HIF-1α Confers Aggressive Malignant Traits on Human Tumor Cells Independent of Its Canonical Transcriptional Function

Young-Gun Yoo¹, Jared Christensen¹, and L. Eric Huang¹,²

Abstract

Hypoxia is known to favor tumor survival and progression. Numerous studies have shown that hypoxia-inducible factor 1α (HIF-1α), an oxygen-sensitive transcription factor, is overexpressed in various types of human cancers and upregulates a battery of hypoxia-responsive genes for the growth and survival of cancer cells. Although tumor progression involves the acquisition of genetic and/or epigenetic changes that confer additional malignant traits, the underlying mechanisms of these changes remain obscure. We recently identified an alternative mechanism of HIF-1α function by which HIF-1α suppresses DNA repair by counteracting c-Myc transcriptional activity that maintains gene expression. Here, we show that this HIF-α–c-Myc pathway plays an essential role in mediating hypoxic effects on malignant progression via genetic alterations, resulting in the formation of malignant tumors with aggressive local invasion and epithelial–mesenchymal transition. We show an absolute requirement of the HIF-α–c-Myc pathway for malignant progression, whereas the canonical transcription function of HIF-1α alone is insufficient and seemingly dispensable. This study indicates that HIF-1α induction of genetic alteration is the underlying cause of tumor progression, especially by the hypoxic microenvironment.

Introduction

Hypoxia—deficiency in the amount of oxygen reaching the tissues—is frequently observed in human cancers. Although the accelerated proliferation of tumor cells contributes to oxygen deficiency by outstripping blood vessel growth, the aberrant vasculature with poor blood flow compounds the issue. As a result, cell death becomes increasingly conspicuous as the stage of the malignancy progresses. On the contrary, overwhelming evidence indicates hypoxia promotes tumor growth, progression, and resistance to therapies (1). Recent studies on the hypoxia-inducible factors (HIF), have corroborated further the essential role of hypoxia in tumor growth and progression (2, 3).

HIFs are heterodimeric transcription factors consisting of HIF-α and ARNT (4, 5). Although both subunits are expressed constitutively at the transcriptional and translational levels, the oxygen-responsive HIF-α, which is regulated through ubiquitin-proteasomal degradation, has been the focus of intensive investigations (6). In particular, HIF-1α and HIF-2α (also known as EPAS1), the two best studied members of the HIF-α family, harbor in the oxygen-dependent degrada-tion (ODD) domain 2 proline residues that are marked by hydroxylation for the recognition of E3 ubiquitin ligase (7–13). Hypoxic signaling stabilizes HIF-α by inhibiting prolyl hydroxylation and, in turn, ubiquitin-proteosomal degradation, rendering HIF-α capable of dimerizing with ARNT, binding to the hypoxia-responsive DNA element, and recruiting the transcription coactivator p300/CBP for transcriptional activation of a host of hypoxia-responsive genes (6). Both HIF-1α and HIF-2α are overexpressed frequently in human cancers, and this canonical HIF-α–ARNT pathway accounts for the upregulation of numerous cancer-relevant genes such as VEGFA, PGK1, and LOX for tumor angiogenesis, glycolysis, and metastasis, respectively (2, 14, 15).

Tumor initiation and progression require genetic alterations (16). Both tumor cells and their hypoxic microenvironment exert selective pressure for gene mutations and genetic instability (17, 18). We recently reported that HIF-1α, but not HIF-2α, is responsible for inhibiting DNA repair, leading to genetic instability (19, 20). However, neither HIF-1α DNA binding nor transactivation domain is required for gene repression; rather, it is the subregion of the HIF-1α PAS (Per-ARNT-Sim) domain PAS-B that is both necessary and sufficient to inhibit DNA repair by counteracting c-Myc transcriptional activity that maintains gene expression (21). Although the identification of this HIF-1α–c-Myc pathway has begun to shed light on the role of the hypoxic response in genetic alteration, whether such mechanism is responsible for malignant progression remains to be shown. In this study, we
show that the HIF-1α–c-Myc pathway is not only essential to the acquisition of malignant traits by tumor cells but also functions independent of the HIF–α–ARNT pathway. In contrast, the canonical HIF–α–ARNT pathway alone is insufficient to confer malignant traits.

