Differential Angiogenic Capability and Hypoxia Responses in Glioma Stem Cells

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2009
ABSTRACT

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Abstract

Malignant gliomas are highly lethal cancers characterized by florid angiogenesis. Glioma stem cells (GSCs), enriched through CD133 (Prominin1) selection, are highly tumorigenic and therapy resistance. However, the mechanism through which GSCs promote tumor growth was largely unknown. As we noticed that tumors derived from GSCs contain widespread tumor angiogenesis, necrosis, and hemorrhage, we examined the potential of GSCs to support tumor angiogenesis. We measured the expression of a panel of angiogenic factors secreted by GSCs. In comparison with matched non-GSC populations, GSCs consistently secreted markedly elevated levels of vascular endothelial growth factor (VEGF), which were further induced by hypoxia. In an in vitro model of angiogenesis, GSC-conditioned medium significantly increased endothelial cell migration and tube formation compared with non-GSC glioma cell–conditioned medium. The proangiogenic effects of GSCs on endothelial cells were specifically abolished by the anti-VEGF neutralizing antibody bevacizumab, which is in clinical use for cancer therapy. Furthermore, bevacizumab displayed potent antiangiogenic efficacy in vivo and suppressed growth of xenografts derived from GSCs but limited efficacy against xenografts derived from a matched non-GSC population. As hypoxia is a key regulator of angiogenesis, I further examined hypoxic responses in GSCs to determine the molecular mechanisms underlying their angiogenic drive. I demonstrated that
multiple hypoxia response genes, including the hypoxia-inducible factors (HIFs)-1α and -2α (EPAS-1) were differentially expressed in GSCs in comparison to non-stem glioma cells and normal neural progenitors. GSCs preferentially induced HIF2α and HIF2α-regulated genes under hypoxia in comparison to non-stem glioma cells. In contrast, neural progenitor/stem cells did not induce HIF2α in response to hypoxia suggesting that the HIF2α hypoxic response is not a general stem cell response. Targeting HIF1α or HIF2α in GSCs using short hairpin RNA (shRNA) inhibited neurosphere formation efficiency, indicating a requirement for HIFs in cancer stem cell self-renewal. HIF1α and HIF2α were also necessary for VEGF expression in GSCs, but HIF2α was not required in matched non-stem glioma cells. In vivo experiments determined that knockdown of HIFs significantly attenuated the tumorigenic capacity of GSCs and increased survival of immunocompromised mice. Together, our work provides the first evidence that that GSCs can be a crucial source of key angiogenic factors in cancers due to their differential hypoxia responses. It also suggests that anti-angiogenic therapies can be designed to target GSC-specific molecular mechanisms of neoangiogenesis, including the expression and/or activity of HIF2α.
Dedication

This is dedicated to my parents, Dacheng and Wenbin, and my wife Wanli, for their endless love.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ALDH1</td>
<td>Aldehyde Dehydrogenase 1</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukaemia</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>CD133</td>
<td>Cluster of Differentiation 133</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>DFX</td>
<td>Deferrioxamine Mesylate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem (Cell)</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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FISH           In Situ Hybridization
GSC            Glioma Stem Cell
HIF            Hypoxia Inducible Factor
HMVEC          Human Microvascular Endothelial Cell
HRE            Hypoxia-Responsive Element
HSC            Hematopoietic Stem Cells
IgG            Immunoglobulin G
MACS           Magnetic-Activated Cell Sorting
MMP            Matrix Metalloproteinases
NPC            Neural Progenitor Cells
NSC            Neural Stem Cells
NT             Non-Targeting
Olig2          Oligodendrocyte Lineage Transcription Factor 2
PDGF           Platelet Derived Growth Factor
PE             Phycoerythrin
ROS            Reactive Oxygen Species
s.c            Subcutaneous
SEZ            Sub-ependymal Zone
shRNA          Short Hairpin RNA
VEGF  Vascular Endothelial Growth Factor
VEGFR  Vascular Endothelial Growth Factor Receptor
VHL  Von Hippel-Lindau
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1. Background and Overview

1.1 Glioma

1.1.1 General

Gliomas are the most common primary brain tumors (Furnari, Fenton et al. 2007). In 2008, more than 20,000 people are diagnosed with gliomas in the U.S alone. Malignant glioma is also one of the deadliest cancer types and more than 13,000 people died of malignant gliomas in the U.S last year. Although people of any age can develop gliomas, the problem seems to be most common in adults ages 40 to 70. Gliomas can invade brain tissues and spread via the cerebrospinal fluid and cause "drop metastases" to the spinal cord. However, they typically do not spread outside the nervous system. Symptoms of gliomas largely depend on which part of the central nervous system is affected. Generally, they can cause headaches, nausea and vomiting, seizures, and cranial nerve disorders as a result of increased intracranial pressure. If a glioma happens to impair the optic nerve, it can cause visual loss.

1.1.2 Classification

Like many other cancers, pathological evaluations play a pivotal role in glioma diagnosis and classification. Since Bailey and Cushing built the original system in the 1920s, gliomas have been usually classified according to the type of normal central
neural system (CNS) cells they most closely resemble (Bailey and Cushing 1928). For example, the cells of astrocytomas microscopically most closely resembled astrocytes and those of oligodendrogliomas histologically most mimicked oligodendrocytes. However, many gliomas demonstrate the features of mixture cell types, such as oligoastrocytomas, which contain distinct cell populations similar to oligodendrocytes and astrocytes, respectively. Although many researchers have hypothesized that the morphology of tumor cells may suggest the cell of origin, little evidences are available to validate this.

Currently, gliomas are more frequently classified by grades (degree of malignancy), which is determined by a series of pathologic evaluations of the tumor, including its differentiation status. Various grading systems are used world-wide but World Health Organization (WHO) standard is the one most commonly used. The WHO system assigns a grade from 1 to 4, with 1 being the least aggressive and 4 being the most aggressive. Grade I glioma is the most benign one and tumor cells are most differentiated. In contrast, grade IV glioblastoma consists of very poorly differentiated cells and it has the worst patient prognosis (with an average patient survival of less than 1 year)(Furnari, Fenton et al. 2007).

All the above systems are primarily depend on pathologists and histological evidences. Therefore, they remain largely subjective. Because of the recent advances in
large-scale genomic detection methods, many researchers suggest to classify gliomas and other cancers according to their “molecular signatures”, such as genomic mutation signature, mRNA signature, microRNA signature, etc (Golub, Slonim et al. 1999; Louis, Holland et al. 2001; Pomeroy, Tamayo et al. 2002; Nutt, Mani et al. 2003; Sathornsumetee, Cao et al. 2008). These methods are believed to be more objective and there is some evidence suggesting that “molecular signatures” may be more reliable than traditional classification systems in predicting prognosis and patient response to chemotherapy drugs (Nutt, Mani et al. 2003; Shirahata, Iwao-Koizumi et al. 2007).

1.1.3 Treatment and Prognosis

Treatment for gliomas depends on location of the tumor, the cell type and the grade of malignancy. Often, treatment is a combined approach of surgery, radiotherapy and chemotherapy (Chamberlain 2006); (Kim and Glantz 2006). Most patients first receive maximum safe extent of resection followed by conventionally fractionated external-beam radiotherapy. The value of adjuvant chemotherapy has been debated and remains controversial. Temozolomide (TMZ) is the current golden standard chemotherapeutic drug for gliomas (Kim and Glantz 2006). Nowadays, the new standard of care for patients with newly diagnosed glioblastoma is to give TMZ concurrently with radiotherapy, followed by six monthly cycles of TMZ. However, all these traditional treatments of malignant gliomas remain largely palliative.
Recently, molecular biology studies of gliomas have provided new insights into the mechanisms controlling early development and progression of these tumors. Dysregulated cell signaling pathways have been identified and they are now the focus of specific molecularly targeted therapies. For instance, EGFR-PI3K/PTEN-Akt pathway is up-regulated in majority of malignant glioma samples and there are multiple clinic trials going on to test the efficacy of anti-EGFR therapy (Rich, Reardon et al. 2004; Prados, Chang et al. 2009). Besides, some novel targeting strategies such as anti-angiogenesis (new blood vessel growth) therapy are also under active clinic investigation (Ali, McHayleh et al. 2008).

1.2 Stem Cell and Cancer Stem Cell

1.2.1 Stem Cell

Stem cells are functionally defined as cells that are capable of maintaining themselves through self renewal and generating mature cells through well-controlled differentiation process (Reya, Morrison et al. 2001). Stem cells are believed to achieve these two properties by asymmetric division, in which one stem cell gives rise to one stem cell and one differentiated daughter cell. During mammalian development, embryonic stem (ES) cells are the original sources that give rise to other terminal cell types. ES cells are defined as pluripotent stem cells because they can generate literally all
cell types in a human body. Stem cells also exist in adults. However, in comparison to ES cells, these adult stem cells generally have more restricted differentiated ability and they only produce limited types of daughter cells, usually in a specific tissue. For example, stem cells in central nervous system (CNS) normally only produce neurons and glial cells, while hematopoietic stem cells (HSC) are responsible to produce lymphocytes and myeloid cells (Reya, Morrison et al. 2001). Therefore, these adult stem cells are multipotent but not pluripotent (Figure 1).

Figure 1: Hierarchy of Stem Cells
Totipotent or pluripotent stem cells can give rise to multipotent adult stem cells. (Adapted from National Institutes of Health)
While researchers have long proposed the existence of adults stem cells, to identify adult stem cells has been proved to be quite challenging as it is almost impossible to isolate them through morphological criteria. Thanks to the development of monoclonal antibody (mAb) techniques, it now becomes possible to enrich and identify stem cells according to their expression of unique cell surface markers (usually a combination of special markers). Most of our knowledge about adult stem cells nowadays is gained from the studies of hematopoietic stem cells (HSC). Hematopoietic stem cells were first identified in late 1980s (Spangrude, Heimfeld et al. 1988). Since then, similar studies have been done in other tissues and prospective adult stem cell populations have been found in many other organs including brain (Johansson, Momma et al. 1999; Lee, Kessler et al. 2005), heart (Messina, De Angelis et al. 2004), intestine (Barker, van Es et al. 2007) and pancreas (Seaberg, Smukler et al. 2004). Notably, the current cell surface markers are still far from perfect. They are often useful to enrich stem cells but it is still very difficult to identify and study single stem cell by this method. Researchers are still constantly modifying the markers (usually adding new markers) in hope to better separate stem cells from others. For instance, mouse HSC was originally identified as CD34$^{lo-}$ and CD38$^+$ (Spangrude, Heimfeld et al. 1988), but now we know they are actually in a cellular subpopulation of CD34$^{lo-}$, SCA-1$, Thy1$, CD38$^+$,
C-kit⁺, lin⁻ (Forsberg, Bhattacharya et al. 2006). Notably, human HSC seems to have a different marker pattern from the mouse counterpart (Baum, Weissman et al. 1992).

As stem cells have extraordinary capability of differentiating into terminal cell types and replenishing certain tissues, people have been excited about using stem cells to treat degenerative diseases such as Parkinson’s disease or Alzheimer’s disease. However, it is still currently technically challenging as we still do not know exactly how to accurately control the stem cell differentiation and make them give rise to certain lineages in vitro. Besides, there has been a heating debate regarding the ethical issues associated with ES cell research. But this controversy is likely to be solved by the discovery and wide usage of inducible pluripotent stem cells.

1.2.2 Cancer Stem Cell hypothesis

The recent advances in cancer research have resulted in an increasing appreciation of the complexity of tumors. Though tumors had traditionally been thought of as masses of identical neoplastic cells, we now know that there is tremendous heterogeneity with respect to proliferation and differentiation within the malignant cell population. Recent evidence has suggested that within the malignant cell population of some cancers, there is a unique cell subpopulation with the capacity for sustained tumor growth in some in vivo models and they can recapitulate the parental tumor phenotype upon series transplantation (Reya, Morrison et al. 2001; Bao, Wu et al. 2006). Importantly,
these cells usually possess characteristics associated with normal stem cells such as the capability of self renewal and differentiation into multiple cell types (Reya, Morrison et al. 2001; Bao, Wu et al. 2006). Therefore, they were termed as cancer stem cells (CSCs).

Although researchers have not been able to identify CSCs until 1990s, the concept that cancer might arise from a rare population of cells with normal stem cell properties was proposed more than 100 years ago (Sell 2004; Wicha, Liu et al. 2006). CSCs are believed to persist in tumors as a distinct cellular sub-population and cause relapse after treatment. Some researchers also argue that CSCs may contribute to metastasis as well (Hermann, Huber et al. 2007). The concept of CSC changes people’s view of tumorigenesis and supports a hierarchical model instead of the traditional stochastic model (Figure 2). Therefore, the development of specific therapies targeting CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.
Figure 2: Stochastic Model vs Hierarchical Model in Tumorigenesis
The identification of cancer stem cells has challenged the conventional stochastic tumorigenesis model, in which every single tumor cell is equally capable of initiating tumor growth. (Adapted from Reya et al., 2001)

1.2.3 Cancer Stem Cell in Leukemia
Similar to the history of adult stem cells, CSCs were also first identified in the blood system. Using a combination of CD34 and CD38, two cell surface protein markers normally expressed by haematopoietic stem cells, Bonnet and Dick found that only CD34+/CD38− acute myeloid leukaemia (AML) cells had the unique capacity to initiate secondary leukaemias when transplanted from patients into recipient immunodeficient NOD/SCID mice (Bonnet and Dick 1997). These cells could self-renew and gave rise to more differentiated daughter cells. Therefore, these CD34+/CD38− matched the
identification of CSCs. Importantly, consistent with the CSC model, AML CSCs are able to generate secondary and tertiary cancer that recapitulate the phenotypic heterogeneity found in the initial cancer (Bonnet and Dick 1997). However, it is important to notice that not every CD34+/CD38− AML cell is CSC. Rather, it is a cellular pool that enriched for the CSC population. Researchers are actively searching for additional markers that may help further separate CSCs from other cells in the CD34+/CD38− pool.

### 1.2.4 Cancer Stem Cell in Solid Tumors

Soon after the seminal study which identified CSCs in leukaemia, similar populations were successively identified within solid tumors. The evidence that CSCs exist in solid tumors were first reported in breast cancer samples. Clark and his colleagues showed that CD44+/CD24lo− breast cancer cells have stem cell characteristics and as few as 200 of these cells are able to form tumors in NOD/SCID mice. In contrast, 20,000 other cells isolated from the same tumor that do not display this cell surface phenotype failed to form tumors (Al-Hajj, Wicha et al. 2003). Breast CSCs are also able to generate tumors that are phenotypically recapitulate the parental tumors. Following this pioneer work, potent CSCs have also been isolated and characterized in the tumors of the brain, colon, prostate, liver, head and neck, and pancreas. However, the markers used to identify CSCs sometimes differ among different research groups. For example, several recent reports have challenged the use of CD44+/CD24lo− as breast CSC markers
and they utilized ALDH1 instead (Ginestier, Hur et al. 2007). While the debates are still going, these data together might suggest that different tumor samples could have different CSC populations expressing different molecular markers.

Following this pioneering work in breast cancer, researchers have identified potential CSC populations from various tumors. For instance, colon CSCs were found in the CD133+ cancer population (O’Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007), while medulloblastoma CSCs seem to highly express surface marker CD15 (Read, Fogarty et al. 2009). Similar approach has been used to prospectively identify CSC population from the solid tumors of prostate (Collins, Berry et al. 2005), pancreas (Li, Heidt et al. 2007), head and neck (Prince, Sivanandan et al. 2007) and liver (Rountree, Ding et al. 2008).

**1.2.5 Glioma Cancer Stem Cell**

Recent studies of glioblastomas and other central nervous system neoplasms identified tumor subpopulations that share characteristics with normal neural stem cells (Hemmati, Nakano et al. 2003; Singh, Clarke et al. 2003; Singh, Hawkins et al. 2004) (Figure 3).
Figure 3: Isolation Cancer Stem Cells from Brain Tumors
Cancer stem cells in brain tumors can be enriched through cell surface marker CD133. (Adapted from Singh et al., 2004)
Glioma CSCs express neural stem cell markers (Prominin-1/CD133, Sox2, Nestin, Musashi-1), are capable of self-renewal, form neurosphere-like spheroids, and differentiate into multiple nervous system lineages (neurons, astrocytes, and oligodendrocytes) (Bao, Wu et al. 2006). When xenotransplanted into the brains of immunocompromised rodents, human CSC demonstrate greater potential than non-CSC cancer cells to initiate tumor formation, even though non-CSC survive when transplanted intracranially (Singh, Hawkins et al. 2004). The mechanisms responsible for the differential tumorigenic capacity of CSC remain relatively unknown, but our lab have recently demonstrated that CSC contribute to tumor radioresistance. We also reported that glioma CSC up-regulate the expression of several genes such as c-myc and L1CAM to regulate CSCs’ proliferation and invasion (Bao, Wu et al. 2008; Wang, Wang et al. 2008).

Though the cellular origin of CSCs in human glioma is yet to be clarified, several normal murine neural stem/progenitor cell types have been transformed into highly proliferative CSC-like neoplastic cells through manipulation of certain signal pathways (e.g EGFR-PI3K-Akt pathway), indicating that stem/progenitor cells may have the capacity to give rise to CSCs (Bachoo, Maher et al. 2002; Li, Dutra et al. 2009).
1.3 Angiogenesis

1.3.1 Angiogenesis in Tumors

Angiogenesis is a biological process involving the growth of new blood vessels from pre-existing vessels. It usually occurs in several well-defined steps (Yancopoulos, Davis et al. 2000; Carmeliet 2005). First, pro-angiogenic growth factors activate corresponding receptors present on endothelial cells in pre-existing veins. Second, the activated endothelial cells begin to release proteases to degrade the basement membrane which allows endothelial cells to move from the original sites. Last, the endothelial cells migrate, proliferate and form new vessels (Tang and Conti 2004).

