



Published in final edited form as:

Cancer Cell. 2009 June 2; 15(6): 501–513. doi:10.1016/j.ccr.2009.03.018.

Hypoxia-Inducible Factors Regulate Tumorigenic Capacity of Glioma Stem Cells

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Summary

Glioblastomas are lethal cancers characterized by florid angiogenesis promoted in part by glioma stem cells (GSCs). As hypoxia regulates angiogenesis, we examined hypoxic responses in GSCs. We now demonstrate that hypoxia-inducible factor HIF2 α and multiple HIF-regulated genes are preferentially expressed in GSCs in comparison to nonstem tumor cells and normal neural progenitors. In tumor specimens, HIF2 α co-localizes with cancer stem cell markers. Targeting HIFs in GSCs inhibits self-renewal, proliferation and survival *in vitro*, and attenuates tumor initiation potential of GSCs *in vivo*. Analysis of a molecular database reveals that *HIF2A* expression correlates with poor glioma patient survival. Our results demonstrate that GSCs differentially respond to hypoxia with distinct HIF induction patterns and HIF2 α may represent a promising target for anti-glioblastoma therapies.

Significance—Recent evidence supports the presence of cancer stem cell populations that contribute to tumor progression through preferential resistance to radiation and chemotherapy, and promotion of tumor angiogenesis, invasion, and metastasis. Therefore, the elucidation of molecular regulators of cancer stem cells may translate into improved anti-neoplastic therapies. Our work demonstrates that cancer stem cells derived from glioblastomas differentially respond to hypoxia with a distinct induction of HIF2 α . We find that HIFs are critical to cancer stem cell maintenance and angiogenic drive, and that expression of HIF2 α is significantly associated with poor glioma patient survival. These data further suggest that anti-angiogenic therapies can be designed to target

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SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, five tables and seventeen figures and can be found with this article online.

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cancer stem cell specific molecules involved in neoangiogenesis, including HIF2 α and its regulated factors.

Introduction

Cancer stem cells, which have been also described as tumor initiating cells or tumor propagating cells, are tumor cells that self renew and propagate tumors phenotypically similar to the parental tumor. Cancer stem cells from glioblastomas share some characteristics with normal neural stem cells including the expression of neural stem cell markers, the capacity for self renewal and long term proliferation, the formation of neurospheres, and the ability to differentiate into multiple nervous system lineages (neurons, astrocytes, and oligodendrocytes) (Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004; Singh et al., 2004; Vescovi et al., 2006; Bao et al., 2006a). However, brain tumor stem cells exhibit significant distinctions from normal stem cells in frequency, proliferation, aberrant expression of differentiation markers, chromosomal abnormalities, and tumor formation (Quintana et al., 2008; Reya et al., 2001; Vescovi et al., 2006). The potent tumorigenic capacity of cancer stem cells coupled with increasing evidence of radioresistance and chemoresistance suggests that cancer stem cells contribute to tumor maintenance and recurrence and that targeting cancer stem cells may offer new avenues of therapeutic intervention (Wulf et al., 2001; Bao et al., 2006a; Hambardzumyan et al., 2006; Jin et al., 2006; Liu et al., 2006; Blazek et al., 2007; Todaro et al., 2007; Bao et al., 2008). This hypothesis has been recently validated in clinical trial of breast cancer in which patients undergoing treatment with cytotoxic chemotherapy experienced an increase in breast cancer stem cells in the surviving tumor while the use of a targeted therapeutic against the stem cell population stabilized the cancer stem cell population (Li et al., 2008).

While the precise mechanisms responsible for the differential tumorigenic capacity of cancer stem cells have yet to be determined, previous studies have demonstrated that nonstem brain cancer cells can survive xenotransplantation but fail to form tumors (Singh et al., 2004). Although multiple mechanisms may be responsible for lack of tumor initiation, we previously demonstrated that glioma stem cells (GSCs) have a greater ability to promote tumor angiogenesis through secretion of elevated levels of vascular endothelial growth factor (VEGF) (Bao et al., 2006b). However, the upstream regulators responsible for up-regulating VEGF in GSCs remain to be defined. Hypoxia is a well-known regulatory factor for the “angiogenic switch” and regulates stem cell biology (Danet et al., 2003; Gassmann et al., 1996; Ezashi et al., 2005; Parmar et al., 2007; Blazek et al., 2007; Keith and Simon, 2007; Platet et al., 2007). Low oxygen levels promote maintenance of embryonic stem cell pluripotent potential and block differentiation (Ezashi et al, 2005). Moreover, the fraction of brain tumor cells expressing a stem cell marker is increased under hypoxia in vitro (Blazek et al, 2007; Platet et al, 2007). Thus, hypoxia may be a critical component of a cancer stem cell niche (Gilbertson and Rich, 2007; Keith and Simon, 2007). We therefore hypothesized that there are unique hypoxia responses in cancer stem cells which contributes to the tumor initiation and maintenance of cancer stem cells.

Cellular responses to hypoxia are commonly regulated by the hypoxia inducible factor (HIF) family of transcriptional factors (Harris, 2002; Keith and Simon, 2007). HIFs function as heterodimers consisting of an oxygen sensitive HIF α subunit and a constitutively expressed HIF β subunit. Under normoxic conditions, HIF α is ubiquitinated by the Von Hippel-Lindau (VHL) tumor suppressor gene product and then targeted for proteasomal degradation, but under hypoxia the interaction between HIF α and VHL is abrogated. As a result, HIF α is stabilized, dimerizes with HIF β and then binds to hypoxia responsive elements (HREs) in the promoters of hypoxia regulated genes. The HIF dimer activates the transcription of hundreds of downstream genes which modulate cell survival, motility, metabolism and angiogenesis (Harris, 2002). Two HIF α proteins, HIF1 α and HIF2 α , are highly homologous and bind to

similar HRE sequences. As HIF1 α is universally expressed while HIF2 α shows a more restricted expression pattern, relatively few studies have determined the role of HIF2 α in cancer initiation or tumor progression (Covello et al, 2006; Holmquist-Mengelbier et al, 2006; Hu et al, 2006; Raval et al, 2005). However, it is now clear that HIF1 α and HIF2 α can often play nonoverlapping biological roles due to their unique target genes as well as different requirement of oxygen for activation (Holmquist-Mengelbier et al., 2006). The identification of the stem cell regulator Oct4 as a HIF2 α target gene directly links HIF2 α to stem cell biology (Covello et al., 2006). Moreover, in a renal carcinoma model, HIF2 α enhances the transcriptional activity of another stem cell factor c-Myc, while HIF1 α destabilizes Myc complexes (Gordan et al., 2007). Another family member, HIF3 α , lacks the transcriptional activation domain and functions as a dominant negative regulator of the hypoxia response due to sequestration of HIF β s (Kaur et al., 2005). Together, these data differentially link HIFs to stem cell biology and angiogenesis. We therefore sought to determine HIF expression and its biological consequence in the context of the GSC and nonstem glioma cell subpopulations.

Results

mRNA Levels of *HIF2A* and Other Hypoxia Response Genes Are Differentially Expressed in Glioma Stem Cells

To determine if the angiogenic drive of GSCs is regulated by specific molecular responses to hypoxia, we created short term cultures enriched or depleted for cancer stem cells directly from glioblastoma surgical biopsy specimens or xenografts derived from brain tumor specimens (patient characteristics presented in Table S1 and Figure S1) using our previously described methodology (Bao et al., 2006a; Bao et al., 2006b). The neoplastic origin of these cells was confirmed by fluorescent in situ hybridization (FISH) analysis (Figure S2). For these cultures, we validated the enrichment or depletion of cancer stem cells using functional assays, including propagation of tumors with characteristics of the parental sample (Table S2, S3; Figure S2) and stem cell marker expression (Figure S3). Using matched cultures of GSCs or nonstem cells, we compared the mRNA levels of hypoxia-regulated genes in GSCs or nonstem cancer cells under normoxia (20% O₂) and hypoxia (1% O₂). Multiple hypoxia responsive genes were strongly differentially regulated between GSCs and nonstem cells isolated from the same tumor specimens (Table S4), including *HIF2A* (but not *HIF1A*).

Using semi-quantitative real-time PCR, we confirmed a strong basal and hypoxia-induced upregulation of *HIF2A* (but not *HIF1A*) mRNA in GSCs as compared to matched nonstem cancer cells or fetal human neural progenitors (Figures 1A/B, 2A/B, S4A/B, S5A/B, S6A/B). Similar patterns of mRNA expression were detected using the iron chelator, desferrioxamine (DFX) [which induces molecular hypoxic responses with similar kinetics to hypoxia (Wang and Semenza, 1993)] (Figures 1A/B, S4A/B, S5A/B), or atmospheric hypoxia (1% O₂) (Figures 2A/B, S6A/B). Minimal *HIF2A* expression was also detected in normal adult murine neural progenitors under normoxia or hypoxia (Figure S5D). These data demonstrate that *HIF2A*, but not *HIF1A*, is a hypoxia responsive gene dramatically up-regulated in GSCs.

The elevated *HIF2A* mRNA levels in GSCs may result from enhanced transcription or increased mRNA stability. Surprisingly, the half-life of *HIF2A* was shorter in GSCs in comparison to matched nonstem glioma cells (Figure S7A), suggesting that the increase in *HIF2A* mRNA levels is not due to a difference in mRNA stabilization. In contrast, *de novo* mRNA synthesis is required as blocking mRNA transcription by Actinomycin D abrogated the induction of *HIF2A* in GSCs upon hypoxia treatment (Figure S7B). To determine the relative levels of transcription of the *HIF2A* promoter in the tumor subpopulations, we performed RNA Polymerase II chromatin immunoprecipitation. GSCs under both normoxia and hypoxia had a greater enrichment of RNA Polymerase II binding to the *HIF2A* promoter

than nonstem glioma cells (Figure S7C). Together, these data demonstrate that *HIF2A* mRNA is up-regulated in GSCs with enhanced transcription.