Material and Methods

Cell culture and hypoxic treatment

Human osteosarcoma cell line U-2 OS (HTB-96) and U-118 MG (HTB-15) were obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS at 37°C in a 5% CO2 incubator. For the treatment with short-term hypoxia, cells were incubated in a hypoxic chamber (Innova CO-48; New Brunswick Scientific) maintaining 1% O2 and 5% CO2 overnight. For long-term hypoxia, was carried out for 2 weeks in a cycle of 16-hour 1% O2 followed by 8-hour 21% O2. The choosing of repetitive hypoxia treatment was to ensure sustainable HIF-1α levels that otherwise might have been markedly attenuated during prolonged hypoxic treatment. These cells were then cultured in normal conditions for another 2 to 4 weeks before further analysis.

Retroviral transduction

An oxygen-insensitive HIF-1α, HIF-1α(P) (22), with P402A and P564A substitutions was cloned into the retroviral expression vector pBABE-neo (Addgene). Additional mutations were made by site-directed mutagenesis (23) to create HIF-1α(P) mutants VAT (V317L-A321G-T327P; ref. 20) and RBC [R27G (ref. 23)-F99L (ref. 24)-C800S (ref. 25)]. The mutagenic oligonucleotide sequences are listed in Supplementary Table S2.

Polymerase chain reaction

Conventional reverse transcriptase PCR (RT-PCR) was done with primer sequences tabulated in Supplementary Table S2. Genomic DNA was extracted with a DNeasy Blood & Tissue kit (Qiagen) for PCR amplification with primer sequence listed in Supplementary Table S3. For real-time PCR, cDNA was amplified by using the TaqMan Universal Polymerase Chain Reaction Master Mix (Applied Biosystems). Human CA9 (6FAM), PK1 (6FAM), VEGFA (6FAM), MSH2 (6FAM), NBS1 (6FAM), and human ACTB endogenous control (VIC) were purchased from Applied Biosystems.

Tumorogenesis

Male CD-1 nude mice (up to 42 days old) from Charles River Laboratory were used for bilateral, subcutaneous injections. One million cells in 100 μL of PBS were used. Tumor volume was monitored every 3 to 4 days beginning 17 days after injection. Mice were euthanized on day 40 according to the protocol approved by the University of Utah Institutional Animal Care and Use Committee. Hematoxylin-eosin staining was done by the ARUP National Reference Laboratory. Images were examined and photographed with an Axiosave 200 inverted microscope.

Immunoblot

Immunoblot was done as previously described (26). Specific antibodies include HIF-1α, fibronectin, E-cadherin, and ZEB2 from Santa Cruz Biotechnology; MSH2 from BD Biosciences; NBS1 and β-catenin from Cell Signaling Technology; phospho-histone H2AX (γ-H2AX, S139) from Millipore; and β-actin from Sigma. Images were obtained with the Kodak Image Station 4000R. A representative of at least 3 independent experiments with similar results is shown.

Anchorage-independent growth

Cells (1 × 10⁶) were seeded in 6-well plates with a bottom layer of 0.5% Bacto agar in DMEM and a top layer of 0.35% Bacto agar in DMEM. Colonies were counted after 2 weeks and photographed using a Kodak Image Station 4000R. The total number of colonies per well was determined using Kodak Molecular Imaging software. Results in 6 replicates were presented as mean ± SEM.
Cell proliferation and Matrigel invasion

Cell proliferation was assayed in 96-well plates with the CellTitre-Glo Luminescent Cell Viability Assay (Promega). At each time point, cell viability in 6 replicates was recorded with an FLx800 microplate fluorescence reader (Bio-Tek) and presented as mean ± SEM. Cell invasion was determined in triplicates with cell suspensions added to the BD BioCoat Matrigel Invasion Chambers (BD Biosciences) and incubated for 24 hours in a humidified tissue culture incubator. Non-invasive cells were removed by scrubbing with a cotton-tipped swab. The total number of invasive cells was determined according to the manufacturer’s protocol and presented as mean ± SEM.