Angiogenesis is a common and essential process in normal development and wound healing. In addition, it also represents a critical step in tumor formation and is one of the hallmarks of cancer (Hanahan and Weinberg 2000) (Figure 4). The “angiogenic switch” is a fundamental step in the transition of tumors from a dormant state to a malignant state. Most solid tumor larger than 1 mm³ essentially requires angiogenesis in order to continue growing (Folkman 1971; Gimbrone, Leapman et al. 1972). The new blood vessel growth supplies the rapid growing cancer cells with oxygen and nutrition. Angiogenesis may also serves as a waste pathway, taking away the biological end products put out by rapidly dividing cancer cells. Additionally, angiogenesis is also required for the spread of a tumor, or metastasis (Gimbrone, Cotran
et al. 1974; Blood and Zetter 1990; Bello, Giussani et al. 2004). Tumor cells could invade the bloodstream and migrate to other distant sites to form secondary/metastatic tumors. Angiogenesis has been traditionally thought and proven to be critical for later stage tumorigenesis. However, there is new evidence showing that tumor cells are capable of promoting blood vessels growth at a much earlier phase of cancer progression when only about 100 tumors cell exist (Li, Shan et al. 2000). Tumor cells began to induce the migration and proliferation of endothelial cells in vivo even when there were only as few as 100 tumor cells.

Regulation of angiogenesis represents a complex process involving autocrine and paracrine growth factor loops with an interplay between tumor cells and the neovasculature (Jain, di Tomaso et al. 2007). There are a large group of growth factors regulating tumor angiogenesis and the most characterized ones include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and angiopoietins (Ang-1 and Ang-2) (Yancopoulos, Davis et al. 2000; Machein, Knedla et al. 2004; Carmeliet 2005). Specific receptors for these factors are presenting in endothelial cells to mediate the migration and proliferation of blood vessels. The best studied receptors include VEGF receptors (e.g. Flk-1 and Flt-1) and angiopoietin receptor (e.g. Tie-2). Flk-1 is the primary mediator of the mitogenic effects of VEGF and it activates downstream molecules like Raf-Ras-MAPK, PI3K and FAX.
Tie-2 binds to both Ang-1 and Ang-2 and it is generally accepted that Ang-1 is real the agonist while Ang-2 functions more like an antagonist. The binding of Ang-1 to Tie-2 promote vasculature development and tumor angiogenesis. Since Ang-2 functions as an antagonist to interfere the interaction between Ang-1 and Tie-2, it was predicted that overexpress Ang-2 may block tumor angiogenesis. Surprisingly, Ang-2 actually plays a postivie role in angiogenesis and blocking Ang-2 led to decreased tumor angiogenesis (Oliner, Min et al. 2004). The molecular mechanisms are still under investigation. Notably, these molecules are usually regulated by microenvironment such as hypoxia, PH and lactic acid (Dewhirst, Cao et al. 2008).

Given the importance of angiogenesis in tumor progression, targeting blood vessels have been a popular strategy in developing novel anti-cancer drugs. Avastin (bevacizumab), a VEGF neutralizing antibody which specifically block the VEGF pathway has been proved by FDA in treating colorectal cancer patients (Ferrara and Kerbel 2005). Since tumor associated endothelial cells are generally “normal” in comparison to neoplastic cells that bear lots of mutations, recent evidence also suggests that traditional therapies may actually work largely by targeting the genomically stable blood vessels, rather than the genomically unstable tumor cell compartment.
Figure 4: Tumor Angiogenesis
Angiogenesis is critical for tumor growth and the formation of new tumor vessels a complicated process. (Adapted from Carmeliet & Jain)

1.3.2 Angiogenesis in Glioma

Malignant gliomas are highly lethal neoplasms which exhibit striking angiogenesis (Bao, Wu et al. 2006; Jain, di Tomaso et al. 2007). In fact, highly proliferating vasculatures is one of the key characteristics that are commonly used by pathologists to identify glioblastomas multiforme, the most aggressive form of gliomas.
Malignant gliomas usually have elevated expression of multiple pro-angiogenic factors particularly VEGFs, which promote blood vessel formation by endothelial precursors (Plate, Breier et al. 1992; Plate, Breier et al. 1994). VEGFs are secreted or cell surface bound proteins expressed by tumor cells that bind to specific high affinity transmembrane receptors primarily expressed on endothelial cells to promote endothelial cell proliferation, migration, and survival (Yancopoulos, Davis et al. 2000). Anti-angiogenic agents were effective against gliomas in several preclinical studies. For instance, researchers recently demonstrated that a novel anti-angiogenic receptor kinase inhibitor (ZD6474) was highly effective against multiple central nervous system cancer xenografts, including gliomas (Rich, Sathornsumetee et al. 2005). Receptor kinase inhibitors had limited benefit in clinical trials in gliomas, but frequent glioblastoma patient tumor responses were observed using a neutralizing anti-VEGF monoclonal antibody (bevacizumab, Avastin) in combination with the topoisomerase I inhibitor, irinotecan (Stark-Vance 2005; Ali, McHayleh et al. 2008).

1.3.3 Angiogenesis, Stem Cell and Cancer Stem Cell

Traditional views suggest angiogenesis is critical to normal development and cancer biology largely due to the ability of new blood vessels to increase nutrition/oxygen supply while create a channel to discharge metabolic waste. However, increasing evidences have indicated that angiogenesis has more functions than what we
have previously known. It is now clear that vascular structures directly contribute to stem cell maintenance. Normal stem cells are physically located in “stem cell niches”, which are spatially and temporally regulated microenvironments (Gilbertson and Rich 2007; Zhang and Li 2008). The exact organization of a stem cell niche remains largely unknown and they may significantly vary among different tissues and species. Nevertheless, vasculature has been shown to be a critical component of multiple stem cell/progenitor types in mouse and/or human (Gilbertson and Rich 2007; Yoshida, Sukeno et al. 2007; Shen, Wang et al. 2008; Tang, Zeve et al. 2008; Tavazoie, Van der Veken et al. 2008). Endothelial cells can promote the proliferation of hematopoietic stem cells in intro and are likely to also regulate HSCs in in vivo (Kiel and Morrison 2008). Similarly, undifferentiated spermatogonia and adipocyte progenitors are also reported to locate in a vasculature-associated niche (Yoshida, Sukeno et al. 2007; Tang, Zeve et al. 2008). Recently, several groups demonstrated that neural stem cells in sub ventricular zone (SVZ) are preferentially locate close to blood vessels and uniquely poised to receive spatial cues and regulatory signals from vasculatures (Shen, Wang et al. 2008; Tavazoie, Van der Veken et al. 2008). All these data together strongly argue that normal adult stem cells are tightly regulated by vascular structures and located in peri-vascular niches. The precise cocktail of vascular-derived factors that regulate normal stem cells remains to be determined, but brain-derived neurotrophic factor (BDNF), vascular endothelial growth
factor (VEGF) and pigment epithelium-derived factor are likely candidates. It is likely that additional cell types including ependymal and transit progenitor cells contribute paracrine and direct cell–cell contact signals as well (Gilbertson and Rich 2007).

Recently, Calabrese et al provided the first evidence to show that the dependence of stem cells on peri-vascular niche is shared by cancer stem cells (Calabrese, Poppleton et al. 2007). They demonstrated that glioma CSCs are located adjacent to tumor capillaries. Calabrese et al also reported significantly faster tumor initiation and growth when CSCs were co-injected with endothelial cells, which have the capability to secrete factors that promote the self-renewal and proliferation of glioma CSCs. This further underscored the importance of angiogenesis in glioma biology and may help explain the preliminary success of anti-angiogenesis therapy on malignant gliomas. Clinical trials of anti-angiogenic drugs like Bevacizumab and Cediranib have demonstrated promising preliminary results in glioblastoma patients (Rich, Sathornsumetee et al. 2005; Stark-Vance 2005; Ali, McHayleh et al. 2008). It is possible that these drugs might directly disrupt the maintenance of CSCs, thus effectively eliminating the roots of tumor progression.
1.4 Hypoxia and Hypoxia Response

1.4.1 Hypoxia in Tumor

Hypoxia, defined as reduced oxygen tension, is a common physiological phenomenon in both normal development and tumor progression (Harris 2002; Bertout, Patel et al. 2008). Most mammalian tissues exist at 2%–9% O2 level. Hypoxia is usually defined as \( \leq 2\% \) O2, and severe hypoxia (or anoxia) is defined as \( \leq 0.02\% \) O2. In normal organs, hypoxia usually happens in cells that are far away from blood vessels. In tumors, hypoxia seems to happen at a much wider range and even tumor cells that are adjacent to vasculature could actually locate in a hypoxic microenvironment. Many mechanisms count for such a paradoxic phenomenon, including the usually aberrant and poorly functional blood vessels in tumors, high oxygen consumption by tumor cells and extreme variations in microvessel red blood cell flux. Severe hypoxia is toxic to both normal and neoplastic cells; however, tumor cells could adapt to low oxygen concentration environment by additional genomic mutations and therefore hypoxia usually promotes tumor progression (Harris 2002; Kaur, Khwaja et al. 2005; Pouyssegur, Dayan et al. 2006; Bertout, Patel et al. 2008). Using oxygen electrodes to measure the oxygen tension inside of tumors, Vaupel and colleagues first reported that hypoxia was associated with aggressive tumor growth, increased metastasis and poor survival in patients suffering from several tumor types including head and neck cancer, cervical and breast cancers (Brizel, Scully et al. 1996; Nordsmark, Overgaard et al. 1996; Brizel,
Hage et al. 1997; Hockel and Vaupel 2001; Vaupel and Mayer 2007). Brain tumors usually have significant amount of hypoxic region. In glioblastoma multiforme, high rate of necrosis, a well known consequence of prolonged hypoxia, is one of the hallmarks of this highly lethal malignancy (Evans, Kachur et al. 2000).

How exactly hypoxia promotes tumor development is not fully understand yet but there are adequate data demonstrating that hypoxia regulates multiple aspects of cell biology, such as angiogenesis, radio-resistance, metabolism, and invasion/migration (Harris 2002). The major focus of hypoxia research for most of the 20th century was its role in radiotherapy responses. As early as 1909, Schwarz and colleagues already noted that normal cells in hypoxia or anoxia were less sensitive to radiation than those irradiated in the presence of O2. Decades later, researchers found that human tumors also contain radioresistant hypoxic regions and therefore it has been proposed that the combination of radiation with a hypoxia-specific cytotoxin may help improve cancer treatment efficacy (Bristow and Hill 2008).

Hypoxia also affects many other crucial steps in cancer progression. Under hypoxia condition, cells switch their glucose metabolism from aerobic tricarboxylic acid (TCA) cycle to anaerobic glycolysis. It has been well known that cancer cells prefer glycolysis even in the presence of oxygen (Bertout, Patel et al. 2008). One hypothesis to explain this phenomenon is that the glycolytic pathway provide the precursors for
synthesis of glycine, serine, purines, pyrimidines and phospholipids, all of which are essential for rapid cell growth. Elevated angiogenesis is another well characterized biological consequence of tumor hypoxia (Harris 2002). Upon low oxygen stimulation, cells can promote new blood vessels by secreting remarkable level of many pro-angiogenic growth factors, such as VEGF, PDGF-B, etc (Figure 5). Hypoxia may also change the level of cell adhesion molecules and proteinases (like MMPs) to facilitate cancer invasion and migration (Harris 2002).

**Figure 5: Role of Hypoxia in Tumor Angiogenesis**
Hypoxia, the low oxygen tension, is one of best studied factors regulating tumor angiogenesis. (Adapted from Carmeliet & Jain)
1.4.2 Hypoxia Inducible Factors

Cellular responses to hypoxia are complex but the hypoxia-inducible factors (HIFs) represent key mediators (Kaur, Khwaja et al. 2005; Pouyssegur, Dayan et al. 2006). Hypoxia-inducible factors (HIF1, 2, 3) are heterodimers consisting of an alpha (HIF1α, HIF2α, and HIF3α) and a beta subunit, both belonging to the basic-helix-loop-helix Per-aryl hydrocarbon receptor nuclear translocator-Sim (PAS) family of transcription factors. HIF1α, HIF2α, and HIF3α, are induced in response to hypoxia and serve to coordinately activate the expression of target genes whose products facilitate cell survival under conditions of oxygen deprivation (Zhong, De Marzo et al. 1999; Kaur, Khwaja et al. 2005). The HIFα subunits are regulated by oxygen conditions whereas the HIF1β subunits are constitutively expressed (Jaakkola, Mole et al. 2001). HIFα (mostly HIF1α and HIF2α) stability and activity is regulated by two oxygen sensors, termed prolyl-hydroxylase domain (PHD) protein and factor inhibiting HIFα (FIH). PHDs hydroxylate HIFα to initiate binding of the Von Hippel–Lindau (VHL) protein which marks HIFα for destruction by the proteasome under normoxic conditions (Maxwell, Wiesener et al. 1999; Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001) (Figure 6). FIH hydroxylates an asparagine residue in the carboxy-terminal transcriptional activation domain of HIFα to inhibit the binding of cofactors, such as p300, that are required for the transcription of certain HIF-dependent genes (Mahon, Hirota et al. 2001). Thus,
oxygen tension tightly regulates HIFα stability and activity (Pouyssegur, Dayan et al. 2006).

Figure 6: HIF-α Protein Is Regulated by Oxygen Tension
At normoxia condition, HIF-α is hydroxylated by PhD domain proteins and then targeted to proteasome for degradation. However, in hypoxia, the PhD domain proteins are inhibited. HIF-α is therefore released from hydroxylation modification and accumulated. It will bind to HIF-β to form a functional dimer transcription factor in the nuclear.
Although HIFα is regulated by hypoxia, there is little correlation between HIFα expression levels and hypoxia in tumors. The pattern of HIFα or its downstream gene VEGF and localization of hypoxia marker drugs are poorly overlapped. This phenomenon is explained by two mechanisms. First, in tumors, the hypoxia condition is not static but rather cycling (Dewhirst, Cao et al. 2008). Tumor cells may experience frequent hypoxia-reoxygenization all the time. Second, HIFα is also regulated by factors other than hypoxia. Free radicals such as reactive oxygen species (ROS) and nitric oxide also affect HIFα stabilization. Besides, the activation of oncogenes such as EGFR, PI3K and Akt can also activate HIFα. All of these additional mechanisms help explain the relatively puzzling observation that HIFα protein is frequently stabilized in tumor regions even with sufficient oxygen supplies.

HIF1α directly activates transcription of hundreds of hypoxia-inducible genes, including those encoding: erythropoietin (EPO), VEGF, heme oxygenase-1, inducible nitric oxide synthase, and the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase I, and phosphoglycerate kinase 1 (Pouyssegur, Dayan et al. 2006). Less is understood about the transcriptional targets regulated by HIF2α, but there are non-overlapping targets in gene expression studies (Carroll and Ashcroft 2006; Hu, Iyer et al. 2006; Gordan, Bertout et al. 2007; Qing and Simon 2009). The mechanism for differential gene expression is poorly understood as both HIF1α and
HIF2α can bind the same hypoxia-responsive elements (HREs) in the promoter but it appears that gene products regulated by HIF2α contain nearby ETS-binding sites (Elvert, Kappel et al. 2003). As ETS transcriptional family members are overexpressed in human glioma specimens in a grade-dependent manner, HIF2α may have a role in glioma biology.

**1.4.3 Hypoxia and Stem Cell Maintenance**

Local oxygen concentrations can directly influence stem cell self-renewal and differentiation. *In vitro* evidence indicates that hypoxia usually favors an undifferentiated status (Keith and Simon 2007). Low oxygen helps maintain embryonic stem cells and significantly blocked spontaneous cell differentiation (Ezashi, Das et al. 2005). Culturing human bone marrow HSCs under hypoxic conditions promoted their ability to repopulate when they were transplanted into NOD/SCID mice model (Danet, Pan et al. 2003). In fact, bone marrow, where HSCs usually locate, is generally hypoxic in nature. Hypoxia also regulates neural stem cells as low oxygen could promote the proliferation and survival of NSCs. Besides, hypoxia also alters the differentiation program of NSC and it seems low oxygen favors the generation of neurons of dopaminergic phenotype (Studer, Csete et al. 2000). Why hypoxia favors the maintenance of stem cells is largely unknown but one attractive hypothesis is that stem
cells are located in low oxygen environment to reduce the DNA damage resulted from reactive oxygen species (ROS).

Recent reports have identified a few molecular mechanisms by which hypoxia and HIFs directly modify stem cell function. For instance, Notch signaling seems to be essential in hypoxia-mediated blocking of differentiation in myogenic satellite cells and primary neural stem cells (Gustafsson, Zheng et al. 2005). Later, Simon and colleagues reported that HIF2α but not HIF1a can up-regulate the expression of Oct4 and enhance the activity of c-myc (Covello, Kehler et al. 2006; Gordan, Bertout et al. 2007). As both Oct4 and c-myc are famous factors modulating stem cell self-renewal, these data provide important insights into how hypoxia helps maintain stem cells.

Whether hypoxia also controls CSCs are not clear but hypoxia has been shown to promote stem cell-like phenotype in solid tumors. Pahlman and colleagues demonstrated that hypoxic conditions confer a more immature phenotype on human neuroblastoma and breast cancer cells (Jogi, Ora et al. 2002; Axelson, Fredlund et al. 2005). Besides, as hypoxia can directly up-regulates the expression or activity of Oct4 and c-myc, two genes that play pivotal roles in both stem cell biology and cancer biology (Covello, Kehler et al. 2006; Gordan, Bertout et al. 2007), hypoxia may be a functional component of a CSC niche.
2. Glioma Stem Cells Promote Tumor Angiogenesis

2.1 Summary

Malignant gliomas are highly lethal cancers depending on active angiogenesis. Glioma stem cells (GSCs) have much stronger potential to initiate gliomas in immuno-compromised mice in comparison to non-stem glioma cells. Tumors derived from GSC were morphologically distinguishable from those arise from non-stem glioma cells by widespread tumor angiogenesis, necrosis, and hemorrhage. To determine a potential molecular mechanism for GSC to stimulate angiogenesis, we measured the expression of a set of angiogenic factors secreted by GSC and non-stem glioma cell pairs. We found that GSC consistently secreted remarkably elevated levels of vascular endothelial growth factor (VEGF), which were further induced by hypoxia. *In vitro*, GSC-conditioned medium significantly increased migration and tube formation of endothelial cells compared with non-stem glioma cell–conditioned medium. Importantly, the proangiogenic effects of GSC on endothelial cells were specifically abolished by the anti-VEGF neutralizing antibody Avastin (bevacizumab), which is in clinical trail for glioma therapy. Furthermore, Avastin demonstrated strong antiangiogenic efficacy *in vivo* and suppressed growth of xenografts derived from GSC. Together these data suggest that GSC can be a crucial source of key angiogenic factors in tumors and that targeting
proangiogenic factors such as VEGF from GSC populations may be critical for glioma therapy.

