Hypoxia-inducible factor–1 and associated upstream and downstream proteins in the pathophysiology and management of glioblastoma

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Glioblastoma multiforme (GBM) is a highly aggressive brain tumor with an exceptionally poor patient outcome despite aggressive therapy including surgery, radiation, and chemotherapy. This aggressive phenotype may be associated with intratumoral hypoxia, which probably plays a key role in GBM tumor growth, development, and angiogenesis. A key regulator of cellular response to hypoxia is the protein hypoxia-inducible factor–1 (HIF-1). An examination of upstream hypoxic and nonhypoxic regulation of HIF-1 as well as a review of the downstream HIF-1–regulated proteins may provide further insight into the role of this transcription factor in GBM pathophysiology. Recent insights into upstream regulators that intimately interact with HIF-1 could provide potential therapeutic targets for treatment of this tumor. The same is potentially true for HIF-1–mediated pathways of glycolysis-, angiogenesis-, and invasion-promoting proteins. Thus, an understanding of the relationship between HIF-1, its upstream protein regulators, and its downstream transcribed genes in GBM pathogenesis could provide future treatment options for the care of patients with these tumors.

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KEY WORDS • glioblastoma • hypoxia • hypoxia-inducible factor • metabolism • glycolysis

GLIOMASTOMA multiforme (GBM) is a highly aggressive brain tumor that typically develops treatment resistance and results in poor patient outcome. Most solid tumors, including GBM, are thought to contain regions of hypoxia within the body of the tumor.⁸⁸ One of the hallmarks of GBM is intratumoral necrosis, which is thought to be the result of this hypoxia. As with other tumor types, hypoxia in GBM is implicated in conferring resistance to antiangiogenic therapy, chemotherapy, and radiation therapy, and in developing an aggressive tumor phenotype by increased invasion and loss of apoptotic potential.⁸⁸ Of the 3 known hypoxia-inducible transcription factors (HIFs), HIF-1 α is the best characterized. Here we review hypoxia and its role in the aggressive

phenotype of GBM, describe the normal physiology of HIF-1 α , examine it as a therapeutic target in GBM, and review current knowledge regarding important molecules that regulate HIF-1 α and those it regulates in GBM pathophysiology. We also review HIFs as potential therapeutic targets in the treatment of this tumor.

The Role of Hypoxia in GBM

Many studies support the hypothesis that a hypoxic environment is crucial for tumor growth and development and is correlated inversely with patient survival.^{76,229} Furthermore, in some tumor types, hypoxia markers have been shown to be predictive of patient outcome.^{57,83} Thus, determining hypoxia levels may allow us to predict the extent of an aggressive phenotype. For instance, the characteristics of increased metastases,²²⁰ tumor recurrence,⁷⁷ increased invasion,^{33,63} and resistance to chemotherapy^{59,196} and radiation^{59,189,239} have been extensively studied and implicated as hypoxia-mediated changes in solid tumors. These changes that potentially drive hypoxia-mediated malignant progression, including genomic instability, loss of apoptotic potential, oncogene expression, and abnormal angiogenesis have been described in GBM.⁸⁸ Furthermore, the presence of intratumoral necrosis is highly characterized in GBM, such that histological diagnosis of GBM depends on the presence of tumor necrosis and the cluster of cells that surround the necrotic area.⁸⁸ Therefore, it is

Abbreviations used in this paper: α -KG = α -ketoglutarate; CA-IX = carbonic anhydrase-IX; c-Met = c-mesenchymal-epithelial transition; DCA = dichloroacetate; EGFR = epidermal growth factor receptor; FIH-1 = factor inhibiting HIF-1; GBM = glioblastoma multiforme; GLUT-1 = glucose transporter 1; HIF = hypoxia-inducible factor; HKII = hexokinase II; HRE = hypoxia response element; Hsp90 = heat shock protein 90; IDH = isocitrate dehydrogenase; LDH = lactate dehydrogenase; MSC = mesenchymal stem cell; mTOR = mammalian target of rapamycin; PDK = pyruvate dehydrogenase kinase; PHD2 = prolyl hydroxylase domain 2; RACK1 = receptor for activated C kinase 1; shRNA = short hairpin RNA; siRNA = small interfering RNA; SUMO = small ubiquitin-like modifier; VEGF = vascular endothelial growth factor; VHL = von Hippel-Lindau.

reasonable to hypothesize that hypoxia-mediated pathways could have a similar role and effect in this highly aggressive brain tumor.⁸⁹

Chemotherapy is the standard of care for treating GBM,^{156,216} but GBM routinely develops resistance to any given chemotherapy regimen.¹⁹⁷ This response is hypothesized to be hypoxia related.^{26,150} In addition to chemotherapy, radiation therapy is often used in the treatment of GBM;^{123,214} however, just as hypoxia influences radiation resistance in vitro, this response is also observed in GBMs.^{10,46} Antiangiogenic therapy is a more recent option for treatment of patients with GBM. Because GBM is one of the most highly vascularized tumors, with substantial vascular proliferation and angiogenesis,^{47,186,192,224} therapy in the form of the anti-vascular endothelial growth factor (VEGF) antibody drug bevacizumab is typically considered in the treatment of patients with these tumors. Unfortunately, like chemo- and radiation therapy, antiangiogenic therapy is often plagued by the development of resistance as well.^{8,22,37,172} Therefore, because of the involvement of hypoxia in GBM development, targeting hypoxia-regulated proteins such as HIF-1, HIF-2, and HIF-3 and their associated regulators and targets in this highly resistant and aggressive brain tumor could result in significant benefits for cancer patients.