Considering the differential expression of *HIF2A* and *HIF1A* in GSC and nonstem cancer cell subpopulations and normal neural progenitors, we determined the mRNA expression of genes known to be specifically regulated by HIF2 α or HIF1 α (Figures 1C-H, 2C-H, S4C-H, S5C, S6C-H). Genes reported to be HIF2 α dependent [Oct4 (Covello et al., 2006; Keith and Simon, 2007), glucose transporter type 1 (Glut1) (Keith and Simon, 2007), and SerpinB9 (Holmquist-Mengelbier et al., 2006)] were expressed at significantly higher levels in GSCs as compared to matched nonstem cancer cells under hypoxia (Figures 1C/E/G, 2C/E/G, S4C/E/G, S6C/E/G). In contrast, the HIF1 α -regulated gene phosphoglycerate kinase 1 (PGK1) (Keith and Simon, 2007) was strongly up-regulated in nonstem glioma cells under hypoxia as compared to matched GSCs (Figures 1D, 2D, S4D, S6D). Analysis of VEGF, a gene regulated by HIF1 α and/or HIF2 α in a cell specific manner (Harris, 2002; Holmquist-Mengelbier et al., 2006; Keith and Simon, 2007), demonstrated that hypoxia induced a greater increase in VEGF levels in GSCs than in matched nonstem cancer cells (Figures 1H, 2H, S4H, S6H; Table S4). HIF target genes in GSCs were variable relative to normal neural progenitors, with Glut1 demonstrating a consistent elevation in GSCs (Figures 2C-H, 5C).

Glioma Stem Cells Preferentially Express HIF2 α Protein under both Normoxic and Hypoxic Conditions

We interrogated the impact of transcriptional upregulation of *HIF2A* mRNA in GSCs on HIF2 α protein levels. Although HIF1 β and HIF3 α levels did not differ between glioblastoma stem and nonstem cells under normoxia and hypoxia conditions (data not shown), total HIF2 α protein expression was consistently higher in GSCs than in matched nonstem cancer cells (Figures 3A-J, S7D/E) or normal neural progenitors (Figures 3K-M). HIF2 α was highly expressed in GSCs treated with a chemical hypoxia-mimetic (Figures 3A-G, K, L) or grown in a hypoxia chamber under oxygen concentrations ranging from 0.2 to 5% (Figures 3H-J, M). In contrast, HIF1 α expression was only increased by more severe hypoxic conditions induced by the chemical mimetic or $\leq 1\%$ O₂ (Figures 3, S7D/E), which is consistent with a previous report that HIF2 α (but not HIF1 α) accumulates under physiological oxygen levels present in solid tumors (Holmquist-Mengelbier et al., 2006). When oxygen levels were sufficient for HIF1 α induction, HIF1 α was often expressed at higher levels in nonstem cancer cells than matched GSCs (Figures 3, S7D/E). Of note, the induction of HIF2 α and HIF1 α expression was dependent on new protein synthesis and regulated by proteosomal degradation (Figures S7D/E) without cell type specific differences in VHL expression (data not shown). These data suggest that GSCs preferentially express HIF2 α protein under both normoxic and hypoxic conditions to provide cancer stem cells a survival and growth advantage by activating downstream genes even in modest hypoxia conditions.

We further examined whether HIF2 α upregulation in GSCs generally occurs as a stem cell phenotype. Similar to the mRNA data indicating minimal *HIF2A* in human neural progenitors (Figures 2A, S5A, S6A), we found that HIF2 α was almost undetectable in normal human neural progenitor cells resulting in consistent over-expression of HIF2 α in the GSC population (Figures 3K-M). In contrast, HIF1 α accumulated in both human neural progenitors and GSCs under hypoxia (Figures 3K/L). Taken together, these data indicate that HIF1 α upregulation is a shared molecular response in both the normal and cancer stem cell compartments while HIF2 α induction is restricted to cancer stem cells.

To examine the expression pattern of HIF1 α and HIF2 α *in vivo*, we performed immunohistochemistry (IHC) on paraffin embedded primary human glioblastoma surgical biopsy specimens (Figures 4A, S8, S9; Table S5). In the human tumor sections, HIF1 α antibody marked the majority of tumor cells (~60%) arranged about the regions of necrosis in most

samples. In contrast, HIF2 α demonstrated more variable and rare staining, predominately located immediately around regions of necrosis, where it was expressed by 1 to 10% of cells. HIF2 α expression was also frequently observed around proliferating blood vessels where 1% to 10% of tumor cells were stained. Of note, previous studies suggest that the perivascular region is enriched for brain tumor stem cells (Bao et al, 2006b; Calabrese et al., 2007; Christensen et al, 2008). Consistent with these results, we found that CD133 and another potential brain tumor stem cell marker, Olig2 (Ligon et al, 2007), were expressed by 1-10% of tumor cells adjacent to blood vessels (Figures 4A, S8A). We therefore performed immunofluorescence studies on frozen primary human tumor samples to determine if HIF2 α and CD133 co-localized *in vivo*. Indeed, we found that most tumor cells that expressed HIF2 α co-expressed CD133, although not all CD133 positive cells expressed HIF2 α (Figures 4B, S10). FACS analysis of glioma stem and nonstem populations confirmed the co-expression of CD133 and HIF2 α (Figures 4C/D). Together, our data suggest HIF2 α is a molecular immunophenotype specific for glioblastoma tumor stem cells and not a general stem cell phenotype.

HIFs Are Required for Glioma Stem Cell Growth and Survival

As HIF2 α and HIF1 α regulation differs between glioblastoma stem and nonstem cells, we examined the requirement for HIFs in the tumor subpopulations and cancer stem cell biology using a lentiviral shRNA based system. We achieved knockdown efficiency of ~70-95% for both HIF1 α and HIF2 α at the mRNA level (Figure S11A/B), although the efficiency of HIF1 α knockdown was consistently greater than that of HIF2 α at the protein level (Figures 5A, 6B, 7B, S15A). As in previous reports (Keith and Simon, 2007; Holmquist-Mengelbier et al., 2006), HIF2 α knockdown was selectively associated with reduced mRNA levels of Glut1 and SerpinB9 while targeting HIF1 α significantly decreased PGK1 mRNA (Figure S11). These data demonstrate the ability to specifically target HIF2 α and HIF1 α with resulting distinct molecular effects.

To determine the biological consequences of HIF knockdown, we first assessed neurosphere formation in GSCs (Figure 5) as we did not observe neurosphere formation in the nonstem cells (data not shown) similar to our prior report (Bao et al, 2006a). HIF knockdown impaired neurosphere formation not only in primary assays (Figures 5B-E, S12) but also in secondary and tertiary passages (Figures 5F-H), indicating the HIFs are required for proliferation of GSCs *in vitro*. Consistent with this notion, levels of the HIFs appear to also be coordinately regulated through the differentiation status. Growth factor withdrawal induces differentiation and is associated with a decrease in HIF2 α protein levels (Figure S13).

Even though a minority of GSCs with HIF1 α or HIF2 α knockdown retained neurosphere formation potential, the size of the resultant neurospheres was significantly reduced (Figure 5), suggesting the HIFs are required for GSC proliferation or survival. We therefore determined the growth of GSCs and nonstem cells under normoxia or hypoxia when HIF1 α or HIF2 α expression was targeted by lentiviral transduced shRNA (Figures 6A/B). The requirement for HIF2 α in cell growth was restricted to GSCs as no effect of HIF2 α shRNA was observed in matched nonstem cancer cells (Figure 6A/B). In contrast, HIF1 α knockdown resulted in reduced cell growth in both glioblastoma stem and nonstem cells (Figure 6A/B).

Consistent with the cell growth data, we found that targeting HIFs with lentiviral shRNA resulted in decreased cell survival. Loss of HIF2 α in GSCs consistently resulted in an induction of apoptosis determined with Annexin V staining (Figures 6C/D, S14, S15) and caspase activation (Figures 6E, S15C). No requirement for HIF2 α was detected in nonstem glioma cells, even under hypoxia (Figures 6C/D, S14). In contrast, HIF1 α contributed to the survival of both glioma stem and nonstem cells, but HIF1 α shRNA was sometimes less acutely toxic than HIF2 α shRNA (Figures 6C-E, S15). The elevation in apoptosis due to loss of HIF

expression in GSCs was consistent with an increase in the percentage of cells in the sub-G₀ and G₁ phases of the cell cycle and a decrease in cycling and G₂ phase cells (Figure S16). Together these data demonstrate requirements for both HIF1 α and HIF2 α in GSC biology with a specific requirement for HIF2 α in the GSC, but not nonstem, tumor subpopulation for growth and survival. Due to differences in the efficiency of the shRNA constructs (HIF1 α knockdown was consistently more efficient than HIF2 α knockdown), it is not possible to absolutely determine the relative importance of the HIFs in GSCs.

HIF2 α Is Required for VEGF Expression in Glioma Stem Cells but Not Nonstem Cells whereas HIF1 α Is Required in Both Tumor Subpopulations

In addition to their role in tumor initiation, our prior data demonstrate cancer stem cells promote tumor maintenance by enhancing angiogenesis via elevated VEGF (Bao et al, 2006b). As *VEGF* is a known HIF target gene (Kaur et al, 2005), we determined whether HIF2 α and HIF1 α are required for glioblastoma stem and nonstem cell VEGF expression. Knockdown of HIF2 α or HIF1 α in GSCs under hypoxia significantly reduced *VEGF* promoter activity (Figure 7A), mRNA level (Figures 7D/E, S11), and intracellular (Figure 7B) and secreted (Figure 7C) VEGF protein levels. In matched nonstem cancer cells, there was no requirement for HIF2 α in VEGF transcription or protein production (Figures 7A/C). These results strongly suggest that HIF1 α is required in both glioblastoma stem and nonstem cells for the induction of VEGF expression by transcriptionally regulating the VEGF promoter, whereas there is a specific requirement for HIF2 α for VEGF production in GSCs.