### 2.2 Introduction

Angiogenesis, the growth of new blood vessels, is a general phenomenon for most solid tumors and it is critical to tumor progression (Carmeliet 2005). Most solid tumors larger than 1mm$^3$ need to acquire new blood supply in order to keep growing and progression. Regulation of angiogenesis represents a complicated process involving a combination of autocrine and paracrine loops as well as an interaction between tumor cells and the vasculature (Yancopoulos, Davis et al. 2000).

Malignant gliomas are highly lethal neoplasms that exhibit highly active angiogenesis and usually express elevated levels of vascular endothelial growth factors (VEGFs), which promote new blood vessel formation by endothelial precursors (Plate, Breier et al. 1992; Plate, Breier et al. 1994). VEGFs are secreted or are cell surface-bound proteins expressed by tumor cells that bind to specific high-affinity transmembrane receptors (include VEGFR1 and VEGFR2) which are primarily expressed on endothelial cells to promote endothelial cell proliferation, migration, and survival (Hatva, Kaipainen et al. 1995). Given the importance of angiogenesis in glioma, multiple anti-angiogenic agents have been testing in clinical trials to treat malignant gliomas. We previously reported that a novel a VEGF receptor kinase inhibitor (ZD6474) was highly effective
against multiple central nervous system cancer xenografts in vitro, including gliomas (Rich, Sathornsumetee et al. 2005). While receptor kinase inhibitors had limited benefit in clinical trials against gliomas so far (Conrad, Friedman et al. 2004; Reardon, Friedman et al. 2004), frequent responses from patients with glioblastoma (a WHO grade VI glioma) were observed using a neutralizing anti-VEGF monoclonal antibody, bevacizumab (Avastin), in combination with the topoisomerase I inhibitor irinotecan (Stark-Vance 2005; Ali, McHayleh et al. 2008; Sathornsumetee, Cao et al. 2008). In order to improve the efficacy of these treatments, we need to advance our understanding of the molecular mechanisms promoting glioblastoma angiogenesis.

Recent studies of glioblastomas and other brain cancers identified tumor subpopulations termed glioma stem cells (GSCs) that share many characteristics with normal neural stem cells (Singh, Clarke et al. 2003; Galli, Binda et al. 2004; Singh, Hawkins et al. 2004). GSCs express neural stem cell markers (prominin-1/CD133, Nestin, Sox2, and Musashi 1). Like neural stem cells, GSCs are capable of self-renewal, form neurosphere-like spheroids, and differentiate into multiple nervous system lineages (neurons, astrocytes, and oligodendrocytes). More importantly, after xenotransplantation into the brains of immunocompromised mice models, human GSCs generally show greater potential than non-stem glioma cells to initiate tumor formation (Bao, Wu et al. 2006). The mechanisms responsible for the differential tumorigenic capacity of GSCs
remain unknown. As angiogenesis represents a critical regulatory step in both early
tumor development (the "angiogenic switch") as well as late stage progression, we
examined whether GSCs contribute to tumor formation through promoting
angiogenesis.

In this chapter, we discovered that GSCs could enhance glioma angiogenesis
through a VEGF-dependent mechanism. Blocking GSC mediated angiogenesis could
achieve promising anti-tumorigenic effects in vitro.

2.3 Materials and Methods

Chemicals and Reagents

Neutralizing anti-VEGF antibody, bevacizumab (Avastin, Genentech, South San
Francisco, CA), was purchased from the Duke Hospital clinical pharmacy (Durham,
NC). Control mouse IgG and other chemicals were purchased from Sigma unless
otherwise specified.

Patient Biopsy Specimens

Specimens from patients undergoing biopsy for newly diagnosed or recurrent
glioblastoma were collected under a Duke University Institutional Review Board–
approved protocol. Pathologic diagnosis was confirmed by pathologist Dr. Roger McLendon.

**Human Glioma Xenografts**

D456MG xenograft was derived from a biopsy specimen from a 4-year-old child with glioblastoma. D54MG xenograft is the Duke subline of A-172, which was derived from a 54-year-old male with glioblastoma. Xenografts were maintained in the flanks of athymic BALB/c nu/nu mice and early passages were used to minimize genetic drift.

**Purification and Characterization of Glioma Stem Cells**

Matched cultures enriched or depleted for GSCs were isolated from primary human brain tumor patient specimens or human glioblastoma xenografts. Briefly, tumors were disaggregated by Papain Dissociation System (Worthington Biochemical) and filtered by 70µm cell strainer to remove tissue pieces according to the manufacturer’s instructions (Detailed Protocol: http://www.worthington-biochem.com/PDS/default.html). Cells were then cultured in stem cell culture medium supplemented as detailed below for at least four hours to recover surface antigens. Cells were then labelled with an allophycocyanin (APC)- or phycoerythrin (PE)-conjugated CD133 antibody (Miltenyi Biotec), and sorted by fluorescence-activated cell sorting (FACS). Alternatively, cells were separated by magnetic sorting column using
microbead-conjugated CD133 antibodies (Miltenyi Biotech). CD133 positive cells were designated as glioma stem cells whereas CD133 negative cells utilized as non-stem glioma cells.

**Cell Culture**

After sorting, glioma stem cells were cultured as suspension in Neural Basal medium supplied with B27, 20ng/ml EGF and 20ng/ml bFGF. Non-stem glioma cells were cultured in DMEM supplied with 10% FBS. Human Microvascular Endothelial Cells (HMVEC) were cultured in Clonetics® Endothelial Cell Systems (Lonza). All cells were grown in cell incubators at 37°C under 5% CO2 gas mixture.

**Intracranial Tumor Assays**

Glioma stem cell and non-stem glioma cell populations were segregated by FACS or MASC sorting. After short-term culture, cells were implanted into the right frontal lobes of athymic BALB/c nu/nu mice under a Duke University Institutional Animal Care and Use Committee–approved protocol as previously described. Mice were maintained up for 4 weeks or until the development of neurologic symptoms. Brains of euthanized mice were collected, fixed in formalin, paraffin embedded, and sectioned, or were frozen rapidly in liquid nitrogen for sections.
Angiogenesis Antibody Array Analysis

Conditioned media were harvested from matched glioma stem cell and non-stem glioma cell cultures grown in 60-mm plates in Neural Basal medium without growth factors for 24 hours. Hypoxia was induced through maintenance of cells in a closed syringe for 24 hours. TranSignal Human Angiogenesis Antibody Arrays (Panomics) were incubated with 2 mL of conditioned medium for 1.5 hours and then replaced with another 2 mL for 1.5 hours (total, 4 mL conditioned medium, 3 hours of incubation) and processed according to the instructions of the manufacturer. Intensity of VEGF expression was quantified with ImageJ software. Total mean intensity = area x mean intensity.

VEGF Enzyme-Linked ImmunoSorbent Assay (ELISA)

Human VEGF Quantikine ELISA Kits (R&D Systems) were used according to the directions of the manufacturer. Two-hundred microliters of conditioned media were collected for each well and samples were triplicated.

Endothelial Migration Assays

Media conditioned by glioma stem cells or matched non-stem glioma cells for 24 hours were added to the bottom chambers of 24-well tissue culture plates in triplicate.
20,000 HMVEC were added to the upper chambers of Transwell assays (BD Biosciences). In experiments with a VEGF neutralizing antibody, bevacizumab or immunoglobulin G (IgG) control (final, 0.5 mg/mL) was added to conditioned media 30 minutes before addition of endothelial cells. Cells were allowed to invade for 14 hours and then fixed, stained, and quantified.

**Endothelial Tube Formation Assays**

Media conditioned by glioma stem cells or matched non-stem glioma cells for 24 hours were added to 2,000 HMVEC in sextuplicate wells of Matrigel-coated 96-well plates (BD Biosciences). In VEGF neutralizing experiments, bevacizumab or IgG control (final, 0.5 mg/mL) was added to conditioned media 30 minutes before addition to endothelial cell cultures in triplicate. Cells were incubated for 16 hours, imaged, and quantified.

**Xenograft Assays**

Matched stem and non-stem glioma cellular populations were implanted in either subcutaneous (s.c.) or intracranial locations with the specified cell number. After establishment, tumor-bearing animals were treated with either bevacizumab or a control IgG (5 mg/kg i.p. qd) for a total of 21 days (s.c.) or 19 days (intracranial) until harvest.
Mice were euthanized and tumors or tumor-bearing brains were harvested, weighed, and examined.

**Statistical Analysis**

Descriptive statistics were generated for all quantitative data with presentation of means ± SEs. Significance was tested by one-way ANOVA using the SAS Enterprise Guide 3.0 (Cary, NC).

### 2.4 Results

#### 2.4.1 Derivation and characterization of Glioma Stem Cells from Xenograft and human Patient Specimen

Glioma stem cells were originally defined and characterized by their phenotypic similarities to neural stem cells (Singh, Clarke et al. 2003; Singh, Hawkins et al. 2004; Bao, Wu et al. 2006). Our lab generate GSC from either glioma xenografts or fresh human glioma specimen. Glioma xenografts were derived from human patient biopsy specimens that were maintained in immunocompromised mice in either subcutaneous or intracranial locations. Human fresh biopsy specimens are more reliable to validate biological processes directly in patient cancers, while human glioma xenografts permit the purification of large numbers of viable tumor cells, in particularly GSC.
We first characterized the brain tumor samples to ensure they are neoplastic cells and match the initial pathological characteristics. FISH analysis proved that all samples exhibited similar chromosome polysomy, EGFR amplification and PTEN loss as that identified by the pathologist when the fresh primary samples were examined (Table 1; Figure 7).

**Table 1: Characteristics of Brain Tumor Patient Specimens.**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Age</th>
<th>Gender</th>
<th>Tumor Stage</th>
<th>Histopathology</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosome 7</td>
</tr>
<tr>
<td>T3359</td>
<td>31</td>
<td>M</td>
<td>Newly Diagnosed</td>
<td>Glioblastoma</td>
<td>Polysomy 62%</td>
</tr>
<tr>
<td>T3691</td>
<td>59</td>
<td>F</td>
<td>Newly Diagnosed</td>
<td>Glioblastoma</td>
<td>Polysomy 77%</td>
</tr>
<tr>
<td>T3565</td>
<td>32</td>
<td>M</td>
<td>Newly Diagnosed</td>
<td>Anaplastic Astrocytoma</td>
<td>Polysomy 52%</td>
</tr>
<tr>
<td>T3832</td>
<td>75</td>
<td>F</td>
<td>Newly Diagnosed</td>
<td>Glioblastoma</td>
<td>Polysomy 82%</td>
</tr>
<tr>
<td>T3946</td>
<td>65</td>
<td>F</td>
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<td>Glioblastoma</td>
<td>Polysomy 64%</td>
</tr>
<tr>
<td>TB080076</td>
<td>68</td>
<td>F</td>
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<td>Glioblastoma</td>
<td>Polysomy 54%</td>
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<tr>
<td>TB080090</td>
<td>60</td>
<td>F</td>
<td>Recurrent</td>
<td>Glioblastoma</td>
<td></td>
</tr>
</tbody>
</table>

Patient and pathological information associated with brain tumor samples used for the isolation of glioma stem cells and non-stem glioma cells is provided. The age and gender of patients as well as the tumor stage and histopathology are included. FISH (Fluorescence in situ hybridization) data determining Chromosome 7, Chromosome 10, EGFR, and PTEN status are included where performed.
Figure 7: Glioma Stem Cells Had Chromosomal Alterations Identical to the Original Patient Specimen.
FISH (Fluorescence in situ hybridization) experiments were performed on isolated T3565 and T3359 GSCs using a Chromosome 10 centromere probe. Polysomy of chromosome 10 occurs in both samples (76% in T3565 and 86% in T3359) at levels consistent with the original patient specimen (73% in T3565 and 80% in T3359).

As reported before (Singh, Hawkins et al. 2004), we utilized the CD133 cell surface antigen to select for a cell population enriched for GSC (Figure 3). Although the percentage of CD133+ glioma cells varied between samples, we frequently detected between 2% and 10% of glioma cells expressed the CD133 marker. In details, we separate CD133+ and CD133− tumor cells through tumor disaggregation, labeling with CD133 antibody, and FACS or MACS sorting. The CD133+ population was >95% pure in repeated FACS analysis. The tumor cell populations derived from human glioma
xenografts grown in mice were composed nearly exclusively of human tumor cells (>99%) without significant contamination from murine cells through analysis with 3B4 anti-human antibody.

Cultured CD133+ glioma cells from biopsy specimens and xenografts formed neurosphere-like spheroids when grown in stem cell medium (Figure 8A). These spheroids were capable of repeatedly generating new spheroids under limiting passage conditions consistent with self-renewal. They expressed neural stem cell markers (CD133, Nestin, Sox2, and Musashi1) (Figure 8B, 9A, 9B), and when cultured under differentiation culture condition (10% FBS), they underwent multilineage differentiation (neuronal, astrocytic, and oligodendrocytic) (Figure 8C). In contrast, CD133− tumor cells grew in an adherent fashion without spheroid formation and they expressed strong differentiation markers (data not shown). Interestingly, unlike CD133+ glioma cells which can be passed for >20 passages, these CD133− cells underwent senescence rapidly in vitro (data not shown).
Figure 8: Glioma Stem Cells Shared Properties With Normal Neural Stem Cells.
(A) GSCs from glioblastoma xenograft D456MG or primary human specimen T3359 and T3317 formed neurospheres in serum free culture medium. (B) Neurospheres formed from GSCs express neural stem cell makers. (C) GSCs were capable to differentiate into multiple lineages.
Figure 9: Glioma Stem Cells Preferentially Expressed Stem Cell Markers.
GSCs and non-stem glioma cells were isolated from the glioblastoma xenograft D456MG or the glioblastoma patient specimens T3691, T3565, or T3359 short-term passaged in immunocompromised mice. (A) GSCs expressed higher levels of the stem cell markers Olig2 and Sox2 in comparison to matched non-stem glioma cells as analyzed by Western. (B) GSCs expressed elevated levels of the stem cell markers Bmi1, Nanog, and Musashi as determined by Real Time PCR in comparison to matched non-stem glioma cells.
Matching the definition of GSC, these CD133+ glioma cells are highly tumorigenic and constantly form glioma-like lesions in mice upon transplantation. CD133+ cells not only give rise secondary tumor but also capable of forming secondary, tertiary even quaternary tumors (Table 2, 3). Therefore, CD133+ cells were designated as glioma stem cells (GSCs) whereas CD133− cells utilized as non-stem glioma cells.

Table 2: Serial In Vivo Transplantation of Glioma Stem Cells.

<table>
<thead>
<tr>
<th>T3359 Stem</th>
<th>1st Round</th>
<th>2nd Round</th>
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<tr>
<td>Cell number</td>
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</tr>
<tr>
<td>Incidence</td>
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<td>3/3</td>
</tr>
<tr>
<td>Median Survival</td>
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<td>52</td>
</tr>
<tr>
<td>Cell number</td>
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<td>1000</td>
</tr>
<tr>
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<td>3/3</td>
</tr>
<tr>
<td>Median Survival</td>
<td>36</td>
<td>44</td>
</tr>
</tbody>
</table>

An in vivo limiting dilution assay was performed with GSCs isolated from T3359 glioblastoma patient specimen then implanted into the right frontal lobes of immunocompromised mice (n=3 per condition). Animals were allowed to survive until the development of neurologic signs at which time brains were harvested for isolation of GSCs for the second round of injections or sectioned for pathologic review to verify the presence of brain tumors. 500 GSCs in both the first and second round of injections were sufficient to form tumors in all injected mice.
Table 3: Serial In Vivo Transplantation of Primary Glioma Stem Cells.

<table>
<thead>
<tr>
<th></th>
<th>Human Specimen</th>
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<th>Median Survival</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2000 Stem</td>
<td>35</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>T08-0076</td>
<td>2000 Stem</td>
<td>35</td>
<td>3/3</td>
</tr>
<tr>
<td>2nd Round</td>
<td>T08-0090</td>
<td>3000 Stem</td>
<td>25.5</td>
<td>2/2</td>
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<tr>
<td></td>
<td>T08-0076</td>
<td>3000 Stem</td>
<td>40</td>
<td>2/2</td>
</tr>
</tbody>
</table>

2000 GSCs isolated directly from T080090 glioblastoma patient specimen or a T080076 glioblastoma patient specimen were implanted into the right frontal lobes of immunocompromised mice (n=3 per arm). Animals were allowed to survive until the development of neurologic signs at which time brains were harvested for isolation of GSCs or sectioned for pathologic review to verify the presence of brain tumors. 3000 GSCs isolated from the xenografted patient specimen in the first round were then injected into animals and monitored as above (n=2) for the serial transplantation.