The Normal Physiology of HIF-1

The HIFs are all heterodimers composed of a major O₂-sensitive subunit (HIF-1 α , HIF-2 α , or HIF-3 α) and a constitutive HIF-1 β subunit, which bind together in the nucleus to form the HIF-1, HIF-2, or HIF-3 transcriptional activation complexes.¹⁰⁹ Of these 3 α -subunits, HIF-1 α and HIF-2 α are the best understood and seem to have both redundant and unique complementary functions.¹⁰³ However, there are very few studies of HIF-2 in the context of GBM, although there is evidence that it is associated with poor patient outcome, is more active in chronic hypoxia, and plays a role in glioma stem cell maintenance, invasion, and tumorigenesis.^{52,109,129} Because HIF-1 is the best-characterized hypoxia-regulated molecule in GBM, we will limit our discussion to it for the purpose of this review.

HIF-1 is a member of the PAS (per/aryl-hydrocarbon-receptor nuclear translocator [ARNT]/Sim) family of basic helix-loop-helix transcription factors. It is composed of 2 subunits: HIF-1 α , an O₂-sensitive subunit; and HIF-1 β , a constitutively expressed subunit.^{67,233} Under normoxic conditions, the HIF-1 α protein is regulated by 2 independent mechanisms at the posttranscriptional level (Fig. 1).⁹³ One mechanism is characterized by asparagine residue 803 becoming hydroxylated by factor inhibiting HIF-1 (FIH-1), an α -ketoglutarate (α -KG)-dependent dioxygenase with an Fe⁺² catalytic center, which then inhibits interaction between HIF-1 and the nuclear coactivator CBP/p300.^{15,74,140,204} The second mechanism involves hydroxylation of the proline residues Pro402 or Pro564, or both, by the prolyl hydroxylase domain 2 (PHD2), another protein with an Fe⁺² catalytic center, and uses O₂ and α -KG as substrates and generates CO₂ and succinate as byproducts.²⁰⁴ This hydroxylation leads to binding and 26S proteasomal degradation of HIF-1 α by the elongin B- and

elongin C-interacting von Hippel-Lindau (VHL) protein, the substrate recognition component of an E3 ubiquitin ligase.^{203,204} Another regulatory protein, ARD1, has been shown to acetylate lysine residue 532 of HIF-1 α in mouse cells, appearing to promote ubiquitination of murine HIF-1 in a VHL-dependent manner;⁹⁰ however, because ARD1 does not have these properties in human cells, its function remains unclear.⁹

Under hypoxic conditions (1%–2% O₂), both of the above-mentioned regulatory mechanisms of HIF-1 α become inhibited by substrate (O₂) deprivation. Furthermore, sumoylation of lysine residues 477 and 391 by the small ubiquitin-like modifier (SUMO)-1 enhances HIF-1 α stability and upregulation, further augmenting its transcriptional activity.^{6,205} These processes allow HIF-1 α to then dimerize with HIF-1 β , which then binds and activates DNA promoter regions called hypoxia response elements (HREs). These HREs help the cell cope with low O₂ conditions by inducing the transcription of > 100 genes, such as *VEGF*, *erythropoietin*, *glucose transporter 1 (GLUT-1)*, *carbonic anhydrase-IX (CA-IX)*, *enolase*, *lactate dehydrogenase (LDH)*, *tyrosine hydroxylase*, *aldolase A*, *phosphoglycerate kinase (PGK)*, *transferrin* and its associated receptor, and certain growth factors (Fig. 1).^{88,89}

Involvement of HIF-1 in Oncogenesis

The best-known tumor syndrome associated with HIF-1 is autosomal dominant VHL disease. VHL disease results from the loss of the tumor suppressor protein VHL encoded on chromosome 3p.⁹⁸ As shown in Fig. 1, VHL plays a pivotal role in the hypoxia-regulated control of HIF-1. Thus, a loss of VHL results in HIF-1 accumulation and subsequent upregulation of all HIF-1-controlled proteins.^{113,167,190,253} VHL syndrome is characterized by tumors with high vascularity, such as hemangioblastomas, endolymphatic sac tumors, renal cell carcinoma, and pheochromocytomas.⁸⁸ This highly vascular phenotype is thought to be the result of VEGF-mediated angiogenesis, secondary to loss of HIF-1 control via a loss of VHL. This is further supported by studies demonstrating that restoring wild-type VHL restores O₂-dependent expression of HIF-1 α and HIF-1-mediated transcriptional activity.^{111,146}

Further studies demonstrate that in VHL^{-/-} renal cell carcinoma cells, restoring VHL function is sufficient to decrease these cells' ability to form tumors in nude mice.^{24,62} In some neurological tumors such as hemangioblastomas and endolymphatic sac tumors, the VHL/HIF-1 pathway is important for pathogenesis and tumorigenesis; however, with regard to the highly aggressive GBM, few studies have shown the VHL/HIF-1 system to be a model for tumorigenesis. Recently, VHL has been found to regulate the signal transducer and activator of transcription 3 (STAT3), which in turn increases the tumorigenicity and "stemness" of U87 cells grown as neurospheres in vitro and in a mouse flank model.¹⁰¹ VHL has also been implicated in regulation of the microRNA-23b, which is upregulated in GBM, and reduces the expression of VHL, while also being repressed by VHL in a negative feedback loop.²⁵ Thus, overexpression of microRNA-23b in GBM reduces VHL activity and increases HIF-1 protein levels.