As VEGF can support brain tumor angiogenesis through regulation of endothelial cell proliferation and survival (Jain et al, 2007; Plate et al., 1992), we examined if knockdown of HIFs in glioblastoma stem and nonstem cells could significantly impact endothelial cell growth (Figures 7F-H). We performed a coculture experiment, in which glioblastoma cells were cultured in an upper chamber while human microvascular endothelial cells (HMVEC) were planted in the lower wells (Figure 7F). These two chambers were separated by a permeable membrane with 0.4 μ m pores, which prevented physical contact between glioblastoma cells and endothelial cells but allowed transfer of secreted factors. Consistent with our previous report (Bao et al, 2006b), GSCs significantly increased endothelial cell numbers and proliferation in comparison to nonstem cancer cells, as determined by direct cell number counting (Figure 7G and data not shown) and [³H]-thymidine incorporation assay on HMVEC (Figure 7H). Knockdown of either HIF2 α or HIF1 α reduced the paracrine effects of GSCs on endothelial cells, but endothelial cell growth supported by nonstem glioblastoma cells was only affected by targeting HIF1 α (Figure 7H). These data are consistent with the observed differences in HIF requirements for VEGF expression in the tumor subpopulations, and suggest a specific role for HIF2 α in GSC-mediated angiogenesis by affecting endothelial cell growth.

Targeting HIFs in Glioma Stem Cells Decreases Tumorigenic Capacity and Increases the Survival of Mice Bearing Intracranial Xenografts

Considering the *in vitro* requirements for HIF2 α and HIF1 α in GSC proliferation, survival, and VEGF production, we determined the impact of HIF knockdown on GSC tumorigenic capability *in vivo* (Figures 8A-G). When GSCs transduced with nontargeting control shRNA or shRNA targeting HIF2 α or HIF1 α were intracranially implanted into immunocompromised mice, we observed a significant decrease in tumor formation and an increase in the survival of tumor bearing mice when HIF1 α or HIF2 α were targeted (Figures 8A-G). We further found that targeting HIFs can reduce the tumorigenic potential of GSCs in an *in vivo* limiting dilution assay (Figure 8G). As knockdown of HIF2 α increased the survival of tumor bearing mice as well as or significantly more than HIF1 α (Figures 8A-G), but was usually targeted less efficiently at the protein level (Figure 5A, 6B, 7B, S16A), our data may underestimate the importance of HIF2 α for the *in vivo* propagation of GSCs. In fact, tumors arising from

unselected HIF2 α knockdown cells (Figures 8B-E) expressed HIF2 α indicating that these tumors likely originated from unsuccessfully targeted cells (Figure S17). When GSCs underwent puromycin marker selection to confirm successful infection, HIF knockdown cells failed to form any tumors even after six months (Figure 8F).

As brain tumor stem cells usually only account for a small percentage of bulk tumor cells in our studies, we inquired as to whether targeting only the GSC population could impact bulk tumor growth. We therefore employed an *in vivo* mixing experiment in which we prospectively segregated cancer stem cell enriched and depleted tumor populations, genetically manipulated the stem cell population, and xenotransplanted a mixture of stem and nonstem cancer cell populations at a 1:20 ratio (i.e. 5% of the total cancer cells were cancer stem cells similar to the fraction in human glioblastoma specimens) (Figure 8B). As expected, tumor cell mixtures with GSCs transduced with nontargeting shRNA control rapidly formed tumors with a histopathology consistent with a glioblastoma when implanted intracranially into immunocompromised mice. In contrast, tumor cell mixtures that included GSCs transduced with either HIF1 α or HIF2 α shRNA display impaired tumor formation potential indicating that targeting HIFs only in CSCs could have therapeutic benefit (Figure 8B and data not shown). Targeting HIFs likely impairs tumor growth through several mechanisms as many genes are regulated by HIFs as demonstrated above, including regulators of survival. One downstream HIF target that may be important *in vivo* is VEGF. We found that targeting VEGF exclusively in the GSC population in our cell mixing experiments can increase the survival of tumor bearing mice and decrease tumor angiogenesis, suggesting that reducing VEGF production and thus angiogenesis could be one of the potential mechanisms by which targeting HIFs in GSCs decreased tumorigenesis *in vivo* (Figure 8B). Together, our data demonstrate that HIFs are required to maintain the tumorigenic potential of GSCs and that targeting HIF2 α may be a cancer stem cell directed therapy.

Elevated HIF2 α Expression Is Associated with Poor Survival of Glioma Patients

To investigate whether targeting HIF2 α may have a therapeutic benefit for the glioma patient population, we utilized the REMBRANDT (Repository of Molecular Brain Neoplasia Data) database of the National Cancer Institute (<http://caintegrator-info.nci.nih.gov/rembrandt>). We analyzed the data to determine the survival of glioma patients with intermediate, low, or high expression of HIF2A or HIF1A (Figure 8H). We found a significant decrease in the probability of survival with elevated HIF2A expression, with no significant difference in survival with elevated HIF1A expression. As this gene expression database measures mRNA levels, and HIF2A but not HIF1A is regulated by hypoxia at the transcriptional level, the survival information contained in HIF2A levels may be both a surrogate for the presence of hypoxia as well as quantification of GSCs. These data demonstrate HIFs differentially affect patient outcome and strongly support a specific and important role for HIF2 α in gliomas.

Discussion

Glioblastomas are among the most lethal of cancers with current therapies providing only palliation. While the successful cancer cures require eliminating all tumor cells, cancer stem cells may represent particular therapeutic challenges. The direct characterization of cancer stem cells may yield therapeutic targets that are not evident by whole tumor analyses. For example, we recently demonstrated that LICAM, a cell adhesion molecule, was preferentially expressed in brain tumor stem cells and was essential to tumor initiation (Bao et al, 2008). Paramount in the development of cancer stem cell targeting agents must be the recognition that previously unrecognized toxicities may occur if a molecular pathway is shared with normal stem cells. We have therefore sought to identify molecular contributors involved in cancer stem cells without significant expression in the organ specific progenitor compartment, specifically

neural progenitors. Based on these criteria, HIF2 α appears to be an attractive target as it is specifically expressed by brain tumor stem cells but not neural progenitor cells, whereas HIF1 α is shared by these cellular populations. Indeed, HIF1 α is essential in neural development (Tomita et al, 2003) whereas animals with the targeted disruption of HIF2 display defects in other organ systems (Compernelle et al, 2002).

Hypoxia is a well recognized tumor microenvironmental condition that is linked to poor patient outcome and resistance to therapies (Teicher, 1994; Liang, 1996; Semenza, 2004; Chi et al., 2006; Vaupel and Mayer, 2007; Sathornsumetee et al., 2008). Cellular responses to hypoxia are frequently regulated by the HIFs leading to the attempted development of anti-HIF therapies, with limited success to date. Because of our prior work that identified cancer stem cells as a contributor to tumor angiogenesis, we interrogated the HIFs and other hypoxia target genes in brain tumor stem cells. As we expected, all cancer cells responded to acute hypoxia through the increase of HIF1 α protein (Figures 3A-H, 3J). Although these conditions have been widely used in hypoxia studies, some reports suggest that the level of oxygenation may fluctuate and more modest restrictions in oxygen availability may more closely represent actual intratumoral conditions (Inoue and Ohnuma, 1989; Kimura et al., 1996; Cardenas-Navia et al, 2004). A recent report suggested that unlike HIF1 α , which is only stabilized under acute hypoxic conditions, HIF2 α may accumulate under modest hypoxia or even normal physiological oxygen levels (Holmquist-Mengelbier et al., 2006). Indeed, we found under 2-5% oxygen levels that HIF2 α is the dominant hypoxia-inducible factor present in the cancer stem cell population (Figures 3I-L) and that HIF2 α is expressed at wide range of oxygen levels. This indicates that HIF2 α may provide cancer stem cells a growth advantage by activating downstream genes even without hypoxia stimulation *in vitro* and *in vivo*. Our immunohistochemical analysis of glioblastoma surgical specimens revealed that a significant fraction of HIF2 α positive cells are located adjacent to blood vessels (Figures 4, S3). Therefore, it will be of great interest to determine whether HIF2 α functions differentially under various oxygen tensions during tumorigenesis *in vivo*. It is also notable that the role of HIF2 α was likely to be underestimated in previous cancer studies with cell lines or bulk tumor populations as cancer stem cells frequently account for only a restricted fraction of the overall tumor (less than 10% of tumor cells).

Prospective identification of cancer stem cells has been challenging, and the relationship of cancer stem cells to normal stem cells is controversial. In fact, the terminology used to describe the stem cell-like tumor population remains unresolved. Some researchers advocate a description based on the functional assays used to define these cells (i.e. tumor propagation), but others highlight the phenotypic similarities to normal stem cells. We have defined GSCs functionally as current methods for cancer stem cell enrichment from solid cancers remain imperfect. However, we utilized the term cancer stem cell as we note their self renewal and differentiation potentials., Cultures enriched for cancer stem cells with currently known cancer stem cell markers remain heterogeneous, as not every isolated cell is capable of self renewal or tumor propagation. These data suggest that additional cell surface markers or intracellular molecules contribute to the cancer stem cell phenotype. Our data suggest that HIF2 α identifies a subpopulation of CD133 positive cells. The vast majority of HIF2 α positive cells express CD133, but HIF2 α and CD133 do not overlap exclusively: not all HIF2 α positive cells are CD133 positive, and not all CD133 positive cells are HIF2 α positive. Targeting HIF2 α did not uniformly kill all CD133 positive cells suggesting a heterogeneous dependence on HIF2 α in this cancer stem cell population. The role of HIF2 α in tumors which are not driven by CD133 expression (Beier et al, 2007; Zheng et al., 2007; Wang et al., 2008) is still unresolved, but we did not observe HIF2 α expression in a rat glioma cell line in which CD133 negative cells were reported to be tumorigenic (Zheng et al, 2007). We also cannot complete the functional studies required to define cancer stem cells with HIF2 α due to its intracellular localization. Our cancer stem cell cultures therefore remain heterogeneous for HIF2 α expression. HIF2 α does appear

to localize with cancer stem cell markers *in vitro* and *in vivo*, suggesting that HIF2 α positive cells are enriched in a cancer stem cell phenotype. Together, our results suggest that HIF2 α may mark a subpopulation of cancer stem cells essential for tumor growth.