2.4.2 Glioma Stem Cells Initiate Highly Angiogenic Tumors

Recent studies of GSC have suggested that tumorigenesis is dichotomized between the GSC and non-GSC populations (Galli, Binda et al. 2004; Singh, Hawkins et al. 2004). To investigate the tumorigenic capacity of our glioma subpopulations, 10,000 GSCs or non-stem glioma cells derived from a human glioblastoma patient specimen (T3359) or two human glioma xenografts (D456MG and D54MG) were implanted into the right frontal lobes of athymic nude mice. Regardless of tumor source, every brain implanted with the GSCs displayed gross evidence of highly angiogenic and
hemorrhagic tumors, in stark contrast to the brains implanted with non-stem glioma cells, which usually failed to generate tumors (Figure 10A). Pathologic analysis of the brains implanted with GSCs showed large, highly proliferative and vascular tumors with widespread necrosis and hemorrhage (Figure 10B, 10C). No mice implanted with non-stem glioma cells from human biopsy specimens or the majority of xenografts displayed evidence of brain tumor formation. D456MG xenograft-derived non-stem glioma cells could form small tumors, permitting a comparison of vascularity between brains bearing GSC and non-stem glioma cell xenografts. With CD31 immunostaining, we observed that tumors derived from GSCs were markedly more vascular than tumors from non-stem glioma cells (Figure 10D). Therefore, angiogenic potential is a critical difference between the in vivo tumor phenotypes of GSCs and non-stem glioma cells.
Figure 10: Glioma Stem Cells Formed More Vascular And Necrotic Tumors When Grown In Vivo.
(A) Four pairs of representative brains bearing tumors derived from equal number of non-stem glioma cells and GSCs.
(B-C) Pathologic morphology of brain tumors derived from non-stem glioma cell and GSC populations.
(D) CD31 staining to measure the blood vessel density in tumors derived from non-stem glioma cells and GSCs. The densities of CD31 stained blood vessels were quantified. *, p = 0.0002 (Student’s t test).
2.4.3 Glioma Stem Cells Secrete High Level of VEGF

To determine potential molecular mechanisms underlying the differential vascular phenotypes of GSC and non-stem glioma cell derived tumors, we compared the expression of regulators of angiogenesis secreted by GSCs and non-stem glioma cells from multiple samples. We totally measured the protein levels of 18 pro-angiogenic factors in conditioned medium by using angiogenesis antibody array. Patterns of angiogenesis regulator expression differed between specimens but revealed modest changes in angiogenin, interleukin-8 (IL-8), interleukin-6 (IL-6), and basic fibroblast growth factor (bFGF). However, VEGF levels in GSC conditioned media were consistently up-regulated 10- to 20-fold compared with non-stem glioma cell media in populations derived from both patient biopsies and human glioma xenografts (Figure 11A, 11B, 11C). Increased VEGF secretion in GSCs versus non-stem glioma cells was also confirmed through VEGF ELISA (Figure 11D). As hypoxia is known to regulate the expression of many pro-angiogenic factors including VEGF, we measured the levels of these factors upon hypoxia treatment. Both GSCs and non-stem glioma cells had increased VEGF expression in response to hypoxia, but GSCs still expressed elevated VEGF levels relative to non-stem glioma cells under hypoxic conditions. In summary, GSCs consistently expressed higher levels of VEGF than the non-GSC under both normoxic and hypoxic conditions.
Figure 11: Glioma Stem Cells Expressed Elevated Level of VEGF and Hypoxia Induces Further Increased VEGF Expression.

(A-B), The expression of 18 different proangiogenic factors were detected by commercial antibody arrays (Panomics TranSignal Human Angiogenesis Antibody Array).

(C) Quantification of VEGF signal intensity in A and B.

(D) VEGF levels in conditional media from GSC and non-stem glioma cells were quantitatively determined by ELISA.
2.4.4 Glioma Stem Cells Stimulate the Proliferation of Endothelial Cells

VEGF and other angiogenic factors potently regulate new vasculature formation by stimulating the proliferation and migration of endothelial cells or endothelial cell progenitors. In *in vitro* models of angiogenesis, angiogenic factors promote endothelial cell migration and/or induce formation of vascular tubular structures when cultured on Matrigel. To initially assess the effect of glioma cells on tumor angiogenesis, we tested the ability of conditioned media from both GSCs and non-stem glioma cells to modify endothelial cell phenotypes. GSC conditioned medium consistently increased migration of human microvascular endothelial cells (HMVEC) as compared with non-stem glioma cell conditioned medium (*Figure 12A*). The addition of conditioned medium from GSC cultures also promoted stronger tube formation *in vitro* by HMVECs in comparing to that from matched non-stem glioma cells (*Figure 12B*). Together these results suggest that GSCs cells produce proangiogenic factors that can directly modify endothelial cell behavior.
Figure 12: VEGF Neutralizing Antibody Blocked GSC-induced Endothelial Cell Migration and Tube Formation.

(A) VEGF neutralizing antibody, bevacizumab, blocks GSC-induced endothelial cell migration. *, p = 0.0003, GSC (CD133+) conditioned medium versus non-stem glioma cell (CD133-) conditioned medium (ANOVA); **, p = 0.0005, bevacizumab-treated GSC conditioned medium versus IgG control (ANOVA).

(B) VEGF neutralizing antibody blocks GSC-induced endothelial cell tube formation. *, p < 0.0001, GSC conditioned medium versus non-stem glioma cell conditioned medium (ANOVA); **, p < 0.0001 bevacizumab-treated GSC conditioned medium versus IgG control (ANOVA).
2.4.5 Targeting VEGF Significantly Blocks Angiogenesis and Tumorigenesis Mediated by Glioma Stem Cells

Methods to inhibit tumor-associated angiogenesis are under development, including neutralizing anti-VEGF antibodies and low molecular weight VEGF receptor tyrosine kinase inhibitors. One neutralizing anti-VEGF antibody, bevacizumab (Avastin), binds to human VEGF ligand to prevent endothelial cell receptor activation and has shown combinatorial efficacy with irinotecan (CPT-11) in high grade glioma patients in a phase II clinic trial (Stark-Vance 2005). To determine if the increased levels of VEGF in GSC conditioned media represent the mechanism through which GSCs promote in vitro measures of angiogenesis, we first examined the effect of bevacizumab in our endothelial cell assays. Similar to the above studies, conditioned medium from GSCs derived from a human glioblastoma specimen or a human xenograft, mixed with the IgG control antibody, induced a striking increase in HMVEC migration and tube formation (Figure 12). Addition of bevacizumab to neutralize VEGF in GSC conditioned medium blocked its proangiogenic effects on endothelial cell migration and tube formation to levels equal to the effects of non-stem glioma cell conditioned medium. In contrast, bevacizumab displayed only marginal effects on human microvascular endothelial cells exposed to non-stem glioma cell conditioned medium (Figure 12). Thus, the effects of GSCs on endothelial cell behaviors are dependent on VEGF activity.
and bevacizumab specifically blocks the effects of GSCs on endothelial cell behavior in cell culture assays.

To confirm the relevance of the in vitro results, we examined the in vivo effects of inhibiting the VEGF axis through the use of bevacizumab. We quantified the therapeutic effects of bevacizumab on s.c. xenografts derived from matched GSC and non-stem glioma cell populations. Bevacizumab potently inhibited the growth of GSC-derived xenografts with a reduction in tumor weight, vascularity, and hemorrhage. In contrast, the small tumors derived from non-stem glioma cells were not significantly inhibited in growth by bevacizumab (Figure 13). Parallel results were noted in intracranial studies (data not shown).

In summary, these data demonstrated that VEGF is critical to the tumorigenesis from GSCs and blocking VEGF by neutralizing antibody bevacizumab significantly diminished the tumor formation. Therefore, anti-angiogenesis andante-VEGF therapies may be effective against glioblastomas, either by themselves or by combination with other chemotherapy agents.
Figure 13: VEGF Neutralizing Antibody Blocked Tumorigenesis from Glioma Stem Cells and Non-stem Glioma Cells.

(A) Treatment with bevacizumab inhibits the \textit{in vivo} tumor growth of xenografts derived from GSC and blocks the GSC-induced tumor hemorrhage \textit{in vivo}.

(B) Bevacizumab shows limited efficacy against xenografts derived from non-stem glioma cells.

(C) The tumor weights for tumors from (A) and (B) were quantified. *, \( p < 0.001 \), bevacizumab treatment versus control IgG. **, \( p > 0.5 \), bevacizumab treatment versus control IgG.
3. Glioma Stem Cells Upregulate Hypoxia Inducible Factor 2α to Promote Tumor Angiogenesis

3.1 Summary

Hypoxia is a key factor regulating VEGF production and other angiogenesis processes in solid tumors including gliomas. As we have previously found that glioma stem cells (GSCs) promote angiogenesis, we examined whether GSCs have differential hypoxia responses in comparison to non-stem glioma cells. Using real-time PCR, we measured the mRNA level of a set of hypoxia response genes and found that hypoxia-inducible factor 2 alpha (HIF2α) and its downstream targets are significantly increased in GSCs in comparison to non-stem glioma cells. Higher level of HIF2α mRNA results in up-regulated expression of HIF2α protein in GSCs. Both mRNA and protein level of HIF2α could be further induced upon hypoxia treatment in GSCs. In primary tumor specimens, HIF2α co-localizes with GSC markers such as CD133 and Olig2. In contrast, another widely studied HIFa family member, HIF1α, is usually expressed at a higher level in non-stem glioma cells at protein level. The striking up-regulation of HIF2α is partially due to increased mRNA synthesis in GSCs. The up-regulation of HIF2α is not a general stem cell phenomenon as HIF2α is almost undetectable in normal neural progenitor cells. Together, our data show that GSCs specifically up-regulate HIF2α at both normoxia and hypoxia condition in comparison to non-stem glioma cells or normal neural progenitors. We also proved that both HIF2α and HIF1α are required for GSC
mediated VEGF production and angiogenesis. Targeting the expression of either HIF2α or HIF1α using lentiviral short hairpin RNA (shRNA) significantly decreased VEGF secretion from GSCs and abrogated the GSC-mediated endothelial cell proliferation.

3.2 Introduction

We have previously demonstrated that GSCs have a greater ability to promote tumor angiogenesis through secreting elevated level of VEGF. However, the upstream regulators responsible for up-regulating VEGF in GSCs remain to be defined. The regulation of tumor angiogenesis is complicated and many factors could lead to altered angiogenic drive and VEGF secretion. For instance, the activation of multiple oncogenes like Akt, EGFR have been shown to up-regulate VEGF and other pro-angiogenic factors (Rak, Mitsuhashi et al. 1995). Besides, tumor angiogenesis process is tightly linked to microenvironment conditions (Carmeliet 2005). When short in blood supply, tumor cells would be surrounded by microenvironment characterized by hypoxia, low PH and high concentration of lactic acid. All of these conditions are capable of promoting the production of many pro-angiogenic factors including VEGF, which results in new blood vessel growth. Among all of those, hypoxia has been proved to be one of the most important factors. Hypoxia is a common phenomenon in most solid tumors and a well-known regulatory factor for the “angiogenic switch”, which represents a key step in
tumor progression. We therefore hypothesized that there are unique hypoxia responses in GSCs, which contribute to elevated angiogenesis potential of these cells.

Cellular responses to hypoxia are commonly regulated by the hypoxia inducible factor (HIF) family of transcriptional factors. HIFα function as heterodimers consisting of an oxygen sensitive HIFα subunit and a constitutively expressed HIFβ subunit (Kaur, Khwaja et al. 2005). Under normoxic conditions, HIFα is ubiquinated by the Von Hippel-Lindau (VHL) tumor suppressor gene product and then targeted for proteasomal degradation, but under hypoxia the interaction between HIFα and VHL is abrogated. As a result, HIFα is stabilized, dimerizes with HIF and then binds to hypoxia responsive elements (HREs) in the promoters of hypoxia regulated genes. This activates the transcription of hundreds of downstream genes which induce survival, motility, metabolism and angiogenesis (Harris 2002; Bertout, Patel et al. 2008). Two HIFα proteins, HIF1α and HIF2α, are highly homologous and bind to similar HRE sequences. HIF1α is universally expressed in the body but HIF2α shows a more restricted expression pattern and it is only expressed in limited number of tissues such as endothelial cells, heart, lung and central nerve system (Wang, Davis et al. 2005). Therefore, HIF2α was largely treated as a redundant protein of HIF1α and relatively few studies have determined the role of HIF2α in cancer initiation or tumor progression (Raval, Lau et al. 2005; Covello, Kehler et al. 2006; Holmquist-Mengelbier, Fredlund et al.)
2006; Hu, Iyer et al. 2006). However, it is now clear that HIF1α and HIF2α can often play non-overlapping biological roles due to their unique target genes as well as different requirement of oxygen for activation (Holmquist-Mengelbier, Fredlund et al. 2006). Another family member, HIF3α, lacks the transcriptional activation domain and functions as a dominant negative regulator of the hypoxia response due to sequestration of HIFα. As both HIF1α and HIF2α are direct upstream factor regulating VEGF expression level, we examined the possibility that high HIFα activity results in elevated VEGF secretion in GSCs.

In this chapter, we investigated the hypoxia response profiles in GSCs and matched non-stem glioma cells and we have found that HIF2α levels and HIF2α-regulated gene expression are differentially elevated in GSCs in comparison to non-stem glioma cells and normal neural progenitor cells. We also demonstrated that both HIF1α and HIF2α contribute to VEGF secretion and GSC-mediated endothelial cell proliferation. Therefore, elevated HIF2α is one key factor that contributes to the enhanced angiogenesis capability of GSCs.
3.3 Materials and Methods

Isolation of Glioma Stem Cells, Non-stem 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 (Bao, Wu et al. 2006). Normal human neural progenitors were obtained from Lonza and considered exempt as human subjects by the Duke Institutional Review Board.

Tissue Culture and Hypoxia Induction

GSCs were cultured in Neurobasal media with B27 without Vitamin A (Invitrogen), bFGF (20 ng/ml) and EGF (20 ng/ml). After trypsinizing, non-stem glioma 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 Neural Basal medium in order for experiments to be performed in identical medium. Non-stem glioma cells were cultured in Neural Basal medium for at least 24 hours before experiments performed. In order to induce hypoxia, cells were cultured in hypoxia chambers (Sheldon Manufacturing for 0.2% O2, Sanyo for 1%, 2% and 5% O2).
Alternatively, cells were treated by 100 or 200 M hypoxia-mimic chemical deferrioxamine mesylate (DFX, Sigma).

**Real Time Polymerase Chain Reaction**

mRNA was extracted by RNeasy Mini Kit (Qiagen, Inc), followed by cDNA synthesis using SuperScript® III First-Strand Synthesis Kit (Invitrogen, Inc). To investigate single gene expression pattern, individual gene primers and Master Mixes were purchased from SuperArray Bioscience Corporation. For real time PCR array, 384 wells Human Hypoxia Signaling Pathway PCR Array Kits were purchased from SuperArray Bioscience Corporation (APHS-032E).

**Western Blots**

Equal amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore) and detected using an enhanced chemiluminescence system (Pierce Biotechnology). Rabbit Polyclonal Antibodies for human HIF1α (NB100-449), HIF2α (NB100-122) were purchased from Novus Biologicals, Inc. Antibody for α-Tubulin was purchased from Sigma-Aldrich.
Lentiviral Mediated shRNA Targeting

Lentiviral shRNA clones (Mission RNAi) targeting HIF1α, HIF2α VEGF and non-targeting control sequences were obtained from Sigma. Lentiviruses were produced in 293FT cells with packaging 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.

VEGF Transcriptional Reporter Assays

100,000 lentiviral infected glioblastoma stem or non-stem cells were transfected in parallel with 2 μg of empty pGL2basic luciferase reporter vector as control or pGL2 basic containing the full length (2.7 kb) VEGF promoter upstream of luciferase (purchased from ATCC). Transfection efficiency was controlled by simultaneously transfecting 0.1 μg of a renilla luciferase reporter (phRL-null vector; Promega). After transfection, parallel cultures were subjected to either normoxic or hypoxic conditions (200 μM DFX) for 24 hours. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega) with a Fluostar Optima luminometer (BMG Labtech).
Immunohistochemistry

Paraffin-embedded primary human glioblastoma sections or intracranial glioblastoma xenograft underwent IHC staining for CD133 (ab19898/Abcam), Olig2 (kind gift from Dr. John Alberta), HIF2α (MAB3472/Chemicon), HIF1α(NB100-479/Novus), or CD31 (ab28364/Abcam) following protocol as previously described (Sathornsumetee et al., 2008). Citrate buffer (PH 6.1) was used for antigen retrieval in most cases except for HIF2α staining which used Tris-EDTA (PH 9.0). To minimize background, CD133 and HIF2α staining were performed by alkaline phosphatase (AP) conjugated secondary antibody, while Olig2, HIF1α and CD31 staining were performed using horseradish peroxidase (HRP) conjugated secondary antibody.

Immunofluorescence

Immunofluorescence stainings for the GSC differentiation experiment were performed using antibodies for Tuj1, S100 and GFAP (Abcam). Double immunofluorescence staining of CD133 (Ab19898/Abcam) and HIF2α (MAB3472/Chemicon) were performed on primary human glioblastoma frozen sections. Briefly, slides were fixed by 4% paraformaldehyde for 15 minutes at room temperature and blocked by 10% goat serum. Slides were stained by CD133 and HIF2μ antibodies for 16 hours at 4 degree followed by Rhodamine Red-X-AffiniPure Donkey Anti- Rabbit
secondary antibody and Fluorescein (FITC) AffiniPure Donkey Anti Mouse secondary antibody (Jackson-Immuno research). At last, mounting medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was used and pictures were taken by Leica SP5 Confocal microscope (Duke Light Microscopy Core Facility).

3.4 Results

3.4.1 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 malignant glioma surgical biopsy specimens or xenografts derived from glioma specimens using our previously described methodology (Bao, Wu et al. 2006). Using matched cultures of GSCs or non-stem glioma cells, we compared the mRNA levels of hypoxia-regulated genes in GSCs or non-stem glioma cells under normoxia (20% O2) and hypoxia (1% O2). Multiple hypoxia responsive genes were strongly differentially regulated between GSCs or non-stem glioma cells isolated from the same tumor specimens, including HIF2A (but not HIF1A) (Table 4).
Table 4: mRNA Levels of Hypoxia Response Genes Are Differentially Expressed in Glioma Stem Cells and Non-stem Glioma Cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>2D45GMO Stem</th>
<th>2D45GMO Non-stem</th>
<th>T3359 Stem</th>
<th>T3359 Non-stem</th>
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<td>1.05</td>
<td>1.00</td>
<td>0.80</td>
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</tbody>
</table>

63
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 non-stem glioma cells (Figure 14A, 14B, 15A, 15B). Similar patterns of mRNA expression were detected using atmospheric hypoxia (1% O2), or the iron chelator, desferrioxamine (DFX), which induces molecular hypoxic responses with similar kinetics to hypoxia (Wang and Semenza 1993) (Figure 16A, 16B). These data demonstrate that HIF2A, but not HIF1A, is a hypoxia responsive gene dramatically up-regulated in GSCs.

Considering the differential expression of HIF2A and HIF1A in GSC sand non-stem glioma cell subpopulations, we determined the mRNA expression of genes known to be specifically regulated by HIF2α or HIF1α. Genes reported to be HIF2α dependent [Oct4, Glut1 and SerpinB9] (Holmquist-Mengelbier, Fredlund et al. 2006) were expressed at significantly higher levels in GSCs as compared to matched non-stem glioma cells under hypoxia. In contrast, the HIF1α-regulated gene phosphoglycerate kinase 1 (PGK1) was strongly up-regulated in non-stem glioma cells under hypoxia as compared to matched GSCs. Analysis of VEGF, a gene regulated by HIF1α and/or HIF2α in a cell specific manner, demonstrated that hypoxia induced a greater increase in VEGF levels in GSCs than in matched non-stem glioma cells (Figure 14, 15, 16).
Figure 14: D456MG Glioma Stem Cells and and Non-stem Glioma Cells Differentially Expressed Hypoxia Response Genes.