Fig. 1. Schematic representation of hypoxic control of HIF-1 α regulation and downstream HIF-1 α activity. The HIF-1 is composed of 2 heterodimeric subunits, HIF-1 α and HIF-1 β , which are constitutively expressed. Under normoxic conditions, the HIF-1 α protein is regulated by 2 independent mechanisms at the posttranscriptional level. One mechanism is characterized by asparagine residue 803 becoming hydroxylated by FIH-1, an α -KG-dependent dioxygenase with an Fe²⁺ catalytic center, which then inhibits interaction between HIF-1 and the nuclear coactivators CBP/p300. The second mechanism involves hydroxylation of the proline residues Pro402 or Pro564, or both, by the PHD2—another protein with an Fe²⁺ catalytic center—and uses O₂ and α -KG as substrates and generates CO₂ and succinate as byproducts. This hydroxylation leads to binding and 26S proteasomal degradation of HIF-1 α by the elongin B- and elongin C-interacting VHL, the substrate recognition component of an E3 ubiquitin ligase. Under hypoxic conditions (1%–2% O₂), both of these regulatory mechanisms of HIF-1 α become inhibited by substrate (O₂) deprivation. Furthermore, sumoylation of lysine residues 477 and 391 by SUMO-1 enhances HIF-1 α stability and upregulation, further augmenting its transcriptional activity. These processes allow HIF-1 α to dimerize with HIF-1 β , which then binds and activates DNA promoter regions called HREs. These HREs help the cell cope with low O₂ conditions by inducing the transcription of > 100 genes involved in various cellular survival pathways, including those involved in glucose uptake, glycolysis, and angiogenesis. (Figure reproduced with permission of the Department of Neurosurgery, University of Utah.)

Some investigators have found that the role of HIF-1 in tumor formation may not be so straightforward. In some instances, HIF-1 has been shown to function as a tumor suppressor in embryonic stem cells,^{21,138} human astrocytes,¹¹⁰ brain tumor,¹¹ and breast²⁸ and leukemic cells.²¹⁰ This phenomenon is most apparent when cells are grown in vivo. To further complicate matters, one of these studies found that transformed murine Δ HIF-1 α astrocytes had poor tumor growth in a vascular-poor mouse flank model, but were very aggressive when implanted orthotopically.¹¹ Another found that MDA-MB-231 breast cancer cells overexpressing HIF-1 α form significantly smaller tumors than wild-type cells in vivo; however, when those same cells are coinjected with fibroblasts overexpressing HIF-1 α (which by themselves do not form tumors), a substan-

tial increase in tumorigenesis and metastasis is observed.²⁸ It should be noted that in both of these studies HIF-1 α was either completely removed or constitutively expressed in cells prior to implantation. In our own investigations, we have noted that U251 cells with constitutive HIF-1 α short hairpin RNA (shRNA) knockdown are more aggressive orthotopically (unpublished data, 2006) than wild-type U251 cells that are allowed to form tumors prior to silencing HIF-1 α through small interfering RNA (siRNA) injections, which subsequently reduces tumor growth significantly.⁶⁰ This indicates that the timing of HIF-1 activity knockout during tumor growth is an important factor and can cause widely varied results. It also highlights the complicated nature of HIF-1's role in tumor development and the intricate web of intra- and extracellular pathways

involved. Future research must be carefully designed to address these questions of microenvironmental effects.

Involvement of HIF-1 in GBM

The role of HIF-1 in pathogenesis has been studied in many tumor types, such as prostate cancer,²⁹ squamous cell carcinoma,¹²⁵ lung cancer,²³⁴ breast cancer,²⁵⁴ bladder cancer,²²³ pancreatic cancer,⁷⁰ and many other non-CNS tumors.⁸⁸ This also includes brain tumors, which have been shown to correlate with HIF-1 α protein levels, tumor grade, and vascularity.^{49,248} Therefore, it is not surprising that glioma cell lines in vitro and in vivo overexpress HIF-1 α , in both normoxic and hypoxic conditions.^{54,89,102,108} Because of this, directly targeting HIF-1 is a reasonable approach for treatment of GBM.

It has been demonstrated that hypoxia in GBM causes an increase in proangiogenic factors, such as VEGF and stromal-derived factor-1, and recruitment of bone marrow-derived cells, such as vascular progenitor cells, stromal cells, mesenchymal stem cells (MSCs), and monocytes that have the capacity to stimulate endothelial cell recruitment and new blood vessel growth.^{5,8,50} This is enforced by data in GBM showing that HIF-1 promotes angiogenesis and tumor growth through the recruitment of various proangiogenic bone marrow-derived CD45+ myeloid cells, F4/80+ tumor-associated macrophages, and endothelial and pericyte progenitor cells.^{3,39} The recruitment of MSCs by GBM is so pronounced that it has been proposed as a possible delivery route for targeted therapies, such as IFN- β , which has been shown to increase survival significantly in a U87 orthotopic mouse model when delivered via genetically engineered human MSCs that localized specifically to the tumor in vivo.¹⁵⁹

Recruitment, pseudopalisading necrosis, and the abnormal vascular development that results from chaotic angiogenesis are some classic histological markers of GBM.¹⁴ Hypoxia in these pseudopalisading cells surrounding microvascular areas encourages infiltration by inflammatory cells, which can further stimulate the normoxic activation of HIF-1.^{100,192,206} Localized inflammation is an important contributing factor in the development of GBM, as demonstrated by the presence of infiltrating immune cells and the expression of inflammatory cytokines.²²⁵ Because HIF-1 is connected with prominent proinflammatory mediators such as interleukin (IL)-1 β and nuclear factor (NF)- κ B, it is a central player in linking inflammation and tumorigenesis in GBM.²⁰⁶ The *IL-1 β* gene is a target for HIF-1 activation and is a proinflammatory cytokine with pluripotent activity, including the promotion of angiogenesis, tumor growth, and metastasis.²⁴⁹ This link suggests a mechanism for inflammation stimulating HIF-1 activity in the normoxic areas of tumors, particularly in the mesenchymal subtypes of GBM, which show activated Ras and immune cell infiltrates.²⁰⁶