The HIFs function through the transcriptional regulation of a number of important gene products. Besides VEGF, the expression of Oct4, Glut1, and SerpinB9 genes was induced by HIF2 α in our studies. Oct4 is a core regulator in stem cell self-renew and differentiation (Pan et al., 2002; Wang et al, 2006) and very recently validated as a cancer stem cell target (Hu et al., 2008). The glucose transporter Glut1 is frequently up-regulated in cancer cells to facilitate their accelerated metabolism (Macheda et al, 2005; Younes et al, 1995). The proteinase inhibitor SerpinB9 may prevent cytotoxic T cell mediated apoptosis of target cells (Trapani and Sutton, 2003) and can directly inhibit caspases (Young et al, 2000). Indeed, SerpinB9 is up-regulated in some melanoma and leukemia patients and its upregulation predicts poor outcome in high grade melanoma patients (van Houdt et al., 2005). These reports suggest that HIF2 α mediated upregulation of Oct4, Glut1, and SerpinB9 may provide cancer stem cells with advantages in metabolism, proliferation, survival, and escape from immune surveillance.

Normal stem cells reside within highly defined anatomical niches that provide important cues to maintain stem cells in undifferentiated states or promote the acquisition of a more differentiated state. Recent studies suggest that cancer stem cells may also be harbored in specific niches (Gilbertson and Rich, 2007), but many aspects of the cancer stem cell niche are unknown. Our analysis of surgical glioblastoma biopsy specimens suggests that there may be at least two areas enriched for cancer stem cells. We observed GSCs around blood vessels, consistent with prior reports of a perivascular niche for normal stem cells (Tan et al., 2008; Yoshida et al., 2007) and GSCs (Calabrese et al, 2007). However, we also observed GSCs around regions of necrosis, which are hypoxic, suggesting that there may be more than one GSC niche. These results may parallel the hematopoietic stem cell location in the bone marrow, in which these cells are located around the endosteum and vascular sinusoids (Kiel and Morrison, 2008). The regulation of the bone marrow niche is an area of active investigation but it is notable that the bone marrow is maintained at a relatively low oxygen tension relative to the systemic circulation (Parmar et al., 2007). Hypoxia regulates many aspects of tumor biology, contributing to tumor cell proliferation, resistance to anti-neoplastic agents, angiogenic drive, and metastasis/invasion (Pouyssegur et al, 2006). These pro-tumorigenic effects of hypoxia may be due, at least in part, to the promotion of a stem cell-like phenotype in cancer cells in a solid tumor. Hypoxia creates cellular stresses that negatively regulate cell proliferation and survival, but hypoxia is also able to promote normal stem cell maintenance and block differentiation (Ezashi et al., 2005; Keith and Simon, 2007). Together, these data indicate hypoxia may be a functional component of a cancer stem cell niche (Gilbertson and Rich, 2007; Keith and Simon, 2007). Difficultly in reconciling the localization of cancer stem cells to both hypoxic regions and areas around tumor vasculature is resolved with the understanding that angiogenic vasculature is poorly functional and often associated with regions of hypoxia (Jain et al., 2007). In addition, HIF2 α is expressed by the cancer stem cells at oxygen concentrations that approximate normal *in vivo* oxygen levels (2-5% in Figure 3). Thus, cancer stem cells may support the development and maintenance of their own niche by producing angiogenic factors to support blood vessel formation and tumor growth while still being maintained by hypoxia in adjacent regions. However, it remains possible that there are distinct subpopulations of cancer stem cells which are exclusively associated with hypoxic or perivascular regions and may be defined by further elucidation of cancer stem cell markers and molecular profiles.

The dependence of cancer stem cells on a hypoxic and perivascular niche offers potential therapeutic strategies based on vascular targeting. As anti-angiogenic therapies continue to be developed for many cancers, including glioblastomas, efficacy can be improved by increasing

our understanding of the molecular mechanisms by which these agents function. We previously demonstrated that the VEGF neutralizing antibody bevacizumab (Avastin) specifically inhibits the pro-angiogenic effects of GSCs (Bao et al., 2006b), suggesting that anti-VEGF therapies may disrupt the stem cell niche (Calabrese et al., 2007; Gilbertson and Rich, 2007). We now demonstrate that the HIFs, key regulators of VEGF expression and angiogenic drive, promote stem cell maintenance and VEGF expression. Consistent with HIF1 α 's recognition as a molecular cancer target, we determined that HIF1 α is required for the proliferation, survival, and angiogenesis of both the cancer stem cells and nonstem cancer cells. However, we have defined a unique requirement for HIF2 α in the cancer stem cell subpopulation. Notably, *HIF2A* mRNA is significantly transcriptionally up-regulated under normoxia and hypoxia in GSCs in comparison to nonstem cancer cells, whereas HIF1 α protein is usually higher under hypoxia in nonstem cancer cells. We found that targeting HIF2 α in GSCs is as effective as or more effective in vivo than targeting HIF1 α , suggesting that targeting HIF1 α without recognizing the contribution of HIF2 α to hypoxia responses overlooks an important potential compensatory mechanism. It is important to note that comparing the efficacy of targeting HIF1 α and HIF2 α cannot be directly compared in our studies as the efficiency of knockdown was significantly different (HIF1 α was more efficiently targeted). HIF2 α may have additional advantages as a target because the lack of expression in neural progenitor cells as well as its documented role in activating the myc pathway (another stem cell pathway) in contrast to HIF1 α (Gordan et al., 2007). Future studies will be directed towards defining the downstream molecular mechanisms beyond caspase activation and VEGF expression by which the HIFs regulate cancer stem cell survival and tumor growth. Additional studies will be devoted towards defining the upstream mechanisms that regulate HIFs in cancer stem cells.

Our results have direct clinical relevance as we have recently determined that hypoxic markers, including HIF2 α , provide useful biomarkers for predicting patient survival from treatment initiation in a trial of the anti-VEGF antibody bevacizumab in combination with irinotecan (Sathornsumetee et al., 2008). Using this malignant glioma patient cohort, we now find that the expression of HIF2 α in tumor specimens collected at diagnosis can predict patient survival from the time of diagnosis. This conclusion is supported by another independent glioblastoma database from National Cancer Institute, which also suggests that patients with HIF2 α upregulation have significantly shorter survival in comparison to those with lower HIF2 α expression (Figure S6C). Thus, our data supports the development of HIF2 α directed therapies and demonstrates differential molecular responses to hypoxia in the cancer stem cell subpopulation.

Experimental Procedures

Isolation of Glioma Stem Cells, Nonstem Glioma Cells, and Normal Neural Progenitors

Matched cultures enriched or depleted for GSCs were isolated from primary human brain tumor patient specimens or human glioblastoma xenografts as previously described in accordance with a Duke University Institutional Review Board approved protocol concurrent with national regulatory standards with patients signing informed consent (Bao et al, 2006a; Bao et al., 2006b). Briefly, tumors were disaggregated by Papain Dissociation System and filtered by 70 μ m cell strainer according to the manufacturer's instructions. Cells were then cultured in stem cell culture medium supplemented as detailed below for at least four hr to recover surface antigens. Cells were then labelled with APC- or PE-conjugated CD133 antibody, and sorted by fluorescence-activated cell sorting (FACS). Alternatively, cells were separated by magnetic sorting column using microbead-conjugated CD133 antibodies. CD133 positive cells were designated as GSCs whereas CD133 negative cells utilized as nonstem glioma cells. Normal human neural progenitors were obtained from Lonza and the use of these materials is

considered exempt as human subjects by the Duke Institutional Review Board (See Supplemental Experimental Procedures for more details).

Tissue Culture and Hypoxia Induction

GSCs were cultured in Neurobasal media with B27 without Vitamin A (Invitrogen), bFGF (10 ng/ml) and EGF (10 ng/ml). After trypsinizing, nonstem tumor cells were cultured overnight in 10% serum DMEM to allow cell attachment and survival. Then, DMEM medium was removed and the cells cultured in supplemental Neurobasal medium in order for experiments to be performed in identical medias. In order to induce hypoxia, cells were cultured in hypoxia chambers (Sheldon Manufacturing for 0.2% O₂, Sanyo for 1%, 2% and 5%). Alternatively, cells were treated by 100 or 200 μ M hypoxia-mimic chemical deferrioxamine mesylate (DFX, Sigma).

Lentiviral Mediated shRNA Targeting

Lentiviral shRNA clones (Mission RNAi) targeting HIF1 α , HIF2 α VEGF and nontargeting control sequences were obtained from Sigma. Lentiviruses were produced in 293FT cells with packing mix (ViraPower Lentiviral Expression Systems, Invitrogen) according to the manufacturer's instruction. Efficiency of different lentiviral shRNA clones in cells was determined by Western blot analysis and Real Time PCR.

In Vivo Tumor Formation Assays

Intracranial or subcutaneous transplantation of GSCs into nude mice was performed as described in accordance with a Duke University Institutional Animal Care and Use Committee approved protocol concurrent with national regulatory standards (Bao et al., 2006a). Briefly, 72 hr after lentiviral infection, cells were counted and certain number cells were implanted into the right frontal lobes of athymic BALB/c nu/nu mice. In some cases, 48 hr after infection, 1 μ g/ml puromycin was applied to select infected cells for 48 hr before counting. Mice were maintained up to 25 weeks or until the development of neurological symptoms. Brains of euthanized mice were collected, fixed in 4% Paraformaldehyde (PFA), and paraffin embedded.