Cells isolated from D456MG were treated with DFX for the time indicated. Real time PCR was performed with primers specific for HIF2α (A), HIF1α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGFα (F), SerpinB9 (G), and VEGF (H). Data were normalized to beta-Actin. #, p < 0.01 with ANOVA comparison of GSCs to non-stem glioma cells with identical treatments. *, p < 0.01 with ANOVA comparison of GSCs under hypoxia vs. normoxia.
Figure 15: T3946 Glioma Stem Cells and and Non-stem Glioma Cells Differentially Expressed Hypoxia Response Genes.

Cells isolated from D456MG were treated with DFX for the time indicated. Real time PCR was performed with primers specific for HIF2α (A), HIF1α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGFα (F), SerpinB9 (G), and VEGF (H). Data were normalized to beta-Actin. #, p < 0.01 with ANOVA comparison of GSCs to non-stem glioma cells with identical treatments. *, p < 0.01 with ANOVA comparison of GSCs under hypoxia vs. normoxia.
Figure 16: Differential Expression of HIF2A and Hypoxia Response Genes in Glioma Stem Cells Was Similar when Hypoxia Is Induced by a Chemical Mimetic or Low Oxygen Tension.
3.4.2 Glioma Stem Cells Express High Level of HIF2α but Not HIF1α Protein

We interrogated the impact of transcriptional upregulation of HIF2α mRNA in GSCs on HIF2α protein levels. Although HIF1β and HIF3α levels did not differ between GSCs and non-stem glioma cells under normoxia and hypoxia conditions (data not shown), total HIF2α protein expression was consistently higher in GSCs than in matched non-stem glioma cells. HIF2α was highly expressed in GSCs treated with a chemical hypoxia-mimetic or grown in a hypoxia chamber under oxygen concentrations ranging from 0.2 to 5% (Figure 17). In contrast, HIF1α expression was only increased by more severe hypoxic conditions induced by the chemical mimetic or ≤1% O2 (Figure 17A-H), which is consistent with a previous report that HIF2α (but not HIF1α) accumulates under physiological oxygen levels present in solid tumors (Holmquist-Mengelbier, Fredlund et al. 2006). When oxygen levels were sufficient for HIF1α induction, HIF1α was often expressed at higher levels in non-stem glioma cells than matched GSCs (Figure 17A-H). These data suggest that GSCs preferentially express HIF2α protein under both normoxic and hypoxic conditions to provide GSCs a survival and growth advantage by activating downstream genes even in the modest hypoxia conditions.
Figure 17: HIF2α Protein Expression Was Dramatically Increased in Glioma Stem Cells As Compared to Non-stem cells.

GSCs and non-stem glioma cells isolated from glioblastoma xenografts (A-C), a glioblastoma patient specimen passaged short-term in immunocompromised mice (D) or directly isolated from brain tumor patient specimens (E-F) were treated with 200 μM DFX to mimic hypoxia for the indicated times. Nuclear and cytoplasmic fractions (B) or total cell lysate (A, C-F) were separated and analyzed by immunoblotting.

(G-I) Low oxygen tension induced HIF2α protein in GSCs but not non-stem glioma cells. Cell were treated by 0.2% O2 (G), 1% O2 (H) or 2% O2 (I), for indicated time.
3.4.3 HIF2α is Regulated at both Transcriptional and Post-translational Level

Both HIF1α and HIF2α were believed to be primarily regulated at protein stability level. Once hydroxylated, HIFα will be ubiquitinated and degraded by proteosome systems. We tested whether HIF2α is regulated at post-translational level in GSCs. We used cycloheximide (CHX) to block protein biosynthesis and found CHX treatment abrogated the induction of HIF2α protein in GSCs, indicating that protein synthesis machine is required for HIF2α expression (Figure 18A). We then tested whether HIF2α protein is regulated by ubiquitin-proteosome system in GSCs. To examine this, we treated the cells with MG132, which is an inhibitor of proteosome. Of note, upon MG132 treatment, HIF2α protein was stabilized in GSCs even without hypoxia treatment (Figure 18B). This data indicated that HIF2α protein is regulated by proteosomal degradation. However, VHL, the E3 ligase responsive for HIFα degradation, was expressed at very similar levels between GSCs and non-stem glioma cells (data not shown), indicating the proteosome-mediated degradation is not the primary mechanism for the difference of HIF2α protein levels in GSCs and non-stem glioma cells.

Consistent with traditional notion that HIF1α is primarily regulated at post-translational level, the HIF1α mRNA did not change upon hypoxia treatment, nor did it show significant difference between GSCs and non-stem glioma cells (Table 4; Figure 18C).
In contrast, the mRNA of HIF2α was induced by hypoxia treatment and it showed much higher level in GSCs in comparison to matched non-stem glioma cells. The elevated HIF2A mRNA levels in GSCs may result from enhanced mRNA transcription and/or increased mRNA stability. Therefore, we measured the half-life of HIF2A mRNA in both GSCs and non-stem glioma cells. Surprisingly, the half-life of HIF2A was actually shorter in GSCs in comparison to matched non-stem glioma cells (Figure 18C), 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 18D). To determine the relative levels of transcription activity of the HIF2α promoter in the tumor subpopulations, we performed the RNA Polymerase II ChIP assay. GSCs under both normoxia and hypoxia had a greater enrichment of RNA Polymerase II binding to the HIF2A promoter than matched non-stem glioma cells (Figure 18E). As the recruitment of RNA Polymerase II to the promoter is a direct indicator of transcription rate, it proved that GSCs have amplified HIF2A mRNA transcription. Together, these data demonstrate that HIF2A mRNA is up-regulated in GSCs with enhanced transcription.
Figure 18: HIF2A Upregulation Induced by Hypoxia in Glioma Stem Cells Was Due to Enhanced Transcription.

(A) New protein synthesis is necessary for the induction of HIF2α protein expression under hypoxia.

(B) Inhibition of proteosome mediated degradation stabilizes HIFα protein.

(C) HIF2A mRNA degradation is greater in GSCs than matched non-stem glioma cells.

(D) De novo transcription is necessary for stimulation of HIF2A mRNA expression under hypoxia.

(E) Chromosome Immunoprecipitation (ChIP) assay using RNA polymerase II antibody indicates the HIF2α promoter is preferentially activated in GSCs.
3.4.4 HIF2α Co-localizes with Other Glioma Stem Cell Markers

After confirming that HIF2α is up-regulated in GSCs but not non-stem glioma cells \textit{in vitro}, we then moved on to examine the expression pattern of HIF1α and HIF2α \textit{in vivo}. We first preformed immunohistochemistry (IHC) on paraffin embedded primary human glioblastoma surgical biopsy specimens. In the human glioma sections, HIF1α antibody marked the majority of tumor cells (~60%) arranged about the regions of necrosis in most samples. In contrast, HIF2α demonstrated more variable and rare staining, predominately located immediately around regions of necrosis, where it bound to 1 to 10% of cells. HIF2α was also frequently observed around proliferating blood vessels where 1% to 10% of tumor cells were stained (Figure 20A, 22A). Of note, previous studies suggest that the perivascular region is enriched for brain tumor stem cells (Calabrese, Poppleton et al. 2007; Christensen, Schroder et al. 2008). Consistent with these results, we found that CD133 and another potential GSC marker, Olig2 (Ligon, Huillard et al. 2007), were expressed by 1-10% of tumor cells adjacent to blood vessels (Figure 20A, 22A).

The similar expression pattern of HIF2α and cancer stem cell GSC markers CD133 and Olig2 suggests that they may be co-expressed by GSCs. We therefore performed double immunofluorescence staining studies on frozen primary human glioma samples to determine if HIF2α and CD133 co-localized \textit{in vivo}. We found about
70% primary patient samples express HIF2α protein at various levels. In these HIF2α positive samples, we detected that most tumor cells that expressed HIF2α co-expressed CD133, although not all CD133 positive cells expressed HIF2α (Figure 20B). FACS analysis of glioma stem and non-stem populations also confirmed the co-expression of CD133 and HIF2α (Figure 19). Together, our data suggest HIF2α is a molecular immunophenotype specific for GSCs.

Figure 19: HIF2α Co-Expressed with Glioma Stem Cell Marker CD133.

Cells expressing the glioma stem cell marker CD133 also express HIF2α. GSCs and non-stem glioma cells isolated from T3691 (A) or T3359 (B) cells passaged short-term in immunocompromised mice were analyzed for CD133 and HIF2α expression via FACS using anti-CD133-APC and anti-HIF2α-PE.
Figure 20: HIF2α Was Co-Expressed with Glioma Stem Cell Markers in Human Glioblastoma Biopsy Specimens.

(A) Restricted pattern of HIF2α and stem cell marker expression in human brain tumor patient specimens. Immunohistochemical analysis of sections of human brain tumor patient specimens demonstrates the presence of cells expressing the glioma stem cell markers CD133 and Olig2 as well as HIF2α and HIF1α.

(B) Immunofluorescence of cells in human brain tumor patient specimens demonstrates co-localization of CD133 and HIF2α. Majority of HIF2α positive tumor cells were also CD133+. 
3.4.5 Differentiation of Glioma Stem Cells Leads to Reduction of HIF2α Level

GSCs are able to give rise to non-stem glioma cells when they are induced to differentiate. Because HIF2α is significantly up-regulated in GSCs in comparison to non-stem glioma cells, we tested whether GSCs differentiation would lead to the down-regulation of HIF2α protein. We induced the differentiation of GSCs by withdrawing the growth factors EGF and bFGF from the culture medium as previous described. Notably, removal of growth factors resulted in significant morphology change of cells. GSCs began to lose their neurosphere forming capability and exhibit neuron or glia-like characteristics (Figure 21B). This process is also associated with the loss of GSC markers as well as the induction of differentiation markers such as GalC and MAP2, which is determined by both western blots and immunofluorescence staining (Figure 21). All these data confirmed that GSCs were successfully undergoing differentiation. Interestingly, HIF2α protein level significantly decreased during differentiation. To the contrast, HIF1α level was up-regulated at the same time (Figure 21A). This is consistent with previous data which indicate that non-stem glioma cells usually have higher HIF1α induction in hypoxia treatment. Together, these data demonstrate that the HIF2α over-expression in GSCs relies on its “stemness status”. HIF2α was probably controlled by some key factors that also regulate GSC self-renewal and differentiation. Besides, they
also suggest that there is a shift from HIF2α-mediated to HIF1α-mediated cellular hypoxia response during the differentiation of GSCs.

**Figure 21: Differentiation of Glioma Stem Cells Was Associated with Decreased HIF2α and Increased HIF1α Expression.**

(A) GSCs isolated from D456MG xenograft and T3691 patient specimen were induced to differentiate through withdrawal of EGF and bFGF. 10 days later, cells were treated with DFX for 12 hours and harvested for western blot analysis. Increased expression of the oligodendrocyte differentiation marker GalC and the neuronal differentiation marker MAP2 indicated that cells were under differentiation. Differentiated cells have decreased HIF2α levels in comparison to maintained GSCs. In contrast, HIF1α is strongly increased in differentiated cells in comparison to GSCs.

(B) Upon bFGF withdrawal, GSCs demonstrated the typical morphology of glia and neuron cells as well as the expression of differentiation markers including Tuj1 (neurons), S100 (astrocytes), and GFAP (astrocytes).
3.4.6 Glioma Samples Express Various Level of HIF2α

All the data above suggested that HIF2α may serve as a marker for GSCs in malignant gliomas. However, whether it is a universal marker in all glioma patients remains unknown. We therefore examined the expression level of HIF2α in a set of glioma specimens. Using immunohistochemistry, we found that there is an obvious heterogeneity in gliomas in term of HIF2α expression pattern. Briefly, around 70% of glioma samples showed significant HIF2α staining (Table 5; Figure 22B). Among these HIF2α positive samples, the staining intensity remarkably varies among cases and the percentage of HIF2α positive cells showed wide range difference (Figure 22B). To identify the range of variation around necrotic foci, the mean percentage of HIF2α labeled cells for five different necrotic regions per tumor were evaluated when observed in tumor sections. Consistent with the notion that HIF2α is a hypoxia response gene, we found that there was generally higher intensity of HIF2α staining around the necrosis areas, but there greater variation in the percentaged stained in areas of necrosis than in the solid, non-necrotic regions (parenchymal) (Table 5; Figure 22B). Together, these data suggest that HIF2α expression level is highly variable among individual cases and there may be GSCs in some glioma samples that are HIF2α independent.
Figure 22: HIF2α Was Co-Expressed with Glioma Stem Cell Marker Olig2 in Glioblastoma Samples.

(A) HIF2α but not HIF1α is expressed in pattern similar to the glioma stem cell marker Olig2. Olig2 and HIF2α immunostaining were more restricted and more similar than HIF1α immunostaining in adjacent sections from human brain tumor patient specimens.

(B) Representative images of immunohistostaining for HIF2α in multiple glioblastoma samples. Glioblastoma samples express HIF2α at variable levels with a higher percentage of stained cells around regions of necrosis as pictured in samples 2155, 3243, and 890.
Table 5: HIF2α Staining in Primary Glioblastoma Specimens.

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Immunohistostaining of HIF2α was performed on 19 primary glioblastoma specimens. Pathologic review of the resulting slides determined a large variation of HIF2α expression level among samples and additional intratumoral heterogeneity. Data is presented as a single mean percentage of HIF2α labeled cells for parenchymal regions. For necrosis area, tumor cells were counted in the regions about the necrosis, as macrophages entrapped in the necrosis appeared to bind the stain. The values in the table are presented in graphical format to illustrate the variation of the individual data points (black) and the average (red) percentage of HIF2α positive cells in each region.

3.4.7 Glioma Stem Cells Overexpress HIF2α in Comparison to Normal Neural Stem Cells

We found HIF2α level is down-regulated when GSCs were induced to differentiate, indicating HIF2α over-expression may be directly linked to general stem cell biology. Therefore, we further examined whether HIF2α up-regulation in GSCs
occurs as a common stem cell phenotype. We measured the mRNA and protein level of HIF2α in commercially available normal human neural progenitor cells (NPCs). In some experiments, we further FACS sorting these NPCs by CD133 to enrich neural stem cells (NSCs). Surprisingly, in contrast to GSCs, the HIF2A mRNAs in human normal NPCs or NSCs were almost undetectable (Figure 23A, 24A). Similar to the mRNA data, we failed to detect HIF2α protein in normal human NPCs or NSCs resulting in consistent over-expression of HIF2α in the glioma stem cell population (Figure 25). Besides, we isolated normal murine NSCs isolated from postnatal mice and then cultured these cells under normoxia or hypoxia. In either condition, minimal HIF2A mRNA expression was detected, suggesting murine NSCs do not up-regulate HIF2A mRNA either (Figure 23D). In contrast, HIF1α protein was accumulated in both human NPCs and GSCs under hypoxia (Figure 25). We also compared the mRNA level of HIF1α and or HIF2α target genes in human NPCs and GSCs and found that the levels of hypoxia downstream factors in NPCs were variable relative to GSCs, with Glut1 demonstrating a consistent elevation in GSCs (Figure 24, 25C). Taken together, these data indicate that HIF1α up-regulation is a shared molecular response to hypoxia in both normal NPCs and GSCs while HIF2α induction is restricted to GSCs. As HIF2α is not up-regulated in either non-stem glioma cells or normal neural stem cells, it suggests that HIF2α up-
regulation may be a result of synergic actions of stem cell maintenance pathways and cancer related signalings.

Figure 23: Elevated HIF2A Expression in Glioma Stem Cells in Comparison to Normal Neural Progenitors.
(A-C) GSCs were isolated from a D456MG xenograft. Normal fetal neural progenitors were purchased from Lonza Inc. All cells were treated with 200 μM DFX to mimic hypoxia for 8 hours. Real Time PCR analysis was performed with primers specific for HIF2α (A), HIF1α (B), and VEGF (C). #, p < 0.001 by ANOVA comparison of GSCs to neural progenitors with identical oxygen treatment. *, P < 0.001 with ANOVA comparison of GSCs under hypoxia vs. normoxia. ≈, p < 0.001 with ANOVA comparison of neural progenitors under hypoxia vs. normoxia.
(D) Normal adult mouse neural progenitors were isolated from the brains of 2 month old C56B6 animals and cultured under 1% oxygen overnight. Real Time PCR analysis was performed with primers specific for mouse HIF2α, HIF1α, and VEGF. *, p < 0.001 with t-test comparison of adult mouse neural progenitors under hypoxia vs. normoxia.
Figure 24: Glioma Cells and Normal Neural Progenitors Differentially Expressed Hypoxia Response Genes.

Cells were cultured in 20% or 1% oxygen for 24 hours. Real time PCR was performed with primers specific for HIF2α (A), HIF1α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGFα (F), SerpinB9 (G), and VEGF (H). *, p < 0.01 with comparison to both hypoxia treated GSCs. #, p < 0.01 with comparison of hypoxia treated cells to identically prepared normoxia controls.
Figure 25: Glioma Stem Cells Overexpressed HIF2α Protein in Comparison to Normal Neural Progenitor Cells.

(A) T3359 GSCs and CD133+ normal neural progenitors isolated by FACS analysis from a commercially available (Lonza) sample (15167) were treated with DFX for the indicated times. HIF2α protein expression was dramatically increased with hypoxia in GSCs as compared to normal neural progenitors.

(B) HIF2α protein expression was dramatically increased with hypoxia in GSCs as compared to normal neural progenitors. HIF1α protein expression was induced in both GSCs and normal neural progenitors to different extents.