Targeting of HIF-1 as a Treatment for GBM

Several studies have demonstrated that molecular targeting of HIF-1 α that results in decreased HIF-1 α protein levels is a potential therapeutic approach for GBM. For ex-

ample, when the malignant glioma cell line U251, which has elevated VEGF and HIF-1 expression, is transfected with a dominant-negative HIF-1 α expression vector, the HIF-1 α activity and thus VEGF secretion decrease, resulting in growth inhibition.⁸⁹ Similarly, knockdown of HIF-1 α by siRNA results in decreased glioma growth both in vitro and in vivo, and increased survival when used in a continuous delivery system in mice,^{53,61} as well as decreased glioma cell migration and invasiveness in vitro under hypoxia.⁵⁴ Furthermore, data show that by transfecting glioma cells with a constitutive HIF-1 α shRNA expression plasmid to inhibit the expression of HIF-1 α , the cells become more sensitive than controls to the chemotherapeutic drugs doxorubicin and etoposide.²⁶ In addition, the role of HIF-1 in radioresistance has been well documented in many different tumor types, including GBM.^{69,72,149} This role has been verified by in vitro and in vivo studies showing that blockade of HIF-1 activity through different mechanisms results in radiosensitivity.^{64,71,104} This pivotal role of HIF-1 α in promoting both chemo- and radioresistance in tumors indicates that targeting it will improve the outcome of treatment if used as a combined therapy.

In recently approved or currently active Phase II trials, HIF-1 α inhibitors have been shown to decrease its expression through several mechanisms in different tumors (Table 1). In addition, the HIF-1 α inhibitors PX-478 and YC-1¹⁴⁹ show promising results for advanced solid tumor treatment, recently having finished a Phase I clinical trial.¹²² Furthermore, several preclinical research projects developing HIF-1 α inhibitors are underway.¹⁶⁹

In glioma, studies have demonstrated that the HIF-1 α inhibitors 103D5R,²²² zinc,¹⁶⁰ grape seed extract,¹³³ and KC7F2¹⁶¹ decrease HIF-1 α expression. A few specific small-molecule inhibitors of HIF-1 have recently shown progress as potential therapeutic strategies for GBM. Decreased GBM cell invasion under hypoxic conditions occurs through inhibition of HIF-1 α and its major trans-regulating factors by the cyclin-dependent kinase 2,7,9 selective inhibitor SNS-032.⁴ Under normoxia and hypoxic conditions, melatonin exerts antimigratory and antiinvasive effects in GBM cells by blocking HIF-1 α protein expression. Also, under hypoxic conditions in which GBM cells are found to produce reactive oxygen species (ROS), melatonin destabilizes HIF-1 α protein via its antioxidant activity against the ROS.²⁵⁰

The chemotherapy drug temozolomide has been a frontline therapy for patients with GBM tumors.²¹⁵ Unfortunately, because GBM often becomes resistant to most chemotherapy agents,¹⁹⁷ the same holds true for temozolomide;⁷⁵ however, noscapine, a small-molecule inhibitor of HIF-1 α that induces apoptosis in human glioma cells,¹⁶³ may serve as a therapeutic alternative in the treatment of temozolomide-resistant GBM.⁹² Noscapine has also been shown to synergistically potentiate the effects of temozolomide as well as other chemotherapeutic agents¹⁸¹ in the treatment of GBM. Furthermore, conjugating polyethylene glycol solid lipid nanoparticles to noscapine improves its biological half-life, brain delivery, and efficacy in GBM cells, offering a possible approach to regulating the administration of multiple injections of noscapine—yet this warrants further in vivo study.¹³⁹

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TABLE 1: Summary of HIF-1 inhibitors in Phase II trial or approved as of 2012*

Drug	Status	Mechanism of HIF-1 Inhibition	Reference(s)
CCL-779	approved	HIF-1 α translation	38, 141, 230
Camptothecins (CPTs)	approved	HIF-1 α translation	182, 183, 227
2-Methoxyestradiol (2ME2) & analogs	approved	HIF-1 α translation	119, 136
Geldanamycin (GA) & analogs	Phase II	HIF-1 α degradation	84, 137, 177
SCH66336	Phase II	HIF-1 α degradation	68
Apigenin	Phase II	HIF-1 α degradation	48, 170
FK228	approved	HIF-1 α degradation	153
PX-12	Phase II	HIF-1 α degradation/transcriptional activity	237
Bortezomib	approved	transcriptional activity	99
Amphotericin B	approved	transcriptional activity	246

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Upstream Targets of HIF-1 for GBM Therapy

There are many molecules and pathways upstream of HIF-1 (Fig. 2), such as Ras,¹² PI3K/Akt,^{23,30,176} PTEN,^{18,112,256} mammalian target of rapamycin (mTOR),¹⁹⁵ and STAT3,¹³⁵ that regulate its activity, provide opportunities for dysregulation and tumorigenesis, and have potential as therapeutic targets in GBM. Recently, developments have focused on the FIH-1, PHD2, SUMO, the transcriptional cofactor CBP/p300, heat shock protein 90 (Hsp90), receptor for activated C kinase 1 (RACK1), and isocitrate dehydrogenase 1 (IDH-1) in GBM pathogenesis and for potential targeted therapy. Because these upstream regulators intimately interact with HIF-1 α , we chose to expand on them for the purposes of this review.

Factor Inhibiting HIF-1

The FIH-1 is involved in blocking the transcriptional activity of HIF-1 under normoxic conditions via hydroxylation of an asparagine residue.²⁰⁴ Dysregulation of FIH-1 proves important in the tumorigenesis of GBM, supported by the fact that the *FIH-1* gene, which is located at chromosome 10q24, is often deleted in GBM tumors.^{55,81,127,211} Furthermore, compared with normal brain tissues, GBM has a significant reduction of FIH-1 mRNA levels.¹⁸⁸ Further examination of the role of FIH-1 in the pathogenesis of GBM found that even under hypoxic conditions, FIH-1 can inhibit HIF-1-mediated transcription of GLUT1 and VEGF-A in human GBM cells.²³¹ This suggests that FIH-1 has potential as a therapeutic target in treating GBM patients with poor prognosis.

