Cancer Stem Cells in Brain Tumors:
Identification of Critical Biological Effectors

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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

2010
ABSTRACT

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Abstract

Human cancer is a leading cause of morbidity and mortality in the developed world. Contrary to the classical model in which tumors are homogeneously composed of malignant cells, accumulating evidence suggests that subpopulations of highly malignant cells play a dominant role in tumor initiation and growth. These cells have the capacity for prolonged self-renewal and they efficiently generate tumors that phenotypically resemble the parental tumor in transplantation assays. Such characteristics are reminiscent of normal stem cells, and these potently tumorigenic cells have therefore been called “cancer stem cells” (CSCs). Importantly, studies have shown that CSCs are central mediators of therapeutic resistance, tumor angiogenesis, and metastatic or invasive potential. In the case of malignant glioma, poor patient survival and the paucity of effective therapeutic advances have been attributed to inherent CSC growth potential and treatment resistance, respectively. For this reason, there is great interest in elucidating the molecular features of CSCs, with the ultimate hope of developing CSC-directed therapies.

Given the overlap between the malignant characteristics exhibited by CSCs and those promoted by the PI3K/AKT pathway, we hypothesized that AKT activity within CSCs could represent a reasonable therapeutic target for CSC-directed therapies. Indeed, a pharmacological inhibitor of AKT preferentially targeted glioma CSCs versus non-CSCs and was associated with increased
apoptosis and impaired tumorigenesis. These data suggest that interventions targeting AKT could effectively target glioma CSCs.

Quite distinct from the PI3K/AKT pathway, we hypothesized that the pro-survival and pro-growth features of nitric oxide (NO) might also operate in glioma CSCs. Our experiments found that glioma CSCs produced more NO than non-CSCs, which is attributed to inducible nitric oxide synthase (iNOS) expression and activity within the CSCs. Interference with iNOS activity or expression, as well as selective NO consumption, attenuated CSC growth and tumorigenicity. The mechanism behind iNOS-mediated survival appears to involve, at least in part, suppression of the cell cycle inhibitor CDA1. iNOS inhibition decreased glioma growth in murine xenografts and human expression studies demonstrate an inverse correlation between iNOS expression and patient survival.

To more fully evaluate the biological effects of NO in CSCs, we designed a novel strategy to consume NO within mammalian cells through heterologous expression of *E. coli* flavohemoglobin (FlavoHb). This enzyme is a highly specific NO dioxygenase which converts NO to inert nitrate (NO$_3^-$) several orders of magnitude faster than iNOS synthesizes NO. Expression of FlavoHb in mammalian cells is therefore a novel and functional tool to interrogate the role of NO in cellular stress and signaling.

In summary, this doctoral thesis focuses on several molecular characteristics that define malignant CSCs and describes a novel strategy for studying NO, which is one of the CSC-specific molecular effectors.
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List of Abbreviations:

1400W  N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide, dihydrochloride

ABC transporter  ATP (adenosine triphosphate)-binding cassette transporter

AML  Acute myelogenous leukemia

APC  Adenomatosis polyposis coli

bFGF  Basic fibroblast growth factor

BrdU  5-bromo-2-deoxyuridine

BYK191023  2-[2-(4-methoxy-2-pyridinyl)ethyl]-1H-imidazo[4,5-b]pyridine dihydrochloride

CDA1  Cell division autoantigen 1 (also known as TSPYL2, DENNT)

cGMP  Cyclic guanosine monophosphate

CML  Chronic myelogenous leukemia

CMV  Cytomegalovirus promoter

CSC  Cancer stem cell

DAF-2  4,5-diaminofluorescein

DETA-NO  (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate

DMEM  Dulbecco’s modified Eagle’s medium

DMSO  Dimethylsulfoxide

DTT  Dithiothreitol

EGF  Epidermal growth factor

eNOS  Endothelial nitric oxide synthase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FlavoHb</td>
<td>Flavohemoglobin</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cell line</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible factor 1, alpha subunit</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional review board</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemic stem cell</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>REMBRANDT</td>
<td>Repository for Molecular Brain Neoplasia Data (National Cancer Institute, National Institutes of Health)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>SRC</td>
<td>Sarcoma proto-oncogene</td>
</tr>
<tr>
<td>SSEA1</td>
<td>Stage-specific embryonic antigen 1 (also known as CD15)</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
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It is hard to express the gratitude I have for those who have guided, supported, and taught me over the course of my education. My first mentor, Brad Meyer, once advised me that learning has nothing to do with grades or competitiveness; learning “for the sake of learning” was most any intellectual could hope to enjoy. I hope I have applied this over the course of my training, and I aspire to perpetuate this philosophy as I continue in my career. It would be an honor to someday convey similar advice to a developing “intellectual.”