(C) Higher HIF2α protein expression is maintained with a relatively physiological level of oxygen in GSCs as compared to non-stem glioma cells or normal neural progenitors. DFX treated lysates were utilized as a positive control.
3.4.8 Successful Knockdown of HIF1α and HIF2α by shRNA

We already confirmed that HIF2α and HIF1α regulation differed between GSCs and non-stem glioma cells. We next sought to examine the requirement for HIFα in VEGF production in GSCs and GSC mediated angiogenesis. We used a lentiviral shRNA based system to specifically target HIF1α or HIF2α expression. 48 hours after lentivirus infection, mRNAs were harvested and RT-PCR was performed to determine the efficacy of these shRNA constructs. We constantly achieved knockdown efficiency of ~70-95% for both HIF1A and HIF2A at the mRNA level (Figure 26B, 26C, 27A, 27B), although the efficiency of HIF1α knockdown was consistently greater than that of HIF2α at the protein level (Figure 26A, 28B). In order to test the specificity and biological consequences of knocking down HIFα, we measured the mRNA levels of multiple genes that are reported to be specifically regulated by HIF1α or HIF2α. Consistent with previous report, HIF2α knockdown was selectively associated with reduced mRNA levels of HIF2α target genes such as Glut1 and SerpinB9, while targeting HIF1α significantly decreased the mRNA of PGK1, a gene primarily regulated by HIF1α but not HIF2α (Holmquist-Mengelbier, Fredlund et al. 2006; Keith and Simon 2007) (Figure 27). These data validated the ability to specifically target HIF2α and HIF1α with resulting distinct molecular effects.
Figure 26: Specific Knockdown of HIF1α and HIF2α Using ShRNA.

(A) HIF1α and HIF2α protein levels were significantly reduced by specific shRNAs. Lentivirus expressing shRNA directed against HIF1α, HIF2α, or a non-targeting (NT) control shRNA was used to infect both GSCs and non-stem glioma cells from T3359. Cells were harvested 48 hours after infection and 8 hours after treatment with 200 μM DFX.

(B-C) Successful knockdown of HIFα at mRNA level by multiple shRNA constructs. Two different shRNAs were used to target HIF1α and HIF2α in GSCs isolated from T3565 (B) or T3691 (C) mRNA was harvested 48 hours after infection and Real Time PCR was performed using primers specific for HIF1α and HIF2α. *, p<0.01 with ANOVA comparison to non-targeting control (NT)
Figure 27: HIF1α and HIF2α were Differentially Required for Hypoxia Response Genes in Glioma Stem Cells.

GSCs infected with the indicated shRNAs were treated with 200 µM desferrioxamine (DFX) for 8 hours to mimic hypoxia. Real time PCR was performed with indicated primers. PGK1 was a HIF1α dependent gene while Glut1 and SerpinB9 were HIF2α dependent. * p < 0.01 with ANOVA comparison to non-targeting control (NT) under identical oxygen conditions.
3.4.9 HIF1α and HIF2α is Required for Glioblastoma Stem Cell Mediated Angiogenesis

Next, we determined whether HIF2α and HIF1α are required for VEGF expression and angiogenic effects of GSCs and non-stem glioma cells. We first assessed the VEGF transcription in HIFα knockdown cells by using a VEGF promoter based luciferase assay system. Knockdown of HIF2α or HIF1α in GSCs significantly reduced VEGF promoter activity in hypoxia condition (Figure 28A). Consistent with a decreased promoter activity, we observed reduced level of VEGF mRNA in HIF2α knockdown cells (Figure 28D, 28E). We also measured the VEGF protein in GSCs and found that HIFα knockdown led to significant decrease of both intracellular VEGF (as determined by western blots) (Figure 28B) and secreted VEGF protein (as determined by ELISA) (Figure 28AC). We also performed similar experiments in matched non-stem glioma cells. Interestingly, HIF1α showed similar effects as targeting HIF1α reduced VEGF at both mRNA and protein levels. However, there was no requirement for HIF2α as HIF2α knockdown did not change VEGF transcription or protein production in these cells. These results strongly suggest that HIF1α is required in both GSCs and non-stem glioma cells for the induction of VEGF expression by transcriptionally regulating the VEGF promoter, whereas there is a specific requirement for HIF2α for VEGF production in GSCs.
As VEGF supports glioma angiogenesis through regulation of endothelial cell proliferation and survival, we attempted to determine if knockdown of HIFα in GSCs and non-stem glioma cells could significantly impact endothelial cell growth. We therefore performed a co-culture experiment, in which glioblastoma cells were cultured in an upper chamber while human microvascular endothelial cells (HMVEC) were planted in the lower wells. These two parts were separated by a permeable membrane with 0.4 μm pores, which prevented physical contact between glioblastoma cells and endothelial cells but allowed transfer of secreted factors (Figure 29A). Consistent with our previous report, GSCs significantly increased endothelial cell numbers and proliferation in comparison to non-stem glioma cells, as determined by direct cell number counting and [3H]-thymidine incorporation assay on HMVEC (Figure 29). Knockdown of either HIF2α or HIF1α in GSCs reduced the paracrine effects of GSCs on endothelial cells. However, endothelial cell growth supported by non-stem glioma cells was only affected by targeting HIF1α but not HIF2α (Figure 29). These data are consistent with the observed differences in HIFα requirements for VEGF expression in the tumor subpopulations, and suggest a specific role for HIF2α in GSC-mediated angiogenesis by affecting endothelial cell behaviors.
Figure 28: HIFα Knockdown Decreased VEGF Production in Glioma Stem Cells.
(A) HIF2α knockdown prevents hypoxia induced activation of the VEGF Promoter. Lentiviral infected GSCs and non-stem glioma cells were transfected in parallel with empty pGL2 control vector or full length (2.7 kb) VEGF promoter upstream of luciferase. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System *, p < 0.001
(B) HIFα knockdown reduces VEGF protein level in GSCs
(C) HIF2α knockdown reduces VEGF secretion in GSCs, but not non-stem glioma cells.. *, p < 0.01 by ANOVA compared to non-targeting control with hypoxia for the same cell type.
(D-E) HIFα knockdown reduces VEGF mRNA level in GSCs. GSCs isolated from T3565 and T3691 specimens were infected with lentivirus expressing the indicated shRNAs. 48 hours later, mRNA was harvested and RT-PCR performed to determine the level of VEGF. *, p < 0.01 by ANOVA compared to non-targeting control.
Figure 29: HIFα Knockdown in Glioma Stem Cells Reduced Cancer Cell-mediated Endothelial Cell Proliferation.

(A) Representative diagram of the co-culture assay. HMVEC endothelial cells were plated in co-culture plate. Transwell inserts containing 0.4 mm diameter membrane pores were then placed into each well and seeded with glioma cells infected with non-targeting shRNA, HIF1α shRNA, or HIF2α shRNA.

(B) Representative images of co-cultured HMVEC cells after cells fixed with 4% PFA and then stained with toluidine blue are pictured.

(C) HMVEC proliferation was measured through [3H]-thymidine incorporation. *, p < 0.001
4. HIF1α and HIF2α Are Required for Glioma Stem Cell Survival and Tumorigenesis *in vitro* and *in vivo*

4.1 **Summary**

We have previously found that HIF2α levels and HIF2α-regulated gene expression are differentially elevated in GSCs in comparison to non-stem glioma cells or normal neural progenitor cells. We also showed that both HIF2α and HIF1α contribute to GSC-mediated angiogenesis. As hypoxia and HIFα play roles in normal stem cell maintenance, we tried to determine whether HIF2α and HIF1α also affect other aspects of glioma GSC biology. We found that knockdown of HIFα also impaired the self renewal and survival of GSCs *in vitro*, decreased their tumorigenic capacity and increased survival of transplanted immunocompromised mice. While HIF1α has significant roles in both GSCs and non-stem glioma cells, the dependence on HIF2α appeared restricted to GSCs as HIF2α knockdown in non-stem glioma cells has essentially no biological consequences. In addition, by searching glioma patient database, we found that high HIF2α level is correlated with poor patient outcome. Taken together, these data suggest that anti-cancer therapies can be designed to target GSC specific molecular mechanisms underlying hypoxia response, neoangiogenesis and tumor maintenance, including the expression and/or activity of HIF2α.
4.2 Introduction

Hypoxia is a common phenomenon in most solid tumors and modulates wide-range of tumor physiology including angiogenesis, metabolism, survival, proliferation and invasion/metastasis (Harris 2002; Bertout, Patel et al. 2008). While acute hypoxia could cause cell death in both normal tissues and tumors, chronic hypoxia usually favors tumor progression, which results in increased mutations, enhanced metastasis and bad patient outcomes (Harris 2002; Bertout, Patel et al. 2008). Importantly, cells in hypoxia/necrosis areas also tend to be much more resistant to chemotherapy and radiotherapy (Liang 1996; Hockel and Vaupel 2001; Semenza 2004).

The malignant phenotype caused by hypoxia is largely mediated by hypoxia inducible factors (HIFα) (Harris 2002). HIF1α and HIF2α are the best characterized HIF proteins and both of them are directly up-regulated by low oxygen tension. HIF activates transcription of hundreds of hypoxia-inducible genes to regulate various biological functions in tumors, such as cell growth (IGF-2, Interleukin-6, platelet-derived growth factor, etc), angiogenesis (erythropoietin, vascular endothelial growth factor, platelet-derived growth factor, etc), cell metabolism (aldolase A, lactate dehydrogenase, phosphoglycerate kinase 1, etc), and invasion/metastasis (matrix metalloproteinases, alpha-integrins, etc) (Harris 2002).
To study hypoxia in cancer stem cells is extremely interesting, not only because hypoxia usually promotes an aggressive phenotype, but it is proved to help maintain “stemness status” in many normal stem cells (Keith and Simon 2007). Hypoxia helps the self renewal of ES cells (Ezashi, Das et al. 2005) and low oxygen culture seems favor a more undifferentiated status of normal neural stem cells (Studer, Csete et al. 2000). HIF2α seems to be an extremely interesting molecule in cancer stem cell study as it has been directly linked to stem cell biology. Through a genetic model, researchers showed that HIF2α regulates a stem cell regulator, Oct-4 (Covello, Kehler et al. 2006). Moreover, in a renal carcinoma model, HIF2α enhanced the transcriptional activity of another stem cell factor c-Myc, while HIF1α destabilizes c-Myc complexes (Gordan, Bertout et al. 2007). We have demonstrated that GSCs specifically over-express HIF2α in comparison to non-stem glioma cells and both HIF1α and HIF2α modulate GSC angiogenesis potential. However, how hypoxia and HIFα regulate other aspects of GSC biology particularly tumorigenesis remains largely unknown.

In this chapter, we examined the biological consequences after the knockdown of HIF1α and HIF2α expression in GSCs and non-stem glioma cells. We found that targeting HIFα in GSCs inhibits self-renewal, proliferation and survival in vitro, and also attenuates tumor initiation potential of GSCs in vivo.
4.3 Materials and Methods

Neurosphere Formation Assay

Lentivirus expressing shRNA directed against HIF1α, HIF2α, or a non-targeting (NT) control shRNA (Sigma Mission shRNA system) was used to infect GSCs isolated from T3359 derived from a human glioblastoma specimen and passaged short term in immunocompromised mice. Cells were harvested 48 hours after infection and 8 hours after treatment with 200 M DFX. 1, 10, or 100 lentiviral infected GSCs isolated from the glioblastoma patient specimen T3359 passaged short-term in immunocompromised mice were plated into 24-well plates. Cells were monitored every day and pictures were taken 7 or 10 days after plating. In sequential neurosphere formation experiments, single lentiviral infected cell was plated per well and permitted to form neurospheres for 14 days (passage 1). Spheres that formed were dissociated and sequentially plated at one cell per well (passage 2). The process was repeated for passage 3.

CellTiter Assay

5000 lentiviral infected GSCs or non-stem glioma cells isolated from the glioblastoma samples were put into each well of 96-well plates and cultured in normoxia or hypoxia. Hypoxia condition is induced either by hypoxia chamber (2% O2) or 50 μM
chemical mimic desferrioxamine (DFX). Cell titers were determined after the indicated number of days using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega).

**Annexin V Staining**

200,000 lentiviral infected GSCs or non-stem glioma cells isolated from glioma patient specimens passaged short-term in immunocompromised mice were incubated in each well of 6-well plates for 48 hours followed by normoxia or hypoxia treatment for 24 hours. Hypoxia condition is induced either by hypoxia chamber (2% O2) or 50 μM chemical mimic desferrioxamine (DFX). Cell apoptosis was then determined with the Annexin V Apoptosis Detection Kit (Santa Cruz, Inc).

**Caspase-3/7 Activity Assay**

5000 lentiviral infected GSCs isolated from T3565, T3691, or T3956 specimens passaged short term in immunocompromised mice or directly from a T080714 specimen were placed in each well of 96-well plates. 48 hours after infection, Caspase 3/7 activity was analyzed by Caspase-Glo® 3/7 Assay Systems (Promega).

**In Vivo Tumor Formation Assays**

Intracranial or subcutaneous transplantation of GSCs into nude mice was performed as described. Briefly, 72 hours 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 hours after infection, 1 g/ml puromycin was applied to select infected cells for 48 hours 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.

**Mixture Cell Tumor Formation Assay**

T3359 GSCs were infected with non-targeting shRNA or shRNA targeting VEGF, HIF2α, or HIF1α. 72 hours after infection, 5000 infected GSCs were mixed with 95000 matched uninfected non-stem glioma cells to generate the 5% GSCs typical of brain tumor patient specimens. The cell mixtures were then injected into the brains of immunocompromised mice (3 mice for each arm). All animals were allowed to survive until the development of neurologic signs.

**4.4 Results**

**4.4.1 Targeting HIF1α or HIF2α Impaires Self-renewal of Glioma Stem Cells**

To determine the biological consequences of knocking down HIF2α and HIF1α, we first assessed their roles in glioma stem cell self-renewal. Neurosphere formation is one of classic methods to determine the self-renewal capability of both neural stem cells and GSCs *in vitro*. Similar to our prior report, we only saw neurospheres derived from
GSCs but not non-stem glioma cells (data not shown). After infecting GSCs, we measured the percentage of cells that were able to form neurospheres. We found that neurosphere formation was severely impaired upon knockdown of either HIF1α or HIF2α as we observed significantly fewer neurospheres from GSCs targeted by HIFα shRNAs (Figure 30A, 30B, 31). As stem cell self-renewal implies neurosphere formation not only in the first but in sequential passages, we performed series neurosphere formation assay to determine whether HIF1α and HIF2α are needed for continuous neurosphere growth. Notably, HIF1α and HIF2α knockdown impaired neurosphere formation not only in primary assays but also in secondary and tertiary passages (Figure 32), indicating the both HIF1α and HIF2α are required for self-renewal of GSCs at least in vitro.
Figure 30: HIFα Knockdown Altered Glioma Stem Cell Neurosphere Formation.

(A) 1, 10, or 100 lentiviral infected GSCs isolated from T3359 were plated into 24-well plates. Representative images of spheres are shown at the indicated magnifications.

(B) The total number of neurospheres per well is significantly decreased with HIFα targeting. *, p < 0.001 with ANOVA comparison to non-targeting control shRNA.

(C) Neurosphere size is significantly reduced by targeting HIFα expression when the size was quantified using Image J software. *, p < 0.001 with ANOVA comparison to non-targeting control shRNA.

(D) Representative image of neurospheres used for Image J quantification of neurosphere size in C.
Figure 31: HIFα Knockdown Altered Glioma Stem Cell Neurosphere Formation.
GSCs isolated from T3946 or T3691 specimens were infected with lentivirus expressing the indicated shRNAs. 48 hours later, puromycin was added to select infected cells. 48 hours after selection, live cells were plated at 1 cell/well in 24 wells plates in the presence of puromycin. 2 weeks later, neurospheres were counted and representative pictures were taken.
(A-B) Representative images of spheres are shown at the indicated magnifications. While the non-targeting group contains normal neurospheres, none of the HIFα knockdown groups exhibit any neurosphere growth.
(C-D) The percentage of neurosphere contains wells in each group.
Figure 32: Targeting HIFα in Glioma Stem Cells Decreased Neurosphere Formation in Sequential Passages.

(A-B) GSCs from T3359 (A) or T3832 (B) were infected by lentivius with control shRNA, HIF1α shRNA, or HIF2α shRNA. 1 lentiviral infected cell was plated per well and permitted to form neurospheres for 14 days (passage 1). Spheres that formed were dissociated and sequentially plated at 1 cell per well (passage 2). The process was repeated (passage 3). (C) Representative images of neurospheres formed in sequential passage neurosphere formation assays.
4.4.2 HIF1α and HIF2α are Required for Cell Proliferation of Glioma Stem Cells

We noted in neurosphere formation assay, even though a minority of GSCs with HIF1α or HIF2α knockdown retained neurosphere formation potential, the size of the resultant neurospheres was significantly reduced (Figure 30C, 30D, 32C). It suggested the HIF1α and HIF2α are probably required for GSC proliferation. To address this possibility, we determined the growth of GSC and non-stem glioma cells under normoxia or hypoxia when HIF1α or HIF2α expression was targeted by lentiviral transduced shRNA. Using a cell title assay, we found that both HIF2α and HIF1α are indispensable for the growth of GSCs, as knockdown of either one of them blocked GSC proliferation in both normoxic and hypoxic conditions (Figure 33). The requirement for HIF2α in cell growth was restricted to GSCs as no effect of HIF2α shRNA was observed in matched non-stem glioma cells. In contrast, HIF1α knockdown resulted in reduced cell growth in both GSCs and non-stem glioma cells. These data proved that HIF2α are specifically required for the GSCs proliferation while HIF1α plays roles in both GSCs and non-stem glioma cells.
Figure 33: HIFα Knockdown Reduced Glioma Stem Cell Growth.

(A) Targeting HIF2α in GSCs, but not non-stem glioma cells decreases growth. Lentiviral infected cells isolated from T4302 were put into 96-well plates and cultured in the absence or presence of 200 μM DFX. Cell titers were determined using the CellTiter-Glo Luminescent Cell Viability Assay kit. *, p < 0.05. #, p < 0.01.

(B) Cells were plated and quantified as in A but cultured in 20% or 2% oxygen as indicated. *, p < 0.001. #, p < 0.001.