RNA interference

Cells were seeded in 6-well plates and transfected with siRNA duplexes targeting ZEB2 (5’-ACCUGAAAUAGUAUUAAATT-3’ and 5’-UUAUUUCAUAAUGUGGUGC-3; Qiagen) and control si-GL2 with the Oligofectamine reagent (Invitrogen), according to the manufacturer’s protocol.

Microsatellite instability

Genomic DNA was isolated from transduced cells and their tumors with a DNeasy Blood & Tissue Kit (Qiagen). Microsatellite instability was analyzed with a panel of 5 markers (BAT25, BAT26, D2S123, D5S346, and D17S250) at the University of Utah Research Core Facility.

Results

Independent functions of the HIF-1α–ARNT and HIF-1α–c-Myc pathways

The HIF-1α function via the HIF-1α–ARNT pathway has been a prevalent theory of tumor growth and development by which a large group of hypoxia-responsive genes are transcriptionally upregulated. To show a critical role of the HIF-1α–c-Myc pathway in cancer biology, we sought to uncouple the activity of the HIF-1α–c-Myc pathway from that of the HIF-1α–ARNT pathway and tested each pathway individually in malignant progression. Hence, the functions of the HIF-1α–ARNT and HIF-1α–c-Myc pathways were inactivated by site-directed mutagenesis in the context of a stabilized HIF-1α, designated as HIF-1α(PP) (Fig. 1A), in which 2 prolyl hydroxylase sites (P402 and P564) were destroyed to prevent oxygen-dependent proteolysis (11, 12).

Subsequently, the HIF-1α–c-Myc pathway was inactivated through simultaneous substitutions of HIF-1α V317, A321, and T327 in the PAS-B (20), yielding the mutant HIF-1α(PP)+VAT. Likewise, the HIF-1α–ARNT pathway was mutated at R27 (23), P99 (24), and C800 (25) to create HIF-1α(PP)+RFC, a mutant deficient in DNA binding and p300/CBP recruitment. Furthermore, a combinatorial mutant HIF-1α(PP)+RFC+VAT was created to inactivate both functions. These HIF-1α mutants were then expressed in various types of tumor cells by retroviral transduction and selection.

As expected, HIF-1α(PP) could upregulate C49 and PKG1, two classic targets of the HIF-1α–ARNT pathway (Fig. 1B, left), and downregulate the DNA repair genes MSH2 and NBS1 of the HIF-1α–c-Myc pathway (Fig. 1B, right; Supplementary Fig. S1A and B). Likewise, HIF-1α(PP)+RFC could not activate the HIF-1α–ARNT pathway, yet it remained capable of exerting its activity in the HIF-1α–c-Myc pathway. Furthermore, the mutant failed to activate a HIF-1α-mediated reporter gene that requires DNA binding and p300/CBP recruitment (Supplementary Fig. S1C). In contrast, HIF-1α(PP)+VAT was inactive in the HIF-1α–c-Myc pathway but was active in the HIF-1α–ARNT pathway. It is noteworthy that c-Myc protein (Supplementary Fig. S1D) and mRNA (Supplementary Table S1) levels were markedly decreased in the transduced cells with an intact HIF-1α–c-Myc pathway, consistent with HIF-1α counteraction of c-Myc (27, 28). Hence, these HIF-1α mutants by design function independently via the HIF-1α–ARNT and HIF-1α–c-Myc pathways.

DNA damage and genetic alterations via the HIF-1α–c-Myc pathway

The suppression of DNA repair genes by HIF-1α(PP) and HIF-1α(PP)+RFC was correlated with conspicuous DNA double-strand breaks, as indicated by a striking increase in γ-H2AX foci, which were colocalized with those of 53BP1 (ref. 20; Fig. 2A). Elevated levels of γ-H2AX and 53BP1 were confirmed by immunoblot analyses (Supplementary Fig. S2A). In contrast, mutants defective in the HIF-1α–c-Myc pathway, HIF-1α(PP)+VAT and HIF-1α(PP)+RFC+VAT, failed to
produce noticeable DNA damage. All of these cells responded to hydroxyurea-induced DNA damage. Similar results were obtained with various tumor cell lines, such as the human glioblastoma cell U-118 MG and the mouse hepatoma cell Hepa 1-6 (data not shown).