Statistical Analysis

Descriptive statistics were generated for all quantitative data with presentation of means \pm standard error. Significance was tested by one-way ANOVA using the SAS Enterprise Guide 3.0 (Cary, NC) or GraphPad InStat 3.0 software (San Diego, CA). For in vivo studies, Kaplan Meier curves and log-rank analysis were performed using MedCalc software (Belgium).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support was provided by the Childhood Brain Tumor Foundation, the Pediatric Brain Tumor Foundation of the United States, Accelerate Brain Cancer Cure, Alexander and Margaret Stewart Trust, Brain Tumor Society, Goldhirsh Foundation, Duke Comprehensive Cancer Center Stem Cell Initiative Grant (J.R.), NIH grants NS047409, NS054276, and CA116659 (J.R.). J.R. is a Damon Runyon-Lilly Clinical Investigator supported by the Damon Runyon Cancer Research Foundation and a Sidney Kimmel Foundation for Cancer Research Scholar. We thank Z. Su, Y. H. Sun, S. Keir, D. Satterfield, L. Ehinger and J. Faison for technical assistance; M. Cook and B. Harvat for assistance with flow cytometry. We are also grateful to R. Wechsler-Reya for helpful discussions.

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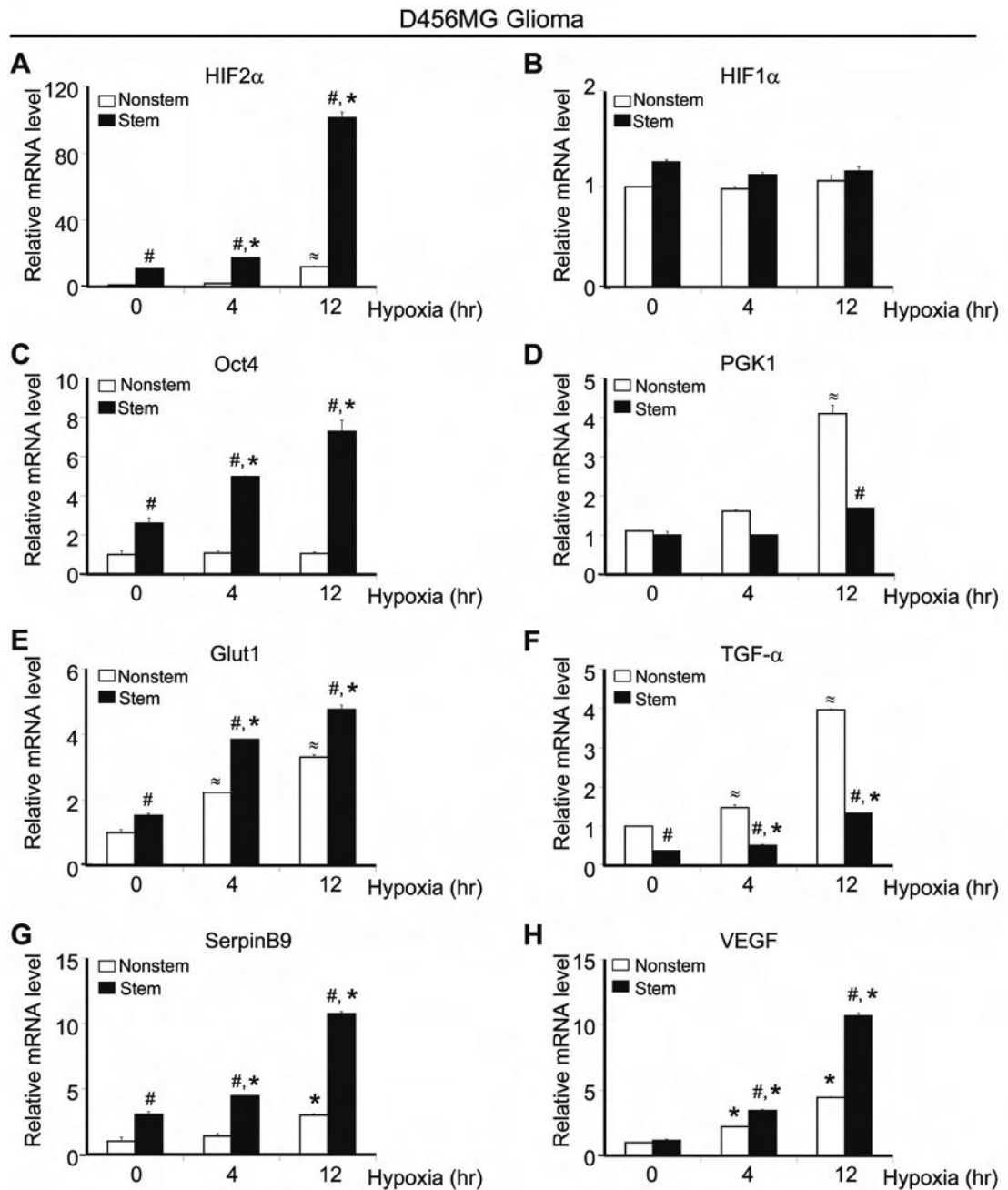


Figure 1. Glioblastoma Stem and Nonstem Cells Differentially Expressed Hypoxia Response Genes
 Glioblastoma stem and nonstem isolated from the glioblastoma xenograft D456MG were treated with desferrioxamine (DFX) to mimic hypoxia for the time indicated. RT-PCR was performed with primers specific for HIF2 α (A), HIF1 α (B), Oct4 (C), phosphoglycerate kinase 1, PGK1 (D), glucose transporter type 1, Glut1 (E), transforming growth factor alpha, TGF- α (F), SerpinB9 (G), and vascular endothelial growth factor, VEGF (H). Data were normalized to GAPDH, Ubiquitin C, and SDHA. #, $P < 0.01$ with ANOVA comparison of stem cells to nonstem cells with identical treatments. *, $P < 0.01$ with ANOVA comparison of stem cells under hypoxia vs. normoxia. ≈, $P < 0.01$ with ANOVA comparison of on-stem cells under hypoxia vs. normoxia.

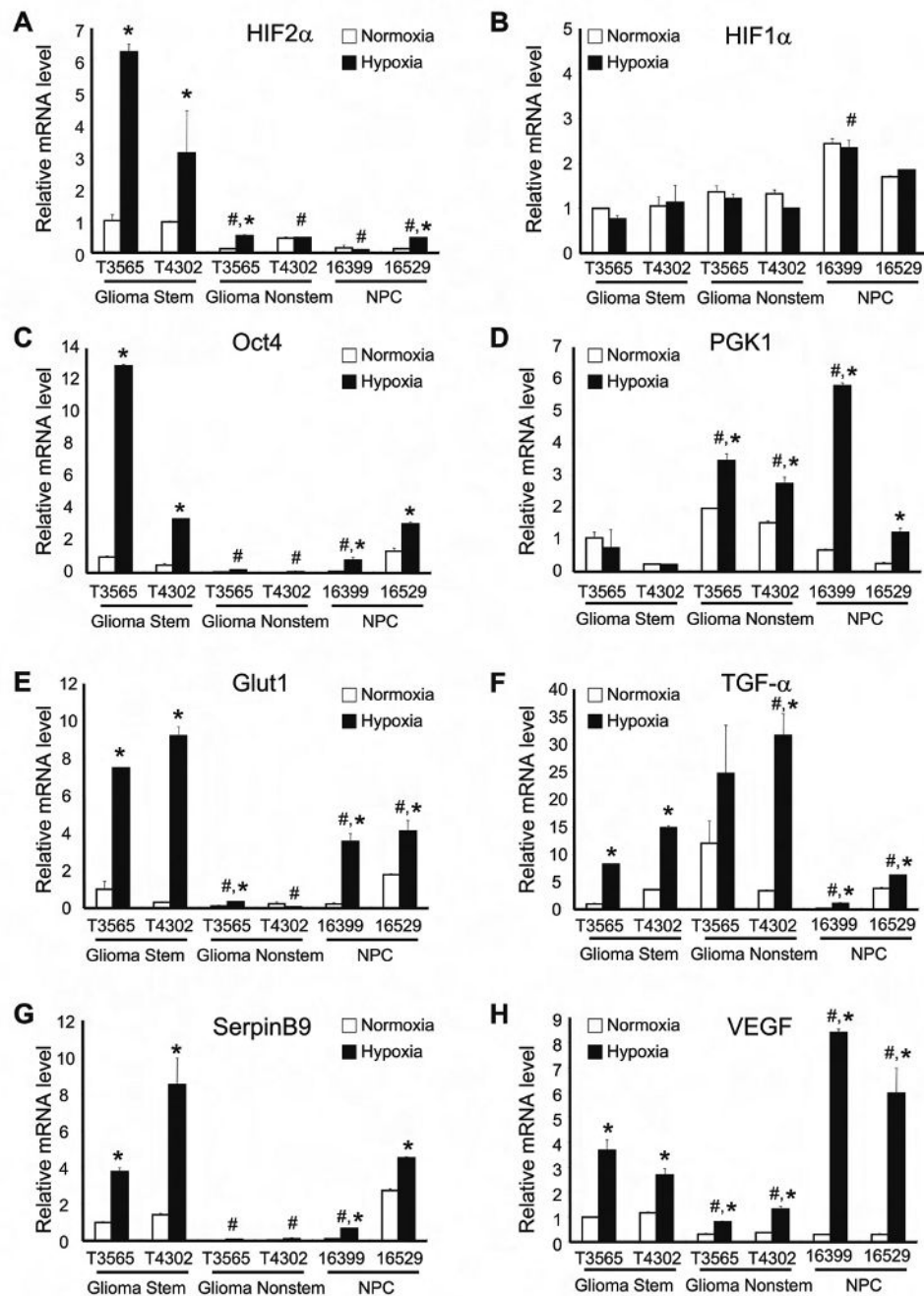


Figure 2. Glioma Stem Cells and Normal Neural Progenitors Differentially Expressed Hypoxia Response Genes

Cells from T3565 and T4302 glioblastoma samples and two different normal neural progenitor cell preparations were cultured in normoxia (20% oxygen) or hypoxia (1% oxygen) for 24 hr. RT-PCR analysis was performed with primers specific for HIF2 α (A), HIF1 α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGF- α (F), SerpinB9 (G), and VEGF (H). Data were normalized to β -actin levels. *, $P < 0.01$ with ANOVA comparison of hypoxia treated cells to identically prepared normoxia controls. #, $P < 0.01$ with ANOVA comparison of indicated hypoxia treated cells to both hypoxia treated GSCs.