Prolyl Hydroxylase Domain 2

The PHD2 protein is an important regulator in the O₂-dependent degradation pathway of HIF-1 α , a mechanism that can become inhibited under hypoxic conditions.²⁰⁴ In GBMs, PHD2 can be induced by hypoxia in vitro and is expressed in hypoxic areas of tumors in vivo. Thus, it most likely remains operative to an extent at low O₂ concentration, and therefore acts as a negative feedback loop to limit the hypoxic HIF-1 response, protecting GBM tumor

cells from hypoxia-induced cell death.⁷³ To the contrary, PHD2 has been further studied for its ability to enhance hypoxia-induced GBM cell death by modulation of *HIF-1* target gene expression of GLUT-1, VEGF-A, and bcl-2 binding protein 3.²¹⁹ Taken together, these data suggest the need for further exploration of the PHD2/HIF-1 pathway, its pathogenesis in GBM, and its use as a novel therapeutic target in the treatment of GBM.

Small Ubiquitin-Like Modifier 1

The SUMO-1 protein conjugates to lysine residues, specifically to lysine residues 477 and 391, during HIF-1 α pathogenesis under hypoxic conditions, thus modifying HIF-1 activity, stability, and subcellular localization.^{6,205} Yang et al.²⁴⁵ showed that SUMO-1-conjugated proteins were significantly elevated in GBM tumor samples. Furthermore, silencing SUMO-1 expression and thus blocking conjugation in GBM cells blocked cell growth, DNA synthesis, and cell survival. It also resulted in cell cycle arrest and enzymatic indication of DNA damage, prompting further research to determine whether SUMO-1 could be a viable GBM therapeutic target upstream of HIF-1.²⁴⁵

CBP/p300

The CBP/p300 proteins are nuclear coactivators that bind to and enable HIF-1 α transcription in the cell nucleus under hypoxic conditions. Thus, it would seem reasonable to target and prevent this molecular interaction between HIF-1 α and CBP/p300 as a potential therapeutic treatment. In fact, studies have effectively demonstrated inhibitors of this interaction in different cancers.^{116,158,187,207} In several glioma cell lines, the inhibitor arylsulfonamide interfered with HIF-1 signaling and disrupted HIF-1 α interaction with the nuclear cofactors CBP/p300, inhibiting in vivo glioma growth,²⁴⁷ further suggesting the potential of the HIF-1 α /CBP/p300 interaction as a therapeutic target in GBM.

Heat Shock Protein 90/Receptor for Activated C Kinase 1

Although much attention is directed at the O₂-dependent regulation of HIF-1, the half-life of HIF-1 is also

Fig. 2. Schematic representation of nonhypoxic and metabolic upstream regulation of HIF-1 α . Translation of HIF-1 α mRNA into protein is dependent on the activity of the mTOR, which in turn is regulated by the activity of upstream tumor suppressor proteins including phosphatase and tensin homolog (PTEN) and tuberous sclerosis proteins TSC-1 (hamartin) and TSC-2 (tuberin), which are part of the receptor tyrosine kinase (RTK), GTPase protein Ras, GTP-binding protein Ras homolog enriched in brain (Rheb), serine/threonine-specific protein kinase (Akt), and phosphoinositide 3-kinase (PI3K) pathway. The protein regulated in development and DNA damage responses 1 (REDD1) is regulated by HIF-1 α and inhibits the tumor suppressor TSC1/TSC2, which in turn upregulates HIF-1 α translation. Proteins shown in *orange* are regulated directly by HIF-1 α and play a role in the cellular uptake of glucose (via GLUT-1), glycolytic enzymes, and the shift to anaerobic glycolysis (Warburg effect). Interestingly, mutations of IDH-1, commonly found in gliomas, alter the conversion of isocitrate to α -KG, with a resultant oncometabolite 2-hydroxyglutarate (2-HG), which has a stabilizing effect on HIF-1 α . PDH = pyruvate dehydrogenase. (Figure reproduced with permission of the Department of Neurosurgery, University of Utah.)

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regulated in an O₂-independent manner. Either Hsp90 competitively binds HIF-1 and stabilizes the protein, or RACK1, an anchoring protein that interacts with elongin C, competitively binds HIF-1 and thus promotes, independently of PHD2 and VHL, HIF-1 ubiquitination and degradation.^{131,204}

Several groups have studied Hsp90 involvement in GBM and have proposed that its inhibition is a possible therapeutic measure for direct reduction of glioma tumor growth.^{58,154,252,255} Furthermore, current research has focused on the ability of Hsp90 knockdown or inhibition to attenuate the effects of radiation^{155,228} and drug therapy¹³ through increased cell sensitization, potentially offering a combinatorial and aggressive approach to GBM treatment by using Hsp90 targeting.

The RACK1 protein has been shown to be a potent molecular enhancer of tumorigenesis in several types of cancers, making it a potentially viable target for suppressing tumor growth, tumor cell proliferation, and tumor-associated angiogenesis.^{20,78,126,232} With regard to malignant glioma, Peng et al.¹⁷¹ showed that RACK1 expression is significantly higher in glioma cell lines and tissues than in normal brain tissues and is associated positively with glioma malignancy. Furthermore, the proliferation and invasion of human glioma cell lines was suppressed by RACK1 downregulation via siRNA, whereas apoptosis of these cells became favored. Moreover, tumor xenograft growth in nude mice became inhibited with forced downregulation of RACK1. Given these effects in vitro and in vivo, it stands to reason that targeting RACK1 in GBM treatment may be an attractive approach.