(C) Western analysis of cells plated for the cell titer assay demonstrated effective knockdown of HIF2α and HIF1α.
**4.4.3 Depletion of HIF1α or HIF2α Results in Elevated Apoptosis**

In previous experiments, we observed significant cell death when we targeted HIF1α or HIF2α in GSCs. Therefore, we sought to determine the roles of HIF1α or HIF2α in the cell survival and apoptosis. Loss of HIF2α in GSCs consistently resulted in an induction of apoptosis determined with Annexin V staining (Figure 32, 33). No requirement for HIF2α was determined in non-stem glioma cells, even under hypoxia. In contrast, HIF1α contributed to the survival of both GSCs and non-stem glioma cells (Figure 32, 33). In order to validate the loss of HIF1α or HIF2α was associated with increased apoptosis, we also measured caspase activity in infected GSCs and confirmed that HIF1α or HIF2α knockdown is associated with increased caspase activity (Figure 34). Interestingly, at this time point, HIF1α shRNA was less acutely toxic than HIF2α shRNA, as they resulted in less increase in caspase activity. In order to check whether HIF2α is more potent to induce cell death than HIF1α, we measured the cell growth and caspase activity in a time course. We found that the loss of the ability to form neurospheres was more rapid in HIF2α (72 hours) than HIF1α targeted cells (108 hours). Accordingly, both HIF2α and HIF1α knockdown groups show significant increases in Caspase3/7 activity, but HIF2α induced apoptosis at an earlier time point (Figure 35). This indicated that HIF2α and HIF1α had similar capability to induce apoptosis but HIF2α inhibition had more acute consequence.
Figure 34: HIFα Knockdown Increased Annexin V Positive Cells in T4302 Glioma Stem Cells.

(A) Lentiviral infected GSCs or non-stem glioma cells were incubated for 48 hours followed by normoxia or hypoxia treatment for 24 hours. Cell apoptosis was then determined with the Annexin V Apoptosis Detection Kit. While knockdown of HIF1α lead to remarkable cell apoptosis in both GSCs and non-stem glioma cells, knockdown of HIF2α increased apoptosis only in GSCs.

(B) Quantification of the percentage of Annexin V positive cells in (A). *, p < 0.05 with ANOVA comparison to non-targeting shRNA of the same cell type. #, p <0.01 with ANOVA comparison of HIF2α shRNA treated GSCs to HIF2α shRNA treated non-stem glioma cells with identical oxygen treatment.
Figure 35: HIFα Knockdown Increased Annexin V Positive Cells in T3359 Glioma Stem Cells.

(A) Lentiviral infected GSCs or non-stem glioma cells were incubated for 48 hours followed by normoxia or hypoxia treatment for 24 hours. Cell apoptosis was then determined with the Annexin V Apoptosis Detection Kit. While knockdown of HIF1α lead to remarkable cell apoptosis in both GSCs and non-stem glioma cells, knockdown of HIF2α increased apoptosis only in GSCs.

(B) Quantification of the percentage of Annexin V positive cells in (A). *, p < 0.05 with ANOVA comparison to non-targeting shRNA of the same cell type. #, p < 0.01 with ANOVA comparison of HIF2α shRNA treated GSCs to HIF2α shRNA treated non-stem glioma cells with identical oxygen treatment.
Figure 36: HIFα Knockdown Increased Caspase3/7 Activity in Glioma Stem Cells.

Targeting HIFs results in increased caspase 3/7 activity in GSCs from multiple samples. Lentiviral infected GSCs isolated from T3565 (A), T3691 (B), T3946(C) or T080714 (D) specimens were placed in each well of 96-well plates. Caspase 3/7 activity was analyzed 48 hours after infection. *, p < 0.05.
Figure 37: HIFα Knockdown Induced Apoptosis and Blocked Neurosphere Formation in Glioma Stem Cells.

T3565 GSCs were infected with shRNA for 72 hours. Cells were then FACS sorted using Annexin V antibody. Annexin positive and negative cells were collected and cultured separately in 24 well plates for up to 108 hours.

(A) Representative images of Annexin V negative cells cultured in the presence of puromycin to select for cells with stable shRNA integration. HIFα targeted cells failed to form neurospheres. The loss of the ability to form neurospheres was more rapid in HIF2α (72 hours) than HIF1α targeted cells (108 hours), but both groups had minimal neurospheres at 108 hours after sorting.

(B) Annexin V negative cells isolated as above were plated in 96 well plates at a density of 5000 cells/well. Caspase 3/7 activity was measured after 24, 72, and 108 hours. Both HIF2α and HIF1α knockdown groups show significant increases in Caspase3/7 activity, but HIF2α induced apoptosis at an earlier time point.
We also measured the change of cell cycle upon targeting HIF1α or HIF2α. Consistent with the elevation in apoptosis with loss of HIFα in GSCs, there was an increase in the percentage of cells in the sub-G0 and G1 phases of the cell cycle. Cycling (S phase) as well as G2 arrested cells decreased at the same time (Figure 36). Together these data demonstrate requirements for both HIF1α and HIF2α in GSC biology with a specific requirement for HIF2α in the GSCs, but not non-stem glioma subpopulation for growth and survival. Due to differences in the efficiency of the shRNA constructs (HIF1α knockdown was consistently more efficient than HIF2α knockdown), it is not possible to absolutely determine the relative importance of the HIFα.
Figure 38: HIFα Knockdown in Glioma Stem Cells Increased Apoptosis and Cell Cycle Arrest.

T3359 GSCs were infected with the indicated shRNAs. 48 hours after infection cells were fixed by ethanol and analyzed for cell cycle.

(A) HIF1α and HIF2α were successfully targeted by shRNAs.
(B) The percentage of SubG0 cells increases with HIFα targeting.
(C) The percentage of cycling, S-phase cells decreases with HIFα targeting.
(D) The percentage of G1 arrested cells increases with HIFα targeting.
(E) Decreased progression through the cell cycle results in a decreased percentage of cells reaching the G2 phase of the cell cycle. *, p < 0.01 with ANOVA comparison to non-targeting control (NT).
4.4.4 Knockdown of HIF1α or HIF2α Delays Tumor Growth and Increased Mice Survival

Considering the requirements for HIF2α and HIF1α in GSC proliferation, survival, and VEGF production in vitro, we determined the impact of HIFα knockdown on GSC tumorigenic capability in vivo. When GSCs transduced with non-targeting control shRNA or shRNA targeting HIF2α or HIF1α were intracranially implanted into immunocompromised mice (Figure 37), we observed a significant decrease in tumor formation (Figure 38A). There was a significant increase in the survival of tumor bearing mice when HIF1α or HIF2α were targeted (Figure 38B-D). We further determined that targeting HIFα can reduce the tumorigenic potential of GSCs in an in vivo limiting dilution assay, indicating that knockdown of HIF1α or HIF2α decreased the number of tumor-initiating cells (Table 6). As knockdown of HIF2α increased the survival of tumor bearing mice as well as or significantly more than HIF1α, but was usually targeted less efficiently at the protein level, our data may underestimate the importance of HIF2α for the in vivo propagation of GSCs. In fact, tumors arising from unselected HIF2α knockdown cells expressed HIF2α indicating that these tumors likely originated from unsuccessfully targeted cells (Figure 39A). When GSCs underwent puromycin selection to ensure successful infection, HIF1α or HIF2α knockdown cells failed to form any tumors even after six months (Figure 39B).
Figure 39: Schematic Representation of Intracranial Injection Experiments.
Figure 40: HIFα Knockdown Suppressed Glioma Stem Cell Mediated Tumor Growth. 

(A) Gross histology demonstrates highly vascular tumors in GSC derived tumors from cells infected with non-targeting shRNA but not HIFα targeting shRNAs. 50,000 viable GSCs were injected into the brains of immunocompromised mice (n = 5 for each of three arms). All animals were sacrificed at 31 days after injection.

(B) Kaplan-Meier analysis indicated significant delay of tumor growth from GSCs with HIFα Knockdown. Infected GSC derived from D456MG were injected into the brains of immunocompromised mice (n=4 for each of three arms). Mice were allowed to survive until the development of neurologic signs at which time animals were sacrificed. *, p < 0.05 by log-rank comparison of HIFα shRNA group to NT group. #, p < 0.03 by log-rank comparison of HIF2α shRNA group to HIF1α shRNA infected group.

(C-D) Experiments were performed as in (B) using GSCs derived from T3691 (C) or T3946 (D). (n=10 for each arm). *, p < 0.05 by log-rank comparison to non-targeting control cells.
Table 6: *In Vivo* Limiting Dilution Assay Demonstrated Glioma Stem Cells Were Less Tumorigenic When HIFα Were Targeted.

<table>
<thead>
<tr>
<th>shRNA</th>
<th>T3359 Stem</th>
<th>D456MG Stem</th>
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<tbody>
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<td></td>
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<tr>
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<td>Incidence</td>
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<tr>
<td></td>
<td>Median Survival</td>
<td>159*</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Incidence</td>
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<td></td>
<td>Median Survival</td>
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</table>

T3359 GSCs were infected with lentivirus with non-targeting shRNA or shRNA targeting HIF2α or HIF1α. 72 hours after infection, 10000, 5000, 1000 or 500 cells were injected into the brains of immunocompromised mice (n=3 for each of twelve arms). Mice were allowed to survive until the development of neurologic signs at which time animals were sacrificed. Similar experiments were performed using GSCs derived from the glioblastoma xenograft D456MG. 72 hours after infection, 50000, 10000, 5000, 1000 or 500 cells were injected into the brains of immunocompromised mice (n=3 for each of fifteen arms). Mice were allowed to survive until the development of neurologic signs at which time animals were sacrificed. * p < 0.03 by log-rank comparison to non-targeting control cells with identical cell number.
Figure 41: Increasing Targeting Efficiency by Antibiotic Selection Further Increased Animal Survival.

(A) Tumors arise from cells with unsuccessful HIF2α knockdown. Hematoxylin and Eosin (H&E) staining demonstrated the presence of tumors in mice exhibiting neurologic signs. Immunohistochemistry revealed the presence of HIF2α expressing cells in tumors, even those derived from GSCs infected with shRNA against HIF2α. 

(B) Tumors do not form from GSCs selected for the incorporation of a puromycin marker associated with HIFα targeting shRNA. (n=6 for each of three arms). Mice were allowed to survive until the development of neurologic signs at which time animals were sacrificed. *, p < 0.03 by log-rank comparison to non-targeting control cells.
As GSCs usually only account for a small percentage of bulk tumor cells in our studies (1%~10%), 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 non-stem glioma cell populations at a 1:20 ratio (i.e. 5% of the total cancer cells were GSCs similar to the fraction in human glioblastoma specimens) (Figure 40). As expected, tumor cell mixtures with GSCs transduced with non-targeting 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 HIF1α or HIF2α shRNA display impaired tumor formation potential indicating that targeting HIFα only in GSCs could have therapeutic benefit (Figure 41). Targeting HIFα likely impairs tumor growth through several mechanisms as many genes are regulated by HIFα 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 glioblastoma stem cell population in our cell mixing experiments can increase the survival of tumor bearing mice and decrease tumor angiogenesis (Figure 41), indicating that reducing VEGF production and thus angiogenesis could be one of the potential
mechanisms by which targeting HIFα in GSCs decreased tumorigenesis \textit{in vivo}. Together, our data demonstrate that HIF2α, HIF1α and VEGF are required to maintain the tumorigenic potential of GSCs and that targeting HIF2α may be a cancer stem cell directed therapy.

![Schematic Representation of Cell Mixture Intracranial Injection Experiments.](image)

Figure 42: Schematic Representation of Cell Mixture Intracranial Injection Experiments.
Figure 43: Targeting VEGF or HIFα Only within the Glioma Stem Cell Subpopulation Increased Animal Survival.

T3359 GSCs were infected with non-targeting shRNA or shRNA targeting VEGF, HIF2α, or HIF1α. 72 hours after infection, 5000 infected GSCs were mixed with 95000 matched uninfected non-stem glioma cells to generate the 5% GSCs typical of brain tumor patient specimens. The cell mixtures were then injected into the brains of immunocompromised mice (n = 3 for each of four arms). All animals were allowed to survive until the development of neurologic signs. *, p < 0.03 by log-rank analysis in comparison to non-targeting control cells using MedCalc software.
4.4.5 HIF2A but Not HIF1A mRNA Is Correlated with Patient Survival

To investigate whether targeting HIF2α may have a therapeutic benefit for the glioma patient population, we analyzed whether HIF2 level is correlated with patient survival. We utilized the Rembrandt (Repository of Molecular Brain Neoplasia Data) database of the National Cancer Institute (http://caintegrator-info.nci.nih.gov/rembrant). We analyzed the data to determine the survival of glioma patients with intermediate, low, or high expression of HIF2A or HIF1A mRNA. Notably, we found a significant decrease in the probability of survival in glioma patients with elevated HIF2A expression (Figure 42A). In contrast, there is no significant difference of the survival in patients with various HIF1A expression (Figure 42B). As this gene expression database measures mRNA levels, and HIF2A but not HIF1A mRNA 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. Interestingly, in another independent study of our lab, high HIF2α protein level also seems to correlated with poor patient outcome (Sathornsumetee, Cao et al. 2008). These data demonstrate HIF2A and HIF1A differentially affect patient outcome and strongly support a specific and important role for HIF2α in gliomas.
Figure 44: HIF2A But Not HIF1A mRNA Level Correlated with Patient Survival.

The Rembrandt (Repository of Molecular Brain Neoplasia Data) database of the National Cancer Institute (http://caintegrator-info.nci.nih.gov/rembrant) was used to determine the survival of glioma patients with intermediate, low, or high expression of HIF2A or HIF1A.

(A) Patients with high HIF2A expression has significantly shorter survival in comparison to patients intermediate HIF2A level (p=0.012).

(B) In contrast, HIF1A high and intermediate patients have similar outcome (p=0.144).
5. Conclusion, Discussions and Future Directions

5.1 Conclusions

The data presented in this thesis demonstrated that glioma stem cells (GSCs) have differential hypoxia responses, which lead to their self-renewal, survival and strong capability of driving angiogenesis.

We first revealed that GSCs are highly capable of promoting endothelial proliferation and migration \textit{in vitro} and initiating glioblastoma-like tumors \textit{in vivo}, which contain abundant and fast-proliferating blood vessels. Such a strong angiogenic drive is partially due to the elevated secretion of a pro-angiogenic factor Vascular Endothelial Growth Factor (VEGF). Blocking VEGF function by a neutralizing antibody Bevacizumab significantly abrogated the ability of GSCs to induce endothelial cells behavior. It also remarkably decreased the sized of tumor formed from GSCs.

We then interrogated the upstream mechanism underlying the increased VEGF production in GSCs. We found that HIF1\(\alpha\) and HIF2\(\alpha\) are differentially expressed by two cellular subpopulations. HIF2\(\alpha\) and its downstream pathways were highly active in GSCs. GSCs overexpress HIF2\(\alpha\) at both mRNA and protein levels in comparison to matched non-stem glioma cells. In contrast, HIF1\(\alpha\) protein is usually expressed at a higher level in non-stem glioma cells. In consistent with this, to induce the
differentiation of GSCs resulted in the loss of HIF2α expression and increased HIF1α level. Interestingly, the up-regulation of HIF2α seems to be a GSC specific phenotype as normal neural stem cell or neural progenitor cells expressed very minimal amount of HIF2α mRNA or protein. By performing double immunofluorescence staining on primary glioblastoma patient specimens, we further demonstrated that HIF2α is generally co-localized with other GSC markers such as CD133 and Olig2 in vivo. This indicates that HIF2α may be used as an additional marker for GSCs.

We finally investigated the biological functions of HIFα in both GSCs and non-stem glioma cells, primarily by using a lentivirus based shRNA construct system. While HIF1α is important for the survival and VEGF secretion in both cell groups, HIF2α is specifically required in GSC biology. Knockdown of HIF2α in GSCs lead to inhibited growth and self-renewal, induced apoptosis, and decreased VEGF secretion and angiogenic drive in vitro. More importantly, targeting HIF2α significantly blocked the GSC-mediated tumor growth in vivo. When we injected GSCs with successful HIF2α knockdown into the animal model, no tumors were observed even six months after implantation, while the non-targeting group consistently gave rise to tumor within two months. Notably, the dependence on HIF2α appeared restricted to GSCs as HIF2α knockdown in non-stem glioma cells has essentially no biological consequences.
5.2 Discussions

5.2.1 Glioma Stem Cells as Targets for Anti-angiogenesis Therapies

Angiogenesis is a hallmark of most solid tumors and anti-angiogenic therapies continue to be developed for many cancers, including glioblastomas. There is preliminary success of anti-angiogenesis (in particular targeting VEGF axis) therapy on malignant gliomas. Clinical trials of anti-angiogenic drugs like Bevacizumab and Cediranib have demonstrated promising preliminary results in glioblastoma patients. However, the determinants of patient response to these agents remain unclear as VEGF levels and microvascular density have not been correlated with patient response in clinical trials of bevacizumab for systemic cancers (Jubb, Hurwitz et al. 2006).