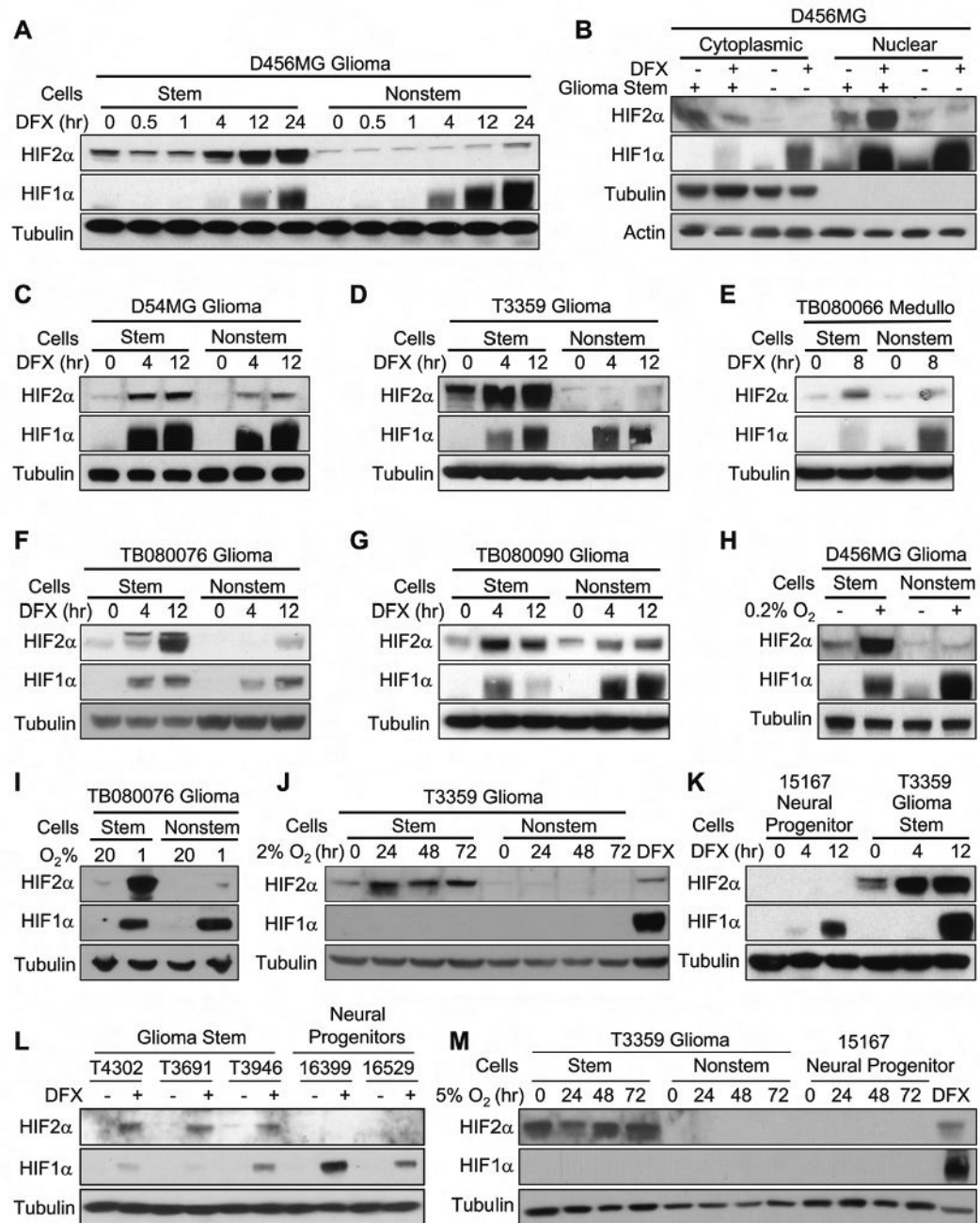


Figure 3. Hypoxia Potently Induced HIF2α Protein Expression in Glioma Stem Cells

(A-G) Glioblastoma stem and nonstem cells isolated from multiple samples were treated with DFX to mimic hypoxia for the indicated times. Nuclear and cytoplasmic fractions (B) or total cell lysate (A, C, E-G) were analyzed. (H) Cells isolated from D456MG were cultured under 20% or 0.2% oxygen for 24 hr and analyzed by immunoblotting. (I) Cells isolated from TB080076 were cultured under normoxia (20% oxygen) or hypoxia (1% oxygen) for 24 hr and analyzed by immunoblotting. (J) Cells isolated from T3359 were cultured under more modest hypoxia (2% oxygen) and total cell lysates were analyzed. (K) T3359 GSCs and CD133+ normal neural progenitors were treated with 100 μM DFX and total cell lysates were analyzed. (L) GSCs (T4302, T3691, and T3946) and the normal human neural progenitors (16399 and

16529) were treated with DFX and analyzed by immunoblotting. (M) Higher HIF2 α protein expression is maintained with a relatively physiological level of oxygen in GSCs. Cells were cultured under physiological level of oxygen (5% oxygen) for the indicated times and total cell lysates collected. In (J) and (M), and DFX treated samples were used as a positive control.

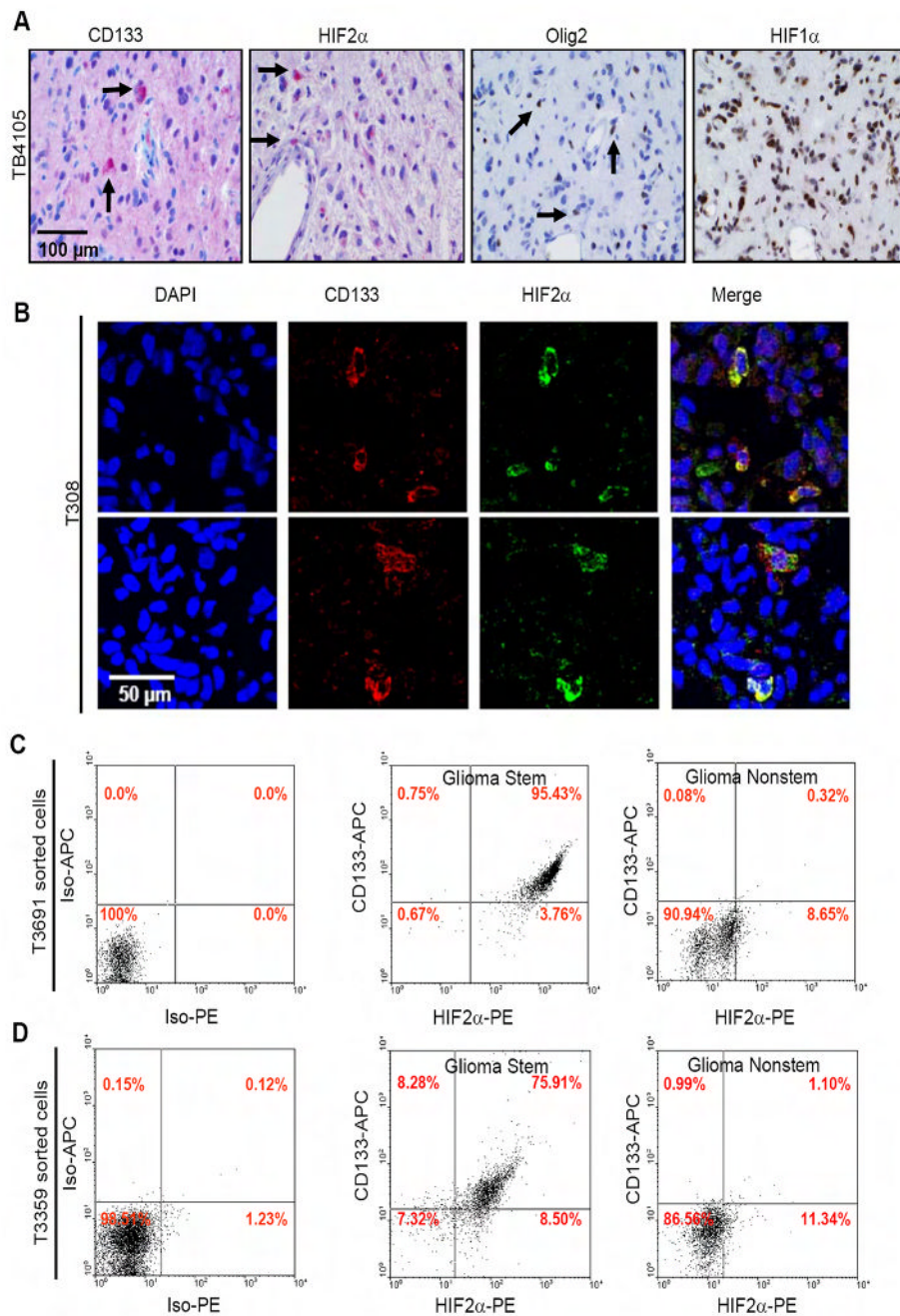


Figure 4. HIF2 α Co-Expressed with Cancer Stem Cell Markers in Human Glioblastoma Biopsy Specimens

(A) Restricted pattern of HIF2 α and stem cell marker expression in human brain tumor patient specimens. (B) Immunofluorescence of cells in human brain tumor patient specimens demonstrates co-localization of CD133 and HIF2 α . (C-D) Cells expressing the cancer stem cell marker CD133 also express HIF2 α . Glioma stem and nonstem cells isolated from T3691 (C) or T3359 (D) were analyzed for CD133 and HIF2 α expression via FACS using anti-CD133-APC and anti-HIF2 α -PE.