Isocitrate Dehydrogenase 1

IDH-1 is a key enzyme in the citric acid cycle, converting isocitrate to α -KG. Mutations in this protein occur in > 80% of WHO Grade II–III and secondary GBMs, but are almost nonexistent (< 5%) in primary tumors.¹⁶⁶ This association is so consistent that IDH-1 mutations have been proposed as a diagnostic molecular marker of secondary GBMs, and it points to differing cells of origin between primary and secondary GBM.¹⁶⁵ The most common of these mutations results in an amino acid substitution at arginine residue 132 (R132H). Using the NADPH-dependent reduction of α -KG, mutant IDH-1 (R132H) produces the 2-hydroxyglutarate metabolite. This metabolite influences several α -KG-dependent dioxygenase enzymes, including the HIF-1 α regulator PHD2,¹⁹³ by acting as a competitive inhibitor (Fig. 2).¹⁹³ Studies implicate ML309, a mutant IDH-1 (R132H) inhibitor,^{34,35} as well as glutaminase²⁰¹ in IDH-1 (R132H) mutant GBM cells as potential therapeutic approaches; however, positive IDH-1 mutation status correlates with a better prognosis in patients with GBM, which indicates that perturbing the wild-type IDH-1 pathway in primary GBM could improve patient outcome.^{174,213} Downregulation of LDHA and underexpression of other essential glycolysis genes may occur in IDH-1 mutant gliomas, contributing to the slow growth and better prognosis in these GBM tumor types.²⁷ This research prompts further investigation into IDH-1, as well as LDHA, and their roles in GBM pathogenesis.

Downstream Targets of HIF-1 for GBM Therapy

During hypoxia, HIF-1 α binds to DNA HREs and induces transcription of many well-characterized genes,²³³ including almost every gene in the glycolytic pathway.⁵¹ Recent studies further implicate a link between aerobic glycolysis, HIF-1, and GBM tumorigenesis. Targeting individual parts of the glycolytic pathway such as LDHA¹²¹ may prove promising in cancer therapy, and knockdown of LDHA in glioma cells by siRNA or the inhibitor sodium oxamate has been shown to decrease those cells' migratory potential.²⁰⁰ The glycolytic enzymes pyruvate dehydrogenase kinase (PDK) and hexokinase II (HKII) are significant markers in the pathogenesis and targeted treatment of GBM. Furthermore, similar to the glycolytic enzymes CA-IX and GLUT-1, other hypoxia-regulated proteins transcribed by HIF-1 α , such as the receptor tyrosine kinase c-Met and VEGF,^{15,106,117,203} are overexpressed in malignant brain tumors.^{88,209,248}

The Warburg Effect

Glucose metabolism via glycolysis is an essential component to GBM tumorigenesis, as evidenced by the observed apoptosis and cell death on withdrawal of glucose from GBM cells.⁸⁷ Thus, modifying cellular metabolism by increasing glycolytic enzymes and therefore the glycolysis rate in tumor cells is desirable. This process is facilitated by activation of HIF-1 α . Aerobic glycolysis is the adaptation of cancer cells through increased glycolysis in the presence of normal blood O₂ tension. Also known as the Warburg effect,²³⁵ it is a necessary step toward an aggressive phenotype.²⁰⁸ A possible link between HIF-1 and this effect in various types of cells^{191,244} has been proposed, prompting the hypothesis that dysregulation of HIF-1 α could increase aerobic glycolysis.¹³² There is also evidence that some products of dysfunctional aerobic glycolysis can stabilize HIF-1. For example, one study found that fumarate appears to be a proto-oncometabolite that functions by binding to glutathione, resulting in the stabilization of HIF-1 α ;²¹⁸ however, a different group has proposed that fumarate inhibits PHD activity directly.¹⁷⁵

Another product, succinate, which accumulates as a result of succinate dehydrogenase inhibition, reduces PHD activity, leading to HIF-1 α stabilization and activation.¹⁹⁹ It is interesting to note that mutations in succinate dehydrogenase are linked to developing renal cell carcinoma, pheochromocytoma, and paraganglioma, all of which are also predominant in VHL disease, as discussed earlier. Furthermore, a complex of the HIF-1 upstream regulator mTOR has been implicated in promoting the Warburg effect in GBM cells.^{144,145} Therefore, increased attention is being given to targeting aerobic glycolysis as a potential route for GBM therapy. One study has shown that reversing the Warburg effect by using methylene blue decreases GBM cell proliferation in vitro.¹⁷⁸ Furthermore, targeting the key metabolic enzymes in glycolysis, some of which we elaborate on below, is a potentially attractive option for GBM therapy.²⁴⁰

Pyruvate Dehydrogenase Kinase

In metabolic oncology, decreased glucose oxidation

in the mitochondria and increased glycolysis provides a proliferative advantage, increased angiogenesis, and resistance to apoptosis in cancer cells. This shift in metabolism is largely mediated by the mitochondrial enzyme PDK. When PDK is activated, it selectively inhibits pyruvate dehydrogenase, a group of enzymes that catalyzes the oxidative decarboxylation of cytosolic pyruvate to mitochondrial acetyl-CoA, the substrate for the Krebs cycle, promoting increased cytosolic glycolysis and lactate production.²²¹ Thus, it is reasonable to hypothesize that inhibition of PDK could modulate this shift in glycolytic metabolism and increase prognostic factors, thereby acting as a potential and attractive therapeutic target in patients with malignant gliomas.⁹¹

Combined targeting of PDK and epidermal growth factor receptor (EGFR) reverses the Warburg effect and triggers regression of GBM.²²⁶ Much research has focused on selective inhibition of PDK and modulation of glycolytic metabolism as a therapeutic target for GBM using the inhibitor dichloroacetate (DCA),¹⁵² demonstrating that it is a viable therapeutic agent in the treatment of glioma.⁴⁰ Recently, a study showed that DCA decreases glycolytic metabolism via PDK metabolism in rat glioma cancer stem cells but not in rat neural stem cells, promising further therapeutic application in glioma.¹⁵⁷ Another study showed that DCA radiosensitized several cancer cell lines in vitro, including LN18 glioma.²⁵⁷ Combining DCA with bevacizumab has been shown to overcome hypoxia-induced anti-VEGF resistance in vivo and has potential as a combinatorial anticancer strategy in GBM.¹¹⁴ Currently, DCA is undergoing a Phase I clinical trial to continue investigation into its efficacy in patients with GBM and other recurrent brain tumors.²⁰²