Our data suggest the limited fraction of GSCs may be critical for tumor growth not only through their capacity for self replication but also through a paracrine effect supporting non-stem glioma cells by inducing neovascularization. It also demonstrate that only a subpopulation of the tumor may be critical for driving VEGF mediated tumor angiogenesis as the effects of a VEGF neutralizing antibody (Bevacizumab) predominantly disrupt the angiogenic effects of GSCs. Researchers have established vascular “normalization” as a potential key response to anti-angiogenic therapies, in addition to the vascular pruning caused through targeting angiogenesis (Jain 2005).
Bevacizumab may be effective as a cancer therapy when combined with chemotherapy by improving chemotherapy delivery specifically to the GSCs that would be expected to be proximal to the tumor vasculature due to VEGF expression. Actually we and others have found that GSCs prefer to locate proximally to blood vessels (Calabrese, Poppleton et al. 2007). Interestingly, there is actually a reciprocal relationship between angiogenesis and GSCs (Figure 43). Not only GSCs promote angiogenesis, but like normal neural stem cells, GSCs are preferentially located in “vascular niches” and blood vessels could secrete growth factors to promote the self-renewal of GSCs. As a result, anti-angiogenesis drugs may work by specifically destroying the microenvironment that is required for the maintenance of GSCs and thus effectively eliminating the roots of tumor progression. While elucidating the exact mechanism requires additional work, our data provide novel insights in the molecular mechanisms by which anti-angiogenic agents may function. Anti-angiogenesis therapies are very promising as it may achieve high efficiency against GSCs, the most tumorigenic population in cancers.
5.2.2 Heterogeneity of Cancer Stem Cells and Glioma Stem Cells

Prospective identification of cancer stem cells has been challenging, and current methods for cancer stem cell enrichment for solid cancers remain imperfect. 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. CD133 has been used widely as a marker for GSCs (Hemmati, Nakano et al. 2003; Singh, Hawkins et al. 2004; Bao, Wu et al. 2006). Our data suggest that HIF2α identifies a subpopulation of CD133 positive cells. The vast majority of HIF2α positive cells express CD133, but HIF2α and CD133 do not overlap exclusively: not all HIF2α positive cells are CD133 positive, and not all CD133 positive cells are HIF2α positive. Targeting HIF2α did not uniformly kill all CD133 positive cells suggesting a heterogenous dependence on HIF2α in this GSC population. The role of HIF2α in tumors which are not driven by CD133 expression (Beier, Hau et al. 2007; Zheng, Shen et al. 2007) is still unresolved, but we did not observe HIF2α expression in a rat glioma cell line in which CD133 negative cells were reported to be tumorigenic. We also cannot complete the functional studies required to define GSC with HIF2α due to its intracellular localization. Our GSC cultures therefore remain heterogeneous for HIF2α expression. HIF2α does appear to localize with GSC markers in vitro and in vivo, suggesting that HIF2α positive cells are enriched in a GSC phenotype. Together, our results suggest that HIF2α may mark a subpopulation of GSCs essential for tumor growth.
5.2.3 Hypoxia, HIF2α and Glioma Stem Cell Biology

Hypoxia is a well recognized tumor microenvironmental condition that is linked to poor patient outcome and resistance to therapies (Teicher 1994; Liang 1996; Hockel and Vaupel 2001; Harris 2002; Brown and Wilson 2004; Semenza 2004; Chi, Wang et al. 2006; Vaupel and Mayer 2007; Sathornsumetee, Cao et al. 2008). Cellular responses to hypoxia are frequently regulated by the HIFα 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 HIFα and other hypoxia target genes in GSCs. As we expected, all cancer cells responded to acute hypoxia through the increase of HIF1α protein. 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, Braun et al. 1996; Cardenas-Navia, Yu et al. 2004). A recent report suggested that unlike HIF1α, which is usually stabilized under more acute hypoxic conditions, HIF2α may accumulate under modest hypoxia or even normal physiological oxygen levels (Holmquist-Mengelbier, Fredlund et al. 2006). Indeed, we found under 2-5% oxygen levels that HIF2α is the dominant hypoxia-inducible factor present in the GSC population and that HIF2α is expressed at wide range of oxygen levels. This indicates
that HIF2α may provide GSCs 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 HIF2α positive cells are located adjacent to blood vessels. Therefore, it will be of great interest to determine whether HIF2α functions differentially under various oxygen tensions during tumorigenesis in vivo. It is also notable that the role of HIF2α was likely to be underestimated in previous cancer studies with cell lines or bulk tumor populations as GSCs frequently account for only a restricted fraction of the overall tumor (less than 10% of tumor cells).

The HIFs function through the transcriptional regulation of a number of important gene products. Besides VEGF, Oct4, Glut1 and SerpinB9 expressions were induced by HIF2α in our studies (Figure 44). Oct4 is a core regulator in stem cell self-renew and differentiation (Pan, Chang et al. 2002; Wang, Rao et al. 2006). In agreement with a previous report which shows Oct4 is a direct target of HIF2α in a renal tumor model (Covello, Kehler et al. 2006), our data support Oct4 regulation by HIF2α in GSCs. Therefore, HIF2α may help maintain GSCs by regulating the level of Oct4. This is significant as Oct4 has been very recently validated as an independent cancer stem cell target (Hu, Liu et al. 2008). Glut1 is the first identified glucose transporter in mammal cells and it permits glucose uptake in many cell types. Glut1 is frequently up-regulated
in cancer cells to facilitate their accelerated metabolism, in particular glycolysis, which is one of the biochemical characteristics of cancer cells (Younes, Brown et al. 1995; Macheda, Rogers et al. 2005). By up-regulating the level of Glut1, HIF2α provides GSCs with advantages in metabolism, proliferation and survival. SerpinB9 is also called proteinase inhibitor 9 (PI-9) and it is an effective endogenous inhibitor of granzyme B, a key factor secreted by cytotoxic T cells to kill target cells by inducing apoptosis (Trapani and Sutton 2003). SerpinB9 can also directly inhibit Caspase enzymes (Young, Sukhova et al. 2000). All these anti-apoptotic effects of SerpinB9 imply that it may play critical roles in cancer stem cell biology. Indeed, SerpinB9 is up-regulated in some melanoma and leukemia patients and its upregulation predicts poor outcome in high grade melanoma patients (van Houdt, Oudejans et al. 2005). Therefore, the induction of SerpinB9 by HIF2 may further help GSCs escape the immune system or other microenvironment stresses.
Figure 46: HIF2α Upregulates Downstream Targets to Promotes More Aggressive Tumor Phenotype.

5.2.4 HIF2α as A Potential Target for Anti-Glioma Stem Cell Therapy

The direct characterization of GSCs may yield novel therapeutic targets that are not evident by whole tumor analyses. For example, we recently demonstrated that L1CAM (Bao, Wu et al. 2008), a cell adhesion molecule, was preferentially expressed in GSCs and was essential to tumor initiation. Paramount in the development of GSCs 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 GSCs without significant
expression in the organ specific progenitor compartment, specifically neural progenitors. Based on these criteria, HIF2α appears to be an attractive target as it is specifically expressed by GSCs but not neural progenitor cells, whereas HIF1α is shared by these cellular populations. Indeed, HIF1α is essential in neural development (Tomita, Ueno et al. 2003) whereas animals with the targeted disruption of HIF2α display defects in other organ systems (Compernolle, Brusselmans et al. 2002).

Consistent with HIF1α’s recognition as a molecular cancer target, we determined that HIF1α is required for the proliferation, survival, and angiogenesis of both the stem cell and non-stem tumor cells. However, we have defined a unique requirement for HIF2α in the cancer stem cell subpopulation. We found that targeting HIF2α in GSCs is as effective as or more effective in vivo than targeting HIF1α, suggesting that targeting HIF1α without recognizing the contribution of HIF2α to hypoxia responses overlooks an important potential compensatory mechanism. It is important to note that comparing the efficacy of targeting HIF1α and HIF2α cannot be directly compared in our studies as the efficiency of knockdown was significantly different (HIF1α was more efficiently targeted). HIF2α 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 HIF1α (Gordan, Bertout et al. 2007).
Our results have direct clinical relevance as we have recently determined that hypoxic markers, including HIF2α, provide useful biomarkers for predicting patient survival from treatment initiation in a trial of the anti-VEGF antibody bevacizumab in combination with irinotecan (Sathornsumetee, Cao et al. 2008). Using this malignant glioma patient cohort, we now find that the expression of HIF2α 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 HIF2α upregulation have significantly shorter survival in comparison to those with lower HIF2α expression. Thus, our data supports the development of HIF2α directed therapies and demonstrates differential molecular responses to hypoxia in the stem cell subpopulation.

5.2.5 Vascular Niche and/or Hypoxia Niche for Glioma Stem Cells

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 include GSCs may also be harbored in specific niches (Gilbertson and Rich 2007), but many aspects of the cancer stem cell niche are unknown. While vasculature seems to be an important niche component for many normal stem cell types, our analysis of surgical glioblastoma biopsy specimens suggests that there may be at least two areas enriched
for GSCs, around blood vessels and regions of necrosis, which are hypoxic. These data raise two interesting questions: is hypoxia a functional component of GSC niche? are there two groups of GSCs that rely on two different niches? (i.e. perivascular and hypoxic).

It has been known that hypoxia can help maintain stem cells. For instance, 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, Mauch 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, Dayan 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, Das et al. 2005; Keith and Simon 2007). Together, these data indicate hypoxia may be a functional component of a cancer stem cell niche.
The answer to the second question seems to be more complicated as perivascular location and hypoxia are not mutually exclusive and it is possible that a perivascular niche actually represents one kind of hypoxia niche (Dewhirst, Cao et al.2008; Sorg, Moeller et al.2005). Difficultly in reconciling the localization of cancer stem cells to both hypoxic regions and areas around tumor vasculature is resolved with the understanding that tumor-associated blood vessels are frequently hypoxic due to many reasons, such as insufficient arteriolar supply, dysregulated microvessels and extreme fluctuation of red blood cell flux, etc (Dewhirst, Cao et al.2008). As a result, these GSCs located next to blood vessels may still rely on hypoxia condition to maintain their self-renewal capability. GSCs 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 is also possible that there are indeed two distinct GSC populations that are located in two different niches (i.e. perivascular and hypoxic). Future work is thus required to further characterize the relationship between oxygen tension around blood vessels and the location of GSCs. For example, it is of great interest to determine that GSCs actually prefer to associate with hypoxic blood vessels or better oxygenized blood vessels.
5.3 Future Directions

5.3.1 Exploration of HIFα Downstream Factors That Mediate Glioma Stem Cell Survival and Tumorigenesis

Consistent with previous report (Kaur, Khwaja et al. 2005), we found that HIF1α and HIF2α are important regulators of VEGF secretion in glioma stem cells. Besides their famous roles in stimulating angiogenesis, our data also demonstrated that both HIF1α and HIF2α are indispensable for the survival of GSCs, and knockdown of either HIFα induced high rate of apoptosis and remarkably delayed the growth of tumors that were initiated by GSCs. However, molecular mechanisms underlying the pro-survival effect of HIF1α and/or HIF2α are unknown.

To study the HIFα downstream factors that are involved in regulating the survival/apoptosis in GSCs, we will initially use a loss of function strategy. After knocking down HIFα in GSCs, we will examine the protein/mRNA levels of genes that are related to cell survival, such as p53, caspases, etc. Once we identify several candidates, rescue experiments will be performed by over-expressing (if they are anti-apoptosis), or knocking down (if they are pro-apoptosis) these genes in combination with HIFα shRNAs to see whether they can improve the survival of GSCs with HIFα knockdown. In my preliminary study, I have found that p53 level was dramatically increased upon HIFα shRNA treatment. It would be interesting to try to rescue cell
death by reduce p53 level and to identify the missing links between HIFα and p53 (how HIFα regulates p53 level). If none of the well known candidates seem relevant, we will utilize large scale screening technique (e.g. microarray) to identify candidates. We expect to have a long list, but we will first focus on these genes that have been reported to be critical factors in apoptosis/survival modulation.

5.3.2 Study of Upstream Factors Leading to HIF2α Up-regulation in Glioma Stem Cells

In order to identify the upstream factors that contribute to high VEGF secretion in GSCs, we studied hypoxia response genes and found that HIF2α is a major molecular contribute to the elevated level of VEGF production. However, why HIF2α level is up-regulated in GSCs are still mysterious. Understanding how HIF2α is up-regulated is important as it may shed lights on a general question: how GSCs are different from non-stem glioma cells? It will also help identify better molecular targets to develop drugs against GSCs.

There are two clues that may guide our experiment designs. First, our data indicate that HIF2α expression is minimal in both non-stem glioma cells and normal neural progenitors/stem cells. Therefore, a very attractive hypothesis would be that HIF2α up-regulation is a consequence of synergic actions of both oncogenes and stemness genes. I have previous knocked down several genes that are involved in
glioma biology including Olig2, Sox2, and c-Myc. However, none of them alone affected the level of HIF2α in GSCs. We will try to knockdown multiple factors to see whether that would result in decreased HIF2α level.

The second clue is that unlike HIF1α, HIF2α is regulated not only at post-translational level but also at transcription level. Our data indicate that HIF2α is regulated at mRNA level in response to hypoxia and the basal transcription rate (in normoxia condition) is much higher in GSCs in comparison to non-stem glioma cells or neural progenitors. There are two major mechanisms that may explain this phenomenon. First, this may be controlled by epigenetic modification, particularly promoter methylation. Alternatively, this may result from transcriptional factors’ activity. The test the epigenetic variation, we will perform DNA methylation assay on HIF2α promoters. As HIF2α promoter is not fully identified yet, we will first start analyzing the methylation status of CpG islands in the 2k base pairs genomic DNA fragment that locates 5’ upstream of HIF2α transcriptional start site. If DNA methylation is one major regulator of HIF2α transcription we would expect to observe relatively low CpG methylation in GSCs in comparison to that in non-stem glioma cells and normal neural progenitors, because DNA methylation usually represses the transcription of target genes.
If the epigenetic factors are not the primary regulator, we will then investigate whether certain transcriptional activators or repressors are involved in regulating HIF2α mRNA level. Briefly, we will clone the HIF2α promoter into PGL4 vector, where HIF2α promoter sequence will drive the transcription of firefly luciferase gene. Full length HIF2α promoter containing PGL4 plasmid will be transfected into GSCs and non-stem glioma cells to examine its activity. If this promoter is functional, we should observe higher luciferase activity in GSCs. Once this is proven, we will create a series of deletion and mutations in the HIF2α promoters to investigate which region of HIF2α promoter is really responsible for the differential expression of HIF2α in GSCs and non-stem glioma cells. If we are able to narrow down the regulatory region, we will then use bioinformatics methods to search the consensus binding sites of transcriptional activators and/or repressors. After that, these transcriptional factors will be co-transfected with HIF2α promoter construct to verify whether they directly affect HIF2α promoter activity. At last, these factors will be knocked down in GSCs or over-expressed in non-stem glioma cells and the endogenous HIF2α mRNA level will be monitored.

5.3.3 Roles of HIF2α in Stem Cells from Breast/Colon Cancers: a Conserved Phenotype?

Cancer stem cell (CSC) population has been isolated from distinct cancer types including these in brain, breast, colon, liver and pancreas. While we have confirmed that HIF2α is specifically up-regulated in glioma stem cells and HIF2α functions are crucial
for the GSC maintenance, it remains unknown that whether HIF2α plays similar roles in other cancer stem cell population.

In order to study the roles of HIF2α in various cancer stem cell populations, we will repeat our HIF2α experiments in cancer stem cells isolated from other tumor types. We will primarily use CSCs in breast cancers and colons cancers as they are among the best characterized and have a large number of available patient specimens. Breast CSC population can be enriched through FACS sorting as they are CD44+/CD24- (Al-Hajj, Wicha et al. 2003). Colon CSC is usually expressing high level of CD133, similar to glioma stem cells (Ricci-Vitiani, Lombardi et al. 2007). Afterwards, CSCs and non-CSCs from the same tumor will be subjected to hypoxia treatment and both the mRNA and protein levels HIF2α will be measured. In order to study the general hypoxia response, the mRNA collected from hypoxia and normoxia groups will be used to do large scale microarray or real time PCR hypoxia arrays.

If HIF2α up-regulation is proved to be a common biology in multiple CSC populations, we will follow that to determine what the universal regulators are for this phenotype. If HIF2α is only specifically increased in glioma stem cells not but CSCs from other cancer types, there will be several interesting questions to ask. For example, how is the hypoxia response in glioma stem cells different from other CSCs? Do HIF2α-less CSCs still have different hypoxia response in comparison to the matched non-stem
cancer cells? Is that HIF1α dependent? etc. This study will help us further understand the difference/similarity among CSCs from different cancer types.

**5.3.4 The Role of HIF2α in Therapy Resistance**

Radiotherapy is one of the most traditional treatments for malignant glioma patients (Chamberlain 2006). Unfortunately, we recently demonstrated that glioma stem cells (GSCs) are more resistant to radiation than non-stem glioma cells (Bao, Wu et al. 2006). Given the strong capability of GSCs in tumor initiation, this data may provide explanation for the high recurrent rate in glioblastoma patients. There are also studies suggesting that cancer stem cells (CSCs) may be more resistant to chemotherapy as well (Liu, Yuan et al. 2006; Li, Lewis et al. 2008). However, the underlying mechanisms of such therapy resistance are largely unknown. Hypoxia has been tightly linked to radiotherapy resistant for decades and therefore, it would be very interesting to investigate whether HIF2α contributes to these resistance effects and whether targeting HIFα would “sensitize” GSCs to therapies.

We will study the radiation responses in GSCs with or without HIF2α/ HIF1α knockdown. Cells will receive HIFα shRNA first and then be subjected to radiation, at the clinic dosage. We will measure the survival/apoptosis rate by direct cell counting and caspase activity, DNA breakage by comet assay, DNA damage response by
ATM/ATR activity assay, and tumorigenesis property by \textit{in vivo} limited dilution assay. We expect to see that HIF2\(\alpha\) and/or HIF1\(\alpha\) contribute to cellular resistance to radiation and GSCs would exhibit severe deficiency in DNA damage repair. The next step would be to confirm these results in other cell types including renal cell lines which usually exhibit robust endogeneous HIF2\(\alpha\) expression because of VHL mutation. Following that, we would try to explore the downstream factors that mediate the radioresistance effect of HIF2\(\alpha\). In general, HIF2\(\alpha\) may increase the expression of proteins (as it does for VEGF) or enhance the activity of proteins (as it does for c-Myc). Therefore, we will measure not only the protein level of these candidates but their activities.

At the same time, gain-of-function experiments could also be done by overexpressing HIF\(\alpha\) in non-stem glioma cells to see whether that will enhance their radiation resistance.
References


Biography

Zhizhong Li was born January 23, 1984 in Sichuan province, China. He spent his first two years with all the relatives in a small city called Long-chang before he moved with his parents to Jian-yang city because of their job relocation. He finished his preschool, preliminary school, middle school and high school all in Jian-yang.

After the annual China College Entrance Exam in 2001, Zhizhong was selected to go to one of the best colleges in China: Tsinghua University (Beijing). In the summer of 2005, four years after he joined the Department of Biological Science and Biotechnology at Tsinghua, he finished 1st place in his class with a GPA of 3.9, received his Bachelor of Science degree and graduated with distinguished honor.

In August 2005, he came to Duke University graduate school to pursue his Ph.D degree in Molecular Cancer Biology. Since then, he has been working with Dr. Jeremy Rich and focused on better understanding angiogenesis and hypoxia response in brain tumors. In his four years in graduate school, he participates in various projects and have published six research papers.
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Duke University  Durham  2005-2009
Ph.D: Pharmacology and Cancer Biology.

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Ph.D Candidate in Dr. Jeremy Rich’s Lab, Angiogenesis and Hypoxia Response in Glioma Cancer Stem Cells.

Tsinghua University, Department of Biological Sciences and Biotechnology  2003-2005
Research Fellow in Dr. Duanqing Pei’s Lab, Embryonic Stem Cell Regulatory Mechanisms.

PUBLICATIONS


EXTRACURRICULAR LEADERSHIP

Duke University Board of Trustees Standing Committee 2008-2009
Graduate/Professional Student Representative

Duke Chinese Alumni Association (NPO) 2007-2009
Executive Board Member

Duke Chinese Students and Scholars Association 2006-2007
President

Global League of Chinese Students and Scholars Association 2006-2008
Chair and Co-founder