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Chapter 1.
Introduction to Cancer Stem Cells

Over the course of my training in the Rich laboratory, I have had the opportunity to participate in writing several reviews and chapters relating to cancer stem cells and their involvement in resistance to therapy. This chapter is based on the manuscripts listed below, with the most substantial portion deriving from the sections I wrote for a book chapter currently under consideration for a book entitled Adult Stem Cells: Physical and Functional Characterizations (Springer).


1.1 History of the Cancer Stem Cell Hypothesis

Over 150 years ago, Rudolf Virchow observed profound cellular diversity in neoplastic growths and theorized that a tumor could arise from mutated embryo-like cells that lie dormant in adult tissue [1]. This pioneer of modern pathology posited that, following an unknown signal, the embryo-like cells “reactivate” and form a growing lesion comprised of primitive cells and their progeny. Along these lines, tumors have long been recognized to contain differentiation patterns reminiscent of stem cell-derived hierarchies [2]. The contribution of “stem-like” cells (i.e., cells that resemble the multipotent, self-renewing stem cells found in normal tissues) to malignant phenotypes is exemplified by the increase in stem-like blastic cancer cells that drives the progression of clinically indolent chronic myelogenous leukemia (CML) to its lethal “blast crisis” phase [3, 4], suggesting that less differentiated cells may promote a more malignant phase of this disease.

Despite these early theories and observations regarding stem-like cells in cancer, a link between stem-like cells and tumor initiation/maintenance remained largely hypothetical until ground-breaking studies performed by John Dick and colleagues [5, 6]. Increased tumorigenicity and augmented stem cell-like characteristics were identified in acute myeloid leukemia (AML) cells that were positive for the hematopoietic stem cell marker CD34 and negative for the lymphocyte differentiation marker CD38 [5]. Importantly, CD34+/CD38- AML cells were several orders of magnitude more tumorigenic than cells not displaying this
cell surface marker profile. These cells demonstrated stem-like characteristics such as prolonged self-renewal and a capacity for producing progeny that display a range of differentiation markers. Collectively, these studies strengthened the notion that AML pathophysiology involves the presence of a cellular hierarchy involving cells with stem-like properties.

Though a series of studies utilized competitive assays of leukemic stem cell (LSC) repopulation to further characterize the properties and cell surface markers of LSCs [7-14] (reviewed in [15]), the potential for highly tumorigenic cancer stem cell (CSC) subpopulations within solid tumors remained speculative until 2003 when a series of groups reported the prospective isolation and characterization of such cells deriving from various brain tumors, including glioblastoma and medulloblastoma [16-18]. Concurrently, identification of stem-like subpopulations with increased tumorigenicity in transplantation assays was reported for malignant breast tumors [19]. Since these initial studies, the isolation of CSCs with potent tumorigenic capacity relative to matched non-stem tumor cells has been reported for a variety of tumors including colon, head and neck, hepatocellular carcinoma, ovarian carcinoma and melanoma [20-35]. While the cell surface molecules employed for isolation of CSC-enriched populations often varies between tissues, the properties of the stem-like tumorigenic subpopulations remain reasonably consistent (see next section 1.2 on “Cancer Stem Cell Criteria”)

3
1.2 Cancer Stem Cell Criteria

1.2.1 Definitive properties of cancer stem cells

Despite their resemblance to normal stem cells, CSCs do not abide by the “rules” governing the behavior of normal stem cells. Therefore, although CSCs resemble normal stem cells in several ways, they are not defined by the exact criteria used to characterize normal stem cells. CSCs are defined by functional characteristics; in order to be a CSC, a cancer cell must be able to: 1) self-renew and, 2) generate a phenocopy of the parental tumor in transplantation assays [36] (Figure 1).

One criterion required of both normal stem cells and CSCs is an ability to self-renew, or generate at least one mitotic daughter cell capable of maintaining the stem cell phenotype exhibited by the parental cell [36]. For normal stem cells, this means that at least one daughter cell must have the capacity for self-renewal, as well as the capacity for multilineage differentiation. For CSCs, self-renewal implies that at least one daughter cell retains the capacity for self-renewal as well as the ability to generate tumors that resemble the parental tumor in transplantation assays.