Hexokinase II

Hexokinase II is an enzyme that phosphorylates glucose to create glucose-6 phosphate for the initial step of glycolysis. In GBM, HKII is a key mediator of the Warburg effect, enabling aerobic glycolytic metabolism. As a result, HKII expression is elevated in GBM compared with normal brain and correlates with a poorer overall survival of patients with GBM; this finding is further supported by exogenous expression of HKII in GBM cells, which leads to therapeutic resistance, increased proliferation, and intracranial growth.^{241,242} Wolf and colleagues showed that mitochondrial oxidative metabolism of glucose was restored in GBM cells after depletion of HKII. This depletion also increased GBM cell sensitivity to radiation and temozolomide. Furthermore, intracranial xenograft GBM cells that are depleted of HKII exhibited decreased expression of VEGF and HIF-1 α , as well as diminished angiogenesis and proliferation, but increased invasion.²⁴¹ In another study, Wolf et al.²⁴² demonstrated that partial epigenetic regulation for preferential expression of HKII occurs in aerobic glycolysis-dependent proliferative states such as the developing embryo and malignant human glioma tissue.²⁴²

A few inhibitors that target HKII in GBM pathogenesis have been examined. In U87MG cells, clotrimazole induced translocation of HKII from the mitochondria to the cytoplasm, which was followed by release of cytochrome

c, suggesting a mechanism for apoptosis in these cells. Furthermore, treatment of these cells with clotrimazole sensitized them to radiation in vitro.¹³⁰ Several recent studies support 3-bromopyruvate as a potentially efficacious and synergistic HKII inhibitor with anticancer activity in GBM,^{42–44} including a safe method for intracranial delivery in an animal model of glioma.²³⁸ Furthermore, tumor-suppressing microRNA-143 overexpression, which can be induced by the drug rapamycin,¹²⁴ directly targets HKII to inhibit glycolysis in GBM.²⁵¹ Taken together, these studies suggest that targeted treatment of HKII may be viable in GBM therapy.

Carbonic Anhydrase-IX

In patients with GBM, hypoxia-inducible overexpression of CA-IX occurs, but its functions in this context remain elusive.¹⁸⁰ In the U251 and LN18 glioblastoma cell lines, the malignant phenotypes of cell attachment and invasion seem correlated with CA-IX expression, because RNA interference to CA-IX strongly reduces these characteristics.¹⁸⁰ It has been hypothesized that overexpression of CA-IX under hypoxic conditions may maintain an acidic extracellular pH, a fundamental property of the malignant phenotype, through enzyme-mediated conversion of CO₂ to a proton, which is extruded in the extracellular environment, and to a bicarbonate ion that is transported to the cytoplasm.^{85,180} Also, because high CA-IX expression is identified as an independent factor for poor survival in patients with GBM, targeted therapy for CA-IX is highly desirable.¹⁸⁰

In cancer, CA-IX expression seems to correlate with poor response to adjuvant treatment.^{179,212} In malignant gliomas, CA-IX expression also predicts survival and radiographic response of patients treated with the chemotherapy drugs bevacizumab and irinotecan.¹⁹⁸ A recent study showed that inhibition of CA-IX enhanced the effect of anti-VEGF therapy with the drug bevacizumab, exhibiting a greater reduction in the growth rate of a U87 GBM cell line xenograft tumor than inhibition of CA-IX or bevacizumab treatment alone.¹⁴⁸ Furthermore, after CA-IX knockdown and RNA-mediated interference, the effects of chemotherapy and radiation were strongly enhanced and accompanied by an increased rate of cell death by apoptosis in U251 and LN18 cell lines.¹⁸⁰ Inhibiting CA-IX enzymatic activity by applying a specific CA-IX inhibitor sulfonamide or molecular inhibition via siRNA led to inhibition of its functional role during GBM tumorigenesis.¹⁹⁴ Thus, experimental data show that targeting this downstream enzyme of HIF-1 transcription as a therapeutic target in GBM shows potential. Although CA-IX is understood as a hypoxia-induced protein, under normoxic conditions CA-IX expression has been shown to be enhanced in GBM cells when an extracellular acidotic environment is present.⁸² These data suggest that further investigation into the relationship between pH metabolism and regulation, O₂ concentration, and GBM expression profile and tumorigenesis may prove fruitful.

Glucose Transporter 1

GLUT-1 is a transmembrane glycoprotein that me-

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diates sodium ion–independent transport of glucose into cells.⁷ Because glucose is unable to cross the blood-brain barrier, glucose transport into the brain is mediated by GLUT family proteins, with the most commonly expressed isoform being GLUT-1.¹⁶⁴ During hypoxia, GLUT-1 becomes overexpressed via transcription by HIF-1 α .^{88,89} Like CA-IX expression, GLUT-1 expression also correlates with poor response to adjuvant treatment,³² as well as a worse prognosis in patients with a wide variety of cancers.^{88,89} Therefore, therapeutic inhibitors of GLUT-1 seem to be a highly attractive approach to cancers that overexpress this gene, as evidenced by a study that shows that inhibition of glucose transporters in lung and breast cancer induces apoptosis.¹⁸⁴ Furthermore, GLUT-1 inhibition in breast and colon cancer cell lines under hypoxia increases chemosensitivity with daunorubicin¹⁹ and vincristine¹⁴² for an erythroleukemia cell line. For brain tumor therapy, implicating GLUT-1 as a target for treatment may prove challenging, because high levels of this protein in the blood-brain barrier help maintain basal glucose levels in the brain, and this would be a major therapeutic problem to overcome.⁴⁵ Furthermore, because of the overexpression of GLUT-1 transporters in GBM during hypoxia, efforts might be focused on developing therapeutic molecules that use the GLUT-1 system. For example, in the T98G GBM cell line, increased expression of GLUT-1 mRNA and protein increased the cytotoxic effects of glycoconjugated nitric oxide donors.²¹⁷