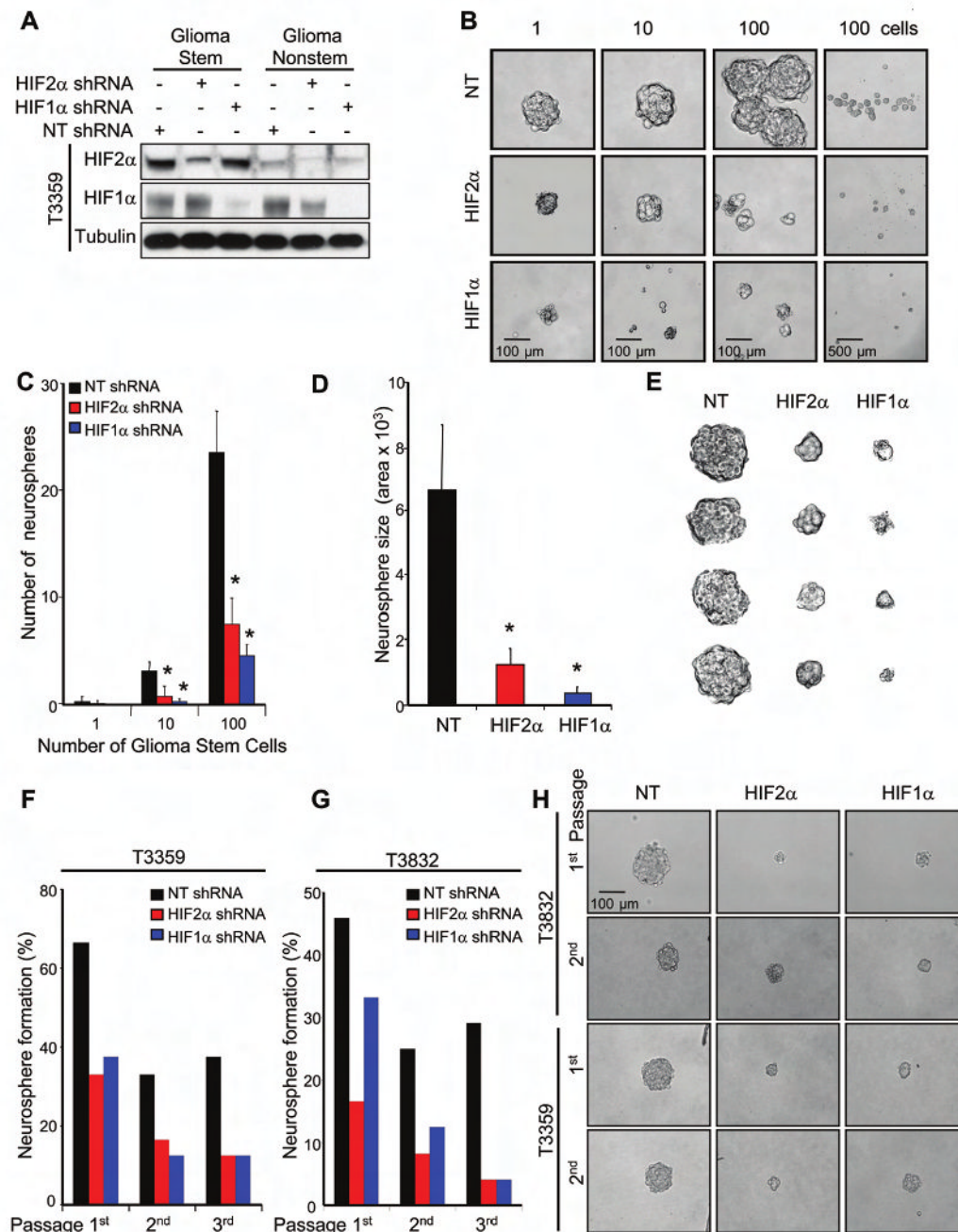


Figure 5. HIF Knockdown Altered Glioma Stem Cell Neurosphere Formation

(A) Specific knockdown of HIF1 α and HIF2 α protein using shRNA. (B-E) 1, 10, or 100 lentiviral infected GSCs isolated from T3359 were cultured in 24-well plates. (B) Representative images of spheres are shown. (C) The total number of neurospheres per well is significantly decreased with HIF targeting. (D) Neurosphere size is significantly reduced by targeting HIF expression. (E) Representative image of neurospheres of neurosphere size in D. (F-G) Targeting HIFs in T3359 or T3832 stem cells decreases neurosphere formation in sequential passages. (H) Representative images of neurospheres formed in sequential passage neurosphere formation assays. *, $P < 0.001$

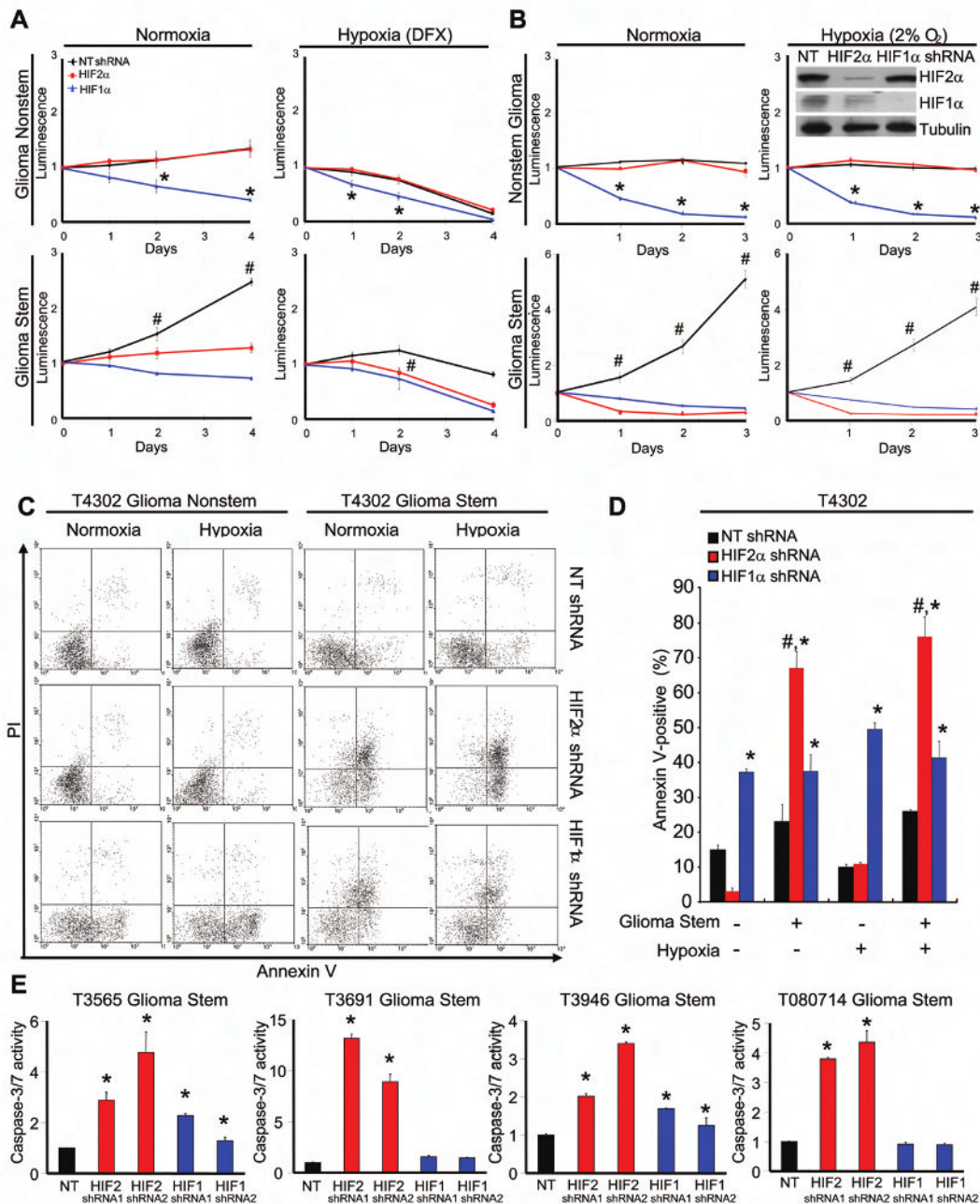


Figure 6. HIF Knockdown Reduced Glioma Stem Cell Growth Due to Elevated Apoptosis
 (A) Targeting HIF2 α in glioblastoma stem, but not nonstem, cells decreases growth. Cell titers were determined using the CellTiter-Glo Luminescent Cell Viability Assay kit. *, $P < 0.05$ with ANOVA comparison of HIF1 α shRNA to either NT shRNA or HIF2 α shRNA. #, $P < 0.01$ with ANOVA comparison of NT shRNA to either HIF1 α or HIF2 α shRNA. (B) Cells were plated and quantified as in A but cultured in 20% or 2% oxygen as indicated. Inset shows effective knockdown with HIF2 α and HIF1 α shRNAs. (C-D) Targeting HIFs leads to increased apoptosis in GSCs as determined by the Annexin V staining. *, $P < 0.05$ with ANOVA comparison to nontargeting shRNA of the same cell type and hypoxia treatment. #, $P < 0.01$ with ANOVA comparison of HIF2 α shRNA treated GSCs to HIF2 α shRNA treated nonstem

cells with identical oxygen treatment. (E) Targeting HIFs results in increased caspase-3/7 activity in GSCs. *, $P < 0.05$