Furthermore, increased microsatellite instability was detected in cells with accumulated DNA damage but not in those without (Fig. 2B; Supplementary Fig. S2B). Chromosomal fragile site instability was also detected in the two most active sites FRA3B and FRA16D, which are harbored within the genomic loci of the tumor suppressor genes FHIT and WWOX, respectively (29), as shown by the loss of FHIT and WWOX expression at mRNA levels (Supplementary Fig. S3A). The lack of gene expression correlated with the loss of the exons at the gene locus (Supplementary Fig. 3B), as previously reported (30, 31). In addition, the tumor suppressor genes RB1 and TP53 were markedly suppressed from cells manifesting chronic DNA damage, even though no mutations were detected in the coding regions by DNA sequencing (Supplementary Table S1; data not shown). Therefore, the HIF-1α–c-Myc pathway inhibits tumor-suppressing activities, at least in part, via genetic alterations.

**Gain of aggressive malignant traits requires the HIF-1α–c-Myc pathway**

In accordance with diminished tumor-suppressing activities, both HIF-1α(PP) and HIF-1α(PP)+RFC cells exhibited a remarkable gain of anchorage-independent growth (Fig. 3A; Supplementary Fig. S3C). They also showed a marked increase in cell proliferation and Matrigel invasion, whereas inactivation of the HIF-1α–c-Myc pathway abrogated such increase (Fig. 3B; Supplementary Fig. S3D). Importantly, these cells, in contrast to the parental and HIF-1α(PP)+VAT cells, all acquired tumorigenicity by forming rapidly growing tumors within 3 weeks in subcutaneous xenograft mouse models (Fig. 3C and D). These tumors were highly aggressive in histology, exhibiting massive hemorrhagic necrosis and invasion of neighboring tissues including the dermal layers and skeletal muscles (Fig. 3E).

We also carried out *ex vivo* characterization of the HIF-1α(PP) and HIF-1α(PP)+RFC tumor specimens by analyzing target gene expression of the HIF-1α–ARNT and HIF-1α–c-Myc pathways in reference to the parental cells. In agreement with the transduced cells, these tumor specimens displayed a similar gene expression pattern, as shown by real-time PCR.
Interestingly, the angiogenic gene VEGFA was not increased in either type of the tumor specimens and transduced cells (Supplementary Table S1). On the other hand, the tumor specimens showed a further increase in microsatellite instability (Fig. 4B) with reference to the original transduced cells (Fig. 2B), suggesting additional genetic alterations by tumor hypoxia.

To corroborate the obligatory role of the HIF-1α–c-Myc pathway in acquiring malignant traits, we took advantage of an isolated HIF-1α PAS-B (referred to as PAS1B hereafter), which has been shown previously to be sufficient to activate the HIF-1α–c-Myc pathway (20). We showed that PAS1B, but not its mutant VAT, yielded identical results as HIF-1α(PP) by inducing DNA damage and acquiring malignant traits (Fig. 5A–C; Supplementary Fig. S4A and B). Furthermore, expression of PAS1B in U-2 OS cells was sufficient to confer tumorigenicity in 10 of 10 nude mice (Supplementary Fig. S4C) and the derived tumors were morphologically as malignant and invasive as those derived from HIF-1α(PP) and HIF-1α(PP)+RFC cells (Fig. 5D). Likewise, PAS1B expression in U-118 MG cells markedly accelerated tumor growth, leading to the invasion of the mouse dermal layer (Supplementary Fig. S4D). In contrast, the parental U-118 MG and control cells formed only tiny, circumscribed tumors confined within the subcutaneous layer.

To ensure that the effect of HIF-1α on malignant progression is physiologically relevant, we subjected U-2 OS cells to 1% O2 treatment for different periods. Although a short-term (overnight) hypoxic treatment with or without days of recovery in normoxia failed to induce tumorigenicity, long-term hypoxia (see the Materials and Methods section) converted the parental cells to 100% tumorigenicity (Supplementary Fig. S5A). However, knockdown of HIF-1α expression (not shown) prior to long-term hypoxia abrogated the tumorigenicity, indicating an essential role of HIF-1α in mediating the hypoxic effect. Moreover, the derived tumors exhibited high degree of malignancy, resembling those of HIF-1α(PP) and HIF-1α(PP)+RFC cells (Supplementary Fig. S5B).