The second CSC-defining criterion involves the ability for CSCs to generate tumors resembling the parental tumor in transplantation assays [37]. In the most pure sense, this would be manifested by the growth of a second tumor in a human patient that is distinct from the primary mass (e.g. growth of a second gliomatous lesion in the contralateral brain hemisphere of a patient with a frontal
lobe glioma) or through regeneration of a tumor after surgical or chemoradiotherapy-based ablation. In the laboratory, this property can be approximated by the propensity of cells to initiate xenografted tumors that recapitulate the heterogeneity and characteristics of the parental human tumor in immunocompromised mouse models.

1.2.2 Common, but not required, properties of cancer stem cells

There are many other characteristics that frequently co-segregate with the required properties of CSCs, though are not fundamentally required (Figure 1). Normal stem cells are defined by a capacity for multi-lineage differentiation – that is, they must be multipotent. In contrast, CSCs have no such absolute requirement for differentiation into discrete cellular lineages. Many tumors contain cells that have aberrant differentiation patterns (i.e. cells may simultaneously express differentiation markers for multiple lineages) [38], while other tumors tend to display cells that exhibit one primary differentiation state [39], and still other tumors may display a panoply of distinctly different tumor cells with characteristics of not only the parental tissue but also differentiation markers resembling other tissues [40, 41].

Just as the original identification of LSCs took advantage of hematopoietic stem cell surface marker expression, CSCs from many types of tumors express cell surface markers typically seen on the surface of normal embryonic or adult stem cells. For example, much work has been invested in determining the cell surface marker expression patterns most effective for isolation of AML stem cells.
[6, 7, 42]. The expression pattern displayed by AML stem cells mimics cell surface markers patterns exhibited by normal hematopoietic stem cells; while human hematopoietic stem cells are contained in the lineage-CD34+CD38-CD90- population, LSC from AML are segregated by CD34+CD38- cell surface marker expression profiles. Similarly, depending on the sample, brain tumor stem cells appear to be effectively segregated into tumorigenic and non-tumorigenic populations based off of cell surface expression of stem cell-related markers such as CD133 (also known as Prominin-1; expressed by hematopoietic stem cells) [16-18], and stage specific embryonic antigen 1 (SSEA1; also known as CD15, expressed by embryonic stem cells) [43, 44].

Stem-like tumorigenic populations within tumors appear to activate transcriptionally-regulated signaling networks typically active within embryonic, induced pluripotent, or adult stem cells, such as the signaling networks associated with the stem transcription factors Oct4 [45], Sox2 [46], or c-Myc [47]. Similarly, CSCs often activate one or more critical stem-related signaling pathways including Notch, Wnt/Beta-catenin, or Hedgehog [36, 48]. Several groups have assessed the gene expression profiles exhibited by CSCs and normal adult or embryonic stem cells demonstrate that overall signatures are often similar, though may cluster into several different global patterns [49-51]. The ability to access stem cell-related signaling pathways provides selective advantages for CSCs, including an ability for sustained proliferation and
enhanced migratory capacity. However, though they may access stem cell signaling programs, CSCs are not defined by this characteristic.

1.3 Technical Aspects Involved in the Isolation and Validation of Cancer Stem Cells

To call a cell a CSC, it must have the functional capacity for self-renewal and the ability to generate tumors that recapitulate the primary mass in transplantation assays. Therefore, to designate an individual cell a CSC, it is necessary to analyze it using functional assays, which fundamentally limits one’s ability to assess the characteristics demonstrated by that particular cell in an unaltered form (i.e., for exploratory experiments outside of the required functional assays). For this reason, CSCs are generally studied in the context of cell populations known as enriched for tumorigenic and self-renewal capacity; single cells are isolated from a primary tumor specimen by mechanical/ enzymatic digestion, then labeled or selected by one of a variety of methods to obtain populations of CSC-enriched cells (Figure 2, Table 1). These cell populations can then be verified as “CSC-enriched” by determining the relative frequency of CSCs in tumorigenesis and self-renewal assays (as described below), and the remainder of these cells evaluated experimentally in vitro or in vivo.