c-Mesenchymal-Epithelial Transition

Immunohistochemical expression of c-mesenchymal-epithelial transition (c-Met) in GBM is an independent predictor of outcomes in patients with these tumors.¹⁶⁸ Our group has recently demonstrated that inhibition of HIF-1 α directly inhibits c-Met.⁶⁰ This protein is a receptor tyrosine kinase that promotes the stem cell phenotype of GBM when activated by HIF-1,³⁶ by influencing the expression of reprogramming transcription factors, which support embryonic stem cells and induce pluripotent stem cell formation from differentiated cells.¹²⁸ Furthermore, activation of c-Met influences cell invasiveness.^{31,41} Thus, targeted treatment of c-Met in GBM seems desirable, as supported by a study showing that when treated with c-Met inhibitors, various lung cancer cell types lose their invasive phenotypes.¹²⁰ In fact, inhibition of c-Met signaling in GBM stem cells disrupts tumor growth and invasiveness both in vivo and in vitro.⁹⁵

Attempts at EGFR inhibition in GBM often induce high levels of c-Met activation as a mechanism of anti-EGFR resistance, further potentiating c-Met as a therapeutic target.⁹⁶ Inhibition of c-Met expression using chimeric transgenes decreases GBM tumor growth and malignancy,² including decreased angiogenesis as well as increased apoptosis in vivo.¹ Studies have also shown that therapeutic antibodies that disrupt the interaction between the ligand of the c-Met receptor, hepatocyte growth factor, and the receptor itself^{117,107,143,151} have a therapeutic effect. Other inhibitors, such as the orally bioavailable SGX523, inhibit glioma stem cell malignancy and intracranial tumor growth.⁶⁵ Enhancing c-Met siRNA delivery via conjugation to cationic solid lipid nanoparticles decreased

GBM cell proliferation in vitro and decreased GBM tumor growth in vivo.⁹⁴ Furthermore, targeting the c-Met pathway potentiates GBM cell response to radiation,^{118,236} and the c-Met antibody inhibitor AMG102 may also potentiate GBM cell radiosensitivity¹⁶ and increase effects of the chemotherapeutic drug reagents temozolomide and docetaxel,⁹⁷ suggesting that combination therapy with c-Met inhibitors may be an attractive approach. As understanding of c-Met and its role in GBM pathogenesis develops, discovering therapies that target c-Met maintenance of the stem cell phenotype are desired, as demonstrated by one study showing that inhibition of c-Met reduces the propagation of glioma stem-like cells in vivo.¹⁸⁵

Vascular Endothelial Growth Factor

Treatment in the form of anti-VEGF therapy with the antibody drug bevacizumab can be used for GBM, but unfortunately resistance often develops.^{8,22,37,172} Efforts to better understand mechanisms of resistance are currently underway. To improve the design of antiangiogenic treatment strategies, we need more details about tumor neovascularization, including VEGF-independent processes.¹¹⁵ Another study describes hypoxia-mediated autophagy as a promoter of GBM tumor cell survival and as a mechanism for antiangiogenic therapy resistance. Because antiangiogenic therapy leads to hypoxia, hypoxia-induced tumor cell autophagy was observed as an adaptive cytoprotective response.⁷⁹ In GBM, downregulation of the *cylindromatosis* (*CYLD*) gene, a tumor suppressor that acts as a de-ubiquitinating enzyme to regulate signaling pathways, may be crucial for hypoxia-mediated inflammation, which is hypothesized to affect the long-term efficacy of anti-VEGF therapy.⁶⁶ Furthermore, a proneural-to-mesenchymal transition as well as upregulation of genes associated with cellular migration, invasion, and inflammation facilitates resistance to anti-VEGF therapy.¹⁷³ Taken together, these data suggest that continued elucidation of mechanisms of antiangiogenic therapy evasion by high-grade GBM is warranted for more effective treatment with this approach.¹³⁴

A more invasive phenotype and expression of matrix metalloproteinase have been found in studies of bevacizumab-treated GBM.^{56,105} The receptor tyrosine kinase c-Met, which is also upregulated in response to therapy with bevacizumab,¹⁴⁷ is hypothesized to mediate this phenotype transition and to have potential as a mediator of antiangiogenic therapy resistance in vivo.⁸⁶ Therefore, dual targeting of c-Met and VEGF activity may prove to be an effective alternative to bevacizumab alone. In gliomas, bevacizumab-induced invasion has been shown to be inhibited by broad-spectrum kinase inhibitors.⁸⁰ Another study cites the potential of cabozantinib, a dual inhibitor of c-Met and VEGFR2 tyrosine kinase with prominent inhibition in vitro, but in vivo studies suggested the need for improvement in delivery of the drug to the tumor and/or surrounding tissue for enhanced effectiveness.¹⁶² Because increased invasion proves to be a mechanism of anti-VEGF resistance, further research is necessary to elucidate the role of c-Met in this process and the implications that this molecular relationship might have for therapy in patients with GBM.

Conclusions

Although a diagnosis of GBM yields a poor prognosis for cancer patients, there are broad, intense research efforts directed at finding suitable therapies for the treatment of patients with this tumor. In tumorigenesis, hypoxia and, most notably, the hypoxia-induced molecule HIF-1 α , play important roles. HIF-1 and its upstream and downstream molecules have been clearly implicated in GBM pathogenesis, suggesting that they are potential therapeutic targets for treatment of these tumors. Although in many instances additional research is warranted, it is clear that HIF-1 and its associated proteins are promising avenues for translational studies. Additional understanding of the role of HIF-1 and its molecular regulators and downstream targets in GBM pathophysiology, and which of those are viable therapeutic targets, may lead to better treatment outcomes and enhanced prognosis for patients with this devastating disease.

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