**Induction of ZEB2 expression by HIF-1α for EMT**

A salient feature observed among the acquired malignant traits was EMT, which is characterized by both a loss of epithelial markers and a gain of mesenchymal markers (32, 33). Although the parental U-2 OS cells expressed the epithelial marker E-cadherin, none was detected in cells with an active HIF-1α–c-Myc pathway (Fig. 6A and B; Supplementary Fig. S6). Another epithelial marker, β-catenin, became distinctly nuclear in these transduced cells. Furthermore, these cells gained expression of the mesenchymal marker fibronectin. It is worthy to note that no EMT was observed in HIF-1α(PP)+VAT cells that were active in the HIF-1α–ARNT pathway, even though several recent studies indicate an important role for HIF-α in EMT (34–37).

Transcriptional repression of CDH1 gene, which encodes E-cadherin, is a key to EMT induction (33). Among a panel of candidate CDH1 transcriptional repressors examined, ZEB2/SIP1, encoding the zinc finger E-box binding homeobox 2 (38), was the one that was markedly upregulated by the HIF-1α–c-Myc pathway (Fig. 6C and D; Supplementary Fig. S7A). Knockdown of ZEB2 gene increased E-cadherin but decreased fibronectin levels in these cells (Fig. 6E). However, only partial reversal of EMT was observed in the knockdown cells with immunofluorescent staining (Supplementary Fig. S8), indicating the involvement of additional CDH1 transcriptional repressors, such as Twist1 (ref. 35; Supplementary Figure 3. Requirement of the HIF-1α–c-Myc pathway for malignant progression and tumorigenicity. A to D, transduced U-2 OS cells as indicated were assayed for anchorage-independent growth (A), Matrigel invasion (B), and tumorigenicity with subcutaneous xenograft in CD-1 nude mice (C). The anchorage-independent colonies and Matrigel-invading cells were quantified and presented as mean ± SEM. The injection sites are marked by arrowheads. Summarized result is in D with a ratio of 5 mice per group. E, hematoxylin-eosin staining of tumor specimens (T) shows hemorrhagic necrosis (N) and invasion of dermal layers (D) and skeletal muscles (M). Two representative images with 200× magnification are shown.
Nevertheless, introduction of the ZEB2 gene in U-2 OS cells was sufficient to decrease E-cadherin, while increasing fibronectin levels, and to induce EMT (Supplementary Fig. S7B and C). Taken together, these results indicate that ZEB2 upregulation by HIF-1α is mediated by the HIF-1α–c-Myc pathway and is responsible for maintaining a mesenchymal phenotype of malignant cells.

Discussion

Tumor growth, as judged by size, volume, and weight, has been the gold standard in experimental cancer research. However, such quantitative change often substitutes for tumor progression, a qualitative measure denoting cumulative gain of malignant traits (18). In this study, we have provided a cogent argument for the role of HIF-1α via the HIF-1α–c-Myc pathway in malignant progression; activation of the HIF-1α–c-Myc pathway confers aggressive malignant traits on tumor cells. In particular, we have shown that the HIF-1α–c-Myc pathway is not only necessary to induce rapid tumor formation but, more importantly, also sufficient to promote malignant behavior, including aggressive local invasion. Notably, expression of PAS1B recapitulates the effects of a full-length HIF-1α in this regard. In contrast, activation of the HIF-1α–ARNT pathway is insufficient to do so in the absence of the HIF-1α–c-Myc pathway. Hence, we define the HIF-1α–c-Myc pathway as an underlying mechanism of malignant progression, which is functionally independent of the HIF-1α–ARNT pathway.

The interplay between HIF-1α and c-Myc was first identified as HIF-1α functional counteraction of c-Myc (19, 23, 39), yet additional transcriptional and posttranslational mechanisms for c-Myc suppression were also reported for hypoxic adaptation by tumor cells (27, 28). Furthermore, c-Myc protein levels were reduced for survival when tumor cells were located distant from the blood vessels or cultured under low-oxygen and glucose-deficient conditions (40). Therefore, lowered c-Myc activity by hypoxia favors tumor survival. However, in Myc-dysregulated tumors HIF-1α has been shown to cooperate with c-Myc to induce angiogenic and glycolytic genes in an inducible MYC lymphoma model (41) and with N-Myc for enhanced aerobic glycolysis in neuroblastomas (42). Therefore, how HIF-1α and Myc interact seems context dependent (21).

