primary adaptive event in the cellular response to hypoxia. More recently the importance of hypoxic regulation of mTOR and eIF2α and control of protein translation (along with the subsequent induction of transcription factors and down-stream genes that occur with the generation of xbp-1, ATF-4 and others) has also been stressed. In contrast, much less attention has been paid to the regulation of mRNA stability in hypoxic cells and the role this stabilization may play in both the hypoxic transcriptome as well as hypoxic phenotypes. The stabilization of several mRNAs, including the pro-angiogenic growth factor VEGF, has been appreciated for years.[116] The 3' untranslated region (3'UTR) of mRNAs is important for the stabilization of many short lived mRNAs, including cytokines where a rapid "on-off" kinetics is important. The 3'UTR regions of these mRNAs typically contain AU rich sequences, which are bound and stabilized by a variety of RNA binding proteins. Investigators have determined that a 40 bp element in the 3' UTR of the VEGF mRNA binds to the RNA binding protein HuR, increasing VEGF mRNA stabilization in hypoxic cells.[117] It has also been reported that HIF-1 may play a role in stabilizing VEGF.[118] Intriguingly, the HuR protein has also been implicated in playing a part in the induction of p53 translation by pVHL,[119] the same tumor suppressor which negatively regulates the HIF-α proteins.

Another form of RNA degradation is termed nonsense mediated RNA decay (NMD) (recently reviewed in ref. 120). In contrast to the degradation mediated by AU rich elements in the 3' UTRs of mRNAs, NMD is responsible for rapidly degrading mRNAs which have a premature termination codon upstream of an exon-exon boundary. During pre-mRNA processing when introns are excised through mRNA splicing, the exon-exon junction is marked by a group of proteins termed the exon junction complex (EJC). Normally the EJC is removed by ribosomes during a scanning round of protein translation mediated by eIF4G.[121] When ribosomes pause at a premature termination codon, the EJC is not removed and instead Rent1/UPF1 is recruited to mRNAs by the translation release factors eRF1 and eRF3. Subsequently Rent1/UPF1 interacts with UPF2 and UPF3, which triggers UPF1 phosphorylation by SMG1 and initiates the degradation of the mRNAs. Although alternative pathways do exist, much of NMD is thought to occur through de-capping (through enzymes dcp1a and dcp2) and exonuclease activity (through xrn-1). Both de-capping enzymes, exonucleases and mRNAs are found concentrated in cells in cytoplasmic processing bodies, suggesting that much of the actual NMD mediated decay may occur there.[122] Indeed in yeast, Rent1/Upf1 tethered to mRNAs can direct those mRNAs to processing bodies.[123]

Bioinformatic studies have suggested that up to 30% of all known human mutations, including those responsible for cystic fibrosis, muscular dystrophy and thalassemia, encode for mRNAs with premature terminations codons upstream of an EJC which should trigger NMD.[124] In additions, NMD promoting mutations in the mRNAs encoding for the tumor suppressors BRCA1 and APC have also been found.[125,126] It is thought that rapid degradation of mutated mRNA

by NMD is protective since a synthesized truncated protein can serve as a dominant negative and block the function of a protein expressed from the non-mutated, allele. Studies have also demonstrated that when Rent1/Upf1 is knocked down up to 10% of the transcriptome in Hela cells is either upregulated or downregulated.[127] It is unclear how many of these are direct targets of NMD, and how many may be targets of transcription factors that are altered by NMD. It is thought that alternative reading frames and alternative splicing events may generate mRNAs that are typically degraded by NMD; these mRNAs would then be stabilized when NMD is inhibited.