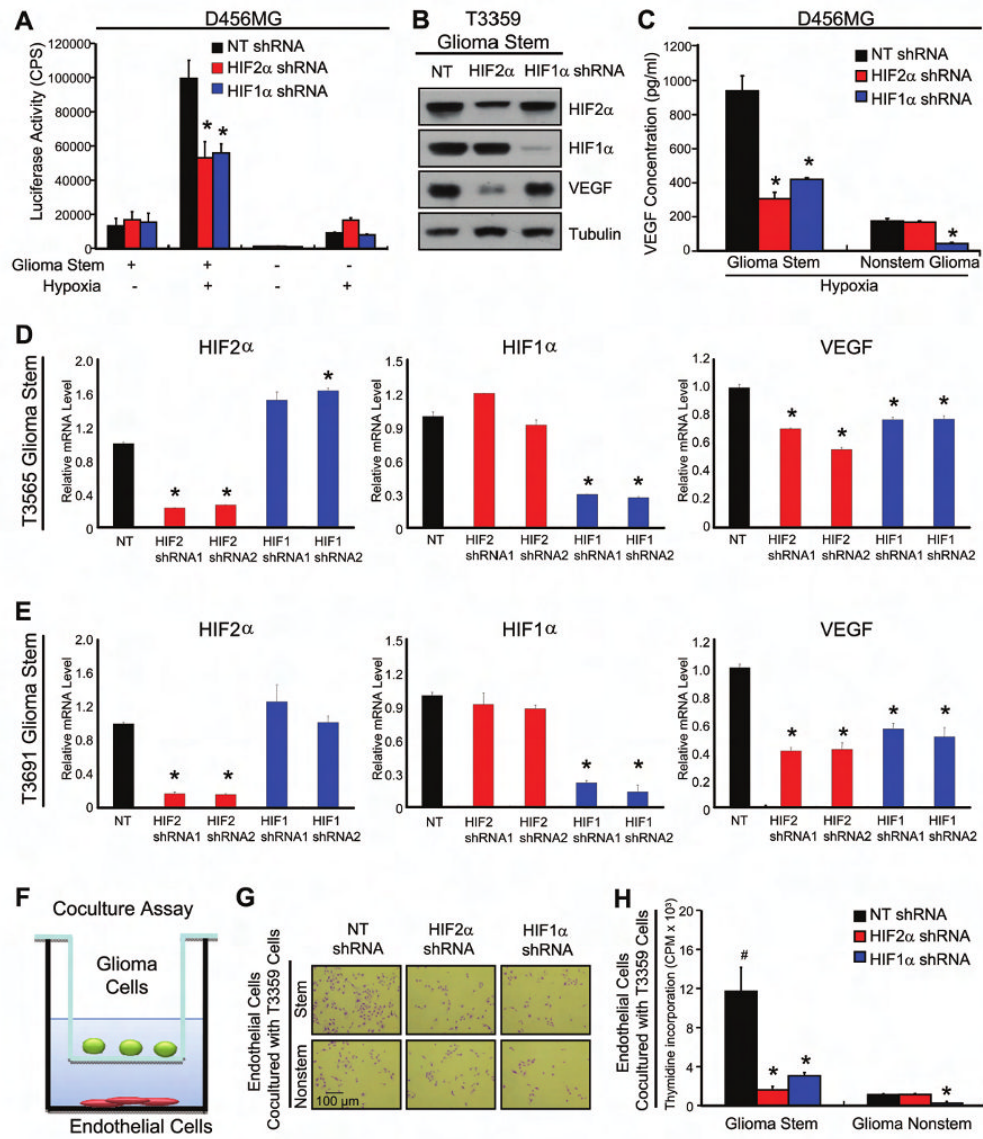


Figure 7. HIF Knockdown Decreased Glioma Stem Cell Mediated Angiogenesis

(A) HIF2 α knockdown prevents hypoxia induced activation of the VEGF Promoter. *, $P < 0.001$ with ANOVA comparison of HIF2 α or HIF1 α shRNA treated stem cells to nontargeting shRNA with hypoxia. (B) HIF knockdown reduces VEGF protein expression in GSCs. (C) HIF2 α knockdown reduces VEGF expression in stem, but not nonstem, glioblastoma cells. *, $P < 0.01$ (D-E) HIF knockdown reduces VEGF mRNA level in GSCs. *, $P < 0.01$ (F-H) HIF2 α knockdown in GSCs reduced cancer cell-mediated endothelial cell proliferation. (F) Representative diagram of the coculture assay. (G) Representative images of cocultured HIVEC cells after cells fixed with 4% PFA and then stained with toluidine blue. (H) HIVEC proliferation was measured through [3H]-thymidine incorporation. *, $P < 0.001$ by ANOVA with comparison of HIF2 α or HIF1 α shRNA infected glioma cells to corresponding nontargeting shRNA infected cells. #, $P < 0.001$ by ANOVA comparison of nontargeting shRNA infected GSCs to nonstem nontargeting control cells.

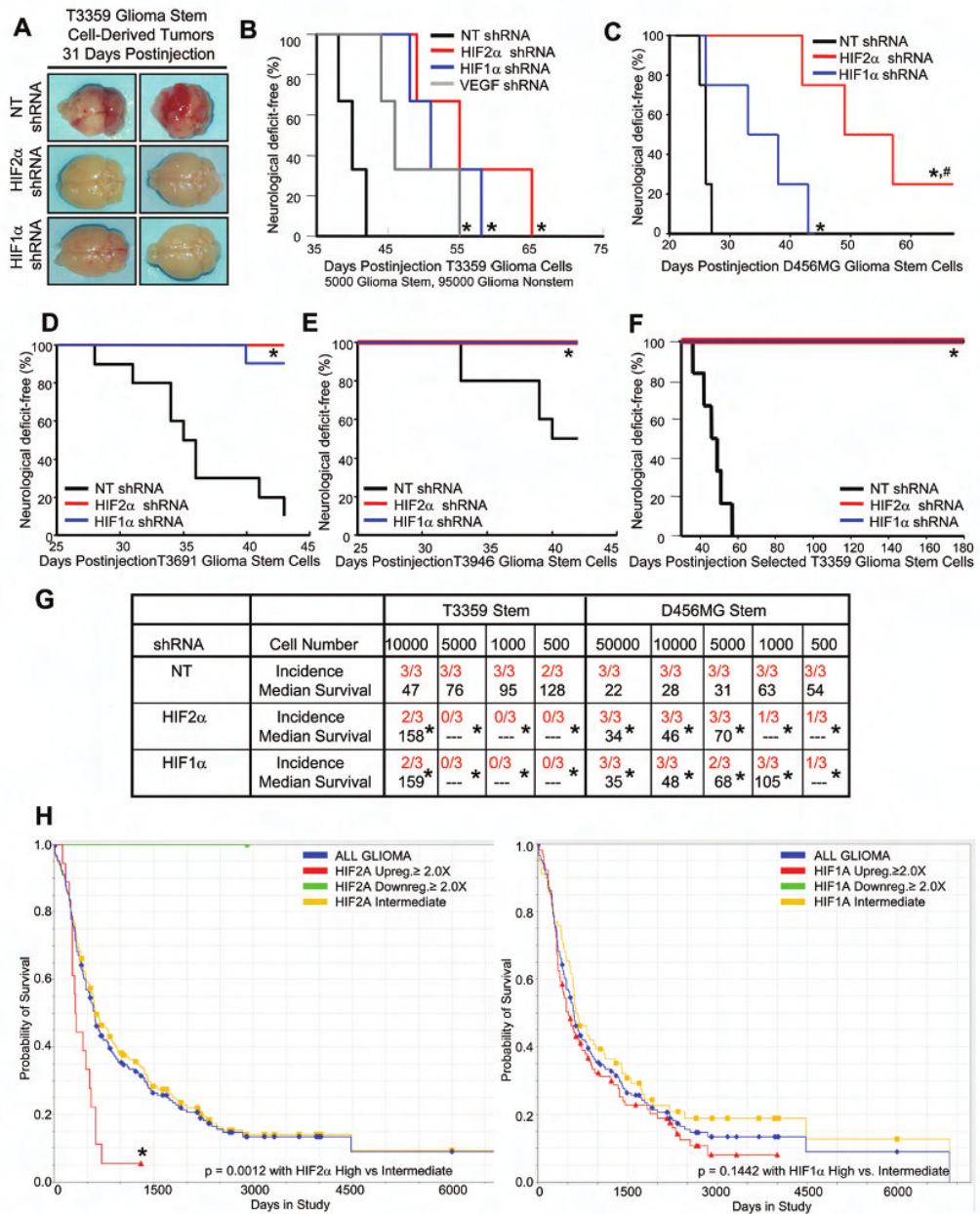


Figure 8. HIF Knockdown Suppressed Cancer Stem Cell Mediated Tumor Growth

(A) Gross histology demonstrates highly vascular tumors in glioblastoma stem derived tumors from cells infected with nontargeting shRNA but not HIF targeting shRNAs. (B) Targeting VEGF or HIFs within the cancer stem cell subpopulation increases survival. 5000 infected GSCs were mixed with 95000 matched uninfected nonstem cells and injected into the mice brains. *, $P < 0.03$. (C-E) Targeting HIFs in the cancer stem cell subpopulation decreases tumorigenesis. *, $P < 0.05$. (F) Tumors do not form from GSCs selected for the incorporation of a puromycin marker associated with HIF targeting shRNA. *, $P < 0.03$. (G) *In vivo* limiting dilution assay demonstrates GSCs are less tumorigenic when HIFs are targeted. *, $P < 0.03$. (H) HIF2α but not HIF1α mRNA level correlates with patient survival (the Rembrandt database of the National Cancer Institute). There was only one patient with more than two fold HIF2A

downregulation. No patients with HIF1A downregulation were observed. Analysis of the NCI TCGA database yields similar results (data not shown).