It seems that the HIF-1α–c-Myc pathway is unique in that substitution of HIF-1α V317, A321, and T327 with the corresponding residues of HIF-2α obliterates the ability of HIF-1α to confer malignant traits, regardless of the status of HIF-1α–ARNT pathway. In keeping with this, HIF-2α does not inhibit DNA repair (20); rather, it stimulates DNA repair and promotes genomic stability in renal clear cell carcinoma cells (43). In this tumor type, a mutually suppressive interaction between HIF-1α and HIF-2α has been reported along with contrasting properties that HIF-1α retards whereas HIF-2α enhances tumor growth (44). Moreover, HIF-2α has been shown to cooperate with c-Myc in increasing cell proliferation (39), to override tumor suppression in renal clear cell carcinomas (45), and to promote an aggressive phenotype in neuroblastomas (46). These findings have led to the notion that HIF-2α is a critical mediator of aggressive phenotypes at least in renal clear cell carcinoma, neuroblastoma, and nonsmall cell lung cancer (47). However, an in-depth study of neuroblastomas shows recently that HIF-1α, but not HIF-2α, is preferentially expressed in tumors with poor prognosis (42).
In addition, genetic evidence supports a crucial role for HIF-1α in the development of cardiac rhabdomyosarcoma and metastasis (48). Although cell-specific expression of HIF-1α and HIF-2α may explain their apparent discrepancies in tumor progression, further understanding of the mechanisms by which HIF-2α promotes tumor progression is warranted.

Both HIF-1α and HIF-2α have been attributed to the hypoxic induction of EMT, and the HIF-1α–ARNT pathway has been shown or implicated for the transcriptional upregulation of CDH1 transcriptional repressors including Twist1, Slug, and ZEB2 (35–37). It remains to be seen, however, that the HIF-1α–ARNT pathway is required for ZEB2 regulation (36, 37). Our results indicate that the HIF-1α–c-Myc pathway is essential to the induction of ZEB2 and in turn EMT. This is further substantiated by the sufficiency of PAS1B in this regard. Although how the HIF-1α–c-Myc pathway upregulates ZEB2 expression is yet to be uncovered, recent studies indicate micro RNA (miR-200) plays a key role in regulating ZEB2 expression (49, 50). Therefore, it will be of great interest to determine whether the HIF-1α–c-Myc pathway affects the miR-200s that target ZEB2 transcripts.

The important role of HIF-1α via the HIF-1α–ARNT pathway has been well documented for its essential role in tumor growth by enhancing glycolysis and angiogenesis (2, 3). Although in this study activation of the HIF-1α–ARNT pathway was insufficient and rather seemingly dispensable for conferring malignant traits, tumor growth was retarded from HIF-1α(PP) cells that had been depleted of HIF-1α afterward (data not shown), further supporting the role of HIF-1α in tumor growth. Notably, all the tumor cells used in this study expressed endogenous HIF-1α, which was labile in normoxic culture but presumably activated by tumor hypoxia. In addition, the surrounding mouse tissue consisting of a Hif1a wild-type microenvironment might have also supported tumor growth. It would be interesting to use an HIF-1α-deficient background of tumor cells and/or of the host mouse to further dissect the individual roles of the HIF-1α–ARNT and HIF-1α–c-Myc pathways, even though the results may not be straightforward because of the feed-forward relationship between tumor growth and malignant progression via the hypoxic microenvironment (18). In light of previous studies (2, 3), we propose that although HIF-1α plays an important role via the HIF-1α–ARNT pathway in cell proliferation and metabolic
In adaptation of tumors, the HIF-1α-c-Myc pathway is the underlying mechanism of malignant progression that drives tumors to advanced stages.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank Kristin Kraus for editorial assistance.

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*Cancer Res* 2011;71:1244-1252. Published OnlineFirst February 8, 2011.

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