The role of NMD to dynamically alter gene expression has not been emphasized because for the most part NMD has been thought of as a constitutive mechanism to rapidly degrade mutated mRNAs and not as a regulated system. However, studies have demonstrated that amino acid starvation inhibits NMD.[127] Intriguingly, amino acid starvation leads to the phosphorylation of eIF2α, the translation factor that is phosphorylated in hypoxic cells.[127] In yeast it is known that the alteration of gene expression requires the expression of Rent1/Upf1,[128] and ROS can also phosphorylate eIF2α. We thus hypothesized that eIF2α phosphorylation promotes inhibition of NMD, and that the phosphorylation of eIF2α in hypoxic cells would inhibit NMD. Indeed we found, by using a mutated β globin transgene whose mRNA is degraded by NMD, that NMD is inhibited in hypoxic cells.[129] We also found that many endogenous mRNAs upregulated with Rent1/Upf1 knock-down and downregulated with Rent1/Upf1 overexpression were stabilized in hypoxic cells. The hypoxic inhibition of NMD was dependent on phosphorylation of eIF2α; in cells that could not phosphorylate eIF2α, hypoxic stabilization of NMD targets did not occur. Because NMD is inhibited with cyclohexamide and other forms or protein translation inhibition, it is important that many of the mRNAs degraded by NMD and stabilized in hypoxic cells are actively translated in these hypoxic cells, ruling out this trivial mechanism for the hypoxic inhibition of NMD. Thus we demonstrated the NMD is regulated and can dynamically alter gene expression in a physiologically relevant condition, and has the potential to be an important determinant of hypoxic gene expression.

Previous analyses had demonstrated that when NMD is disabled though Rent1/Upf1 knock-down, many components of the integrated stress response (e.g., ATF-4 and the ATF-4 targets CHOP and ATF-3) are upregulated.[127] Indeed, we reasoned that if eIF2α phosphorylation leads to the translational upregulation of ATF-4 (and subsequent transcriptional upregulation of CHOP and ATF-3), then an important role of hypoxic inhibition of NMD could be to stabilize these same transcripts. We demonstrated that ATF-4, ATF-3 and CHOP are all bona fide targets of NMD, as these transcripts were stabilized with Rent1/Upf1 knock-down and expression was diminished with overexpression of Rent1/Upf1. As expected based on our previous observations, we found that the stability of these transcripts were increased in hypoxic cells and in a manner dependent on the phosphorylation of eIF2α. The inhibition of NMD was then shown to augment the integrated stress response; when cells were chemically stressed the protein expression of components of the integrated stress response were increased in Rent1/Upf1 knock-down cells as compared to control cells.

The mechanism for hypoxic inhibition of NMD, other than the fact that it is dependent on eIF2α phosphorylation, is less clear. We did demonstrate, however, that in hypoxic cells Rent1/Upf1 is predominantly found in cytoplasmic stress granule, not in processing bodies. Stress granules also contain mRNA, but do not contain the de-capping enzymes or the exonucleases necessary for RNA decay. Stress granules also contain phosphorylated eIF2α, and indeed we demonstrated that their very formation in hypoxic cells was dependent on eIF2α phosphorylation. Thus we hypothesize that mRNAs that might otherwise be degraded by NMD are stuck in stress granules and cannot be transferred to processing bodies for degradation. Alternatively, the sequestration of Rent1 in stress granules in hypoxic cells may be sufficient to repress NMD.

Summary and Perspective

It is clear that mammalian cells have a myriad of responses to cellular hypoxia. Many of these responses are adaptive to the hypoxic environment and are dependent on hypoxic regulation of gene expression. The HIF transcription factors play an important role in this response, but so do a variety of other transcription factors including ones generated by activation of the integrated stress response such as ATF-4 and XBP-1. In addition, the selective stabilization of mRNAs in hypoxic cells, by regulation of NMD and perhaps other mechanisms, is another important component of hypoxic gene regulation. It is likely that a large part of the complexity and cell/tissue specific nature of the hypoxic response is due to the interaction of transcription factors and other genes regulated by all these processes. Because hypoxia is a common feature in many tumors, a better understanding of gene regulation in hypoxic cells offers the promise of future therapeutic options.

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