1.3.1 Isolation of cancer stem cell-enriched populations

Various cell surface markers or labeling techniques have been reported to segregate CSC-enriched populations from CSC-depleted populations, depending on the type and grade of tumor, the tissue from which a tumor derives, the
species of the tumor-bearing animal, and the cytogenetic and gene expression profiles within that tumor (Table 1). Cell surface markers known to enrich for CSCs or non-stem cancer cells are amenable for labeling with fluorophore-conjugated antibodies and sorted using fluorescence activated cell sorting (FACS). Alternatively, magnetic bead-conjugated antibodies and magnetic selection columns are useful for isolation of cells positive for an individual cell surface marker.

Other non-surface marker-based systems are available to isolate CSCs. Partially due to overexpression of ATP-binding cassette type drug transporters (ABC transporters), CSCs from some solid tumors display characteristic dye efflux patterns that permit them to be identified by FACS based on the relative rate of vital dye efflux in combination with cell size and scatter characteristics (e.g., the “side population”) [33, 52-55]. Still other tumors display characteristic enzymatic activities (e.g., aldehyde dehydrogenase activity) that are detectable with fluorescent substrates amenable to FACS [56, 57].

1.3.2 Validation of cancer stem cell-enriched populations

Isolation of cells displaying putative stem cell markers, as discussed above, is not sufficient to demonstrate enrichment for CSCs. Due to a wide range of factors that can influence the efficiency and success of CSC-isolation, it is imperative that populations be validated as CSC-enriched or -depleted by each laboratory for each model system and isolation technique employed.
The classical method for demonstrating enriched tumorigenic capacity is through utilization of “limiting dilution” tumorigenicity analysis for each population [5]. This involves inoculating varying numbers of tumor cells into immunocompromised mice (e.g. 10, 100, 1000 and 10000 cells) and quantifying the number of mice that develop tumors and the length of time to tumor development. From these data, it is possible to back-calculate the approximate number of tumorigenic cells per population. Cancer stem cell-enriched populations will possess more tumorigenic cells than the unsorted tumor. Even though a cell population may be highly tumorigenic, it is also critical that tumors be evaluated to determine if the transplanted tumor maintains the original tumor’s cellular heterogeneity (i.e., the cell surface marker expression, gene expression profiling, and morphologic diversity of cells resembles that of the primary lesion) [37].

Serial transplantation assays are one method by which to validate self-renewal capacity. A transplanted tumor is dissociated, re-sorted for putative stem cell marker populations and re-injected into new animals, again with verification of appropriate cellular diversity in the resultant tumors. Technically speaking, self-renewing CSCs should give rise to tumors able be re-transplanted indefinitely. In vitro serial passage of clonal colonies is one assay adapted from normal stem cell techniques that is used to approximate self-renewal capacity for solid tumor cells [58]. Single cells are plated in stem-cell permissive suspension culture conditions and the efficiency of three-dimensional “tumorsphere”
formation is quantified. As in *in vivo* tumor formation assays, self-renewing cells should be able to permit serial passage of tumorspheres [37]. Though serial evaluations of clonal tumorsphere formation require a capacity for cellular self-renewal, it is important to remember that this assay also requires cellular survival and proliferation. Thus, interventions that decrease tumorsphere formation capacity may not necessarily work via interruption of stem cell-associated self-renewal, but instead may simply impact the survival or proliferation of these cells.

1.4 **Technical considerations for in vitro and in vivo cancer stem cell maintenance**

Optimal and appropriate *in vitro* cell culture conditions are critical to the maintenance of CSCs *ex vivo*. Although standard cell culture conditions for established cell lines employ serum supplementation, recent results have suggested that culturing cells in serum-free, non-adherent conditions in the presence of various growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) facilitates CSC enrichment in certain tumor types in the absence of marker selection, and that these culture conditions are necessary for the maintenance of CSC-enriched populations [59]. Freshly isolated brain tumor cells cultured in stem cell permissive medium (serum-free, growth factor enriched medium) maintain tumorigenic capacity and gene expression profiles that resemble the original tumor [59]. In contrast, culturing cells in serum-containing medium usually results in increased tumor cell
differentiation, a reduction in tumor formation and self-renewal capacity, and divergence from the gene expression profile displayed by the parental tumor [59].

The expression profiles, behaviors, and cellular composition of human tumor cell populations can be altered by in vitro cell culture, and there are clear microenvironmental cues that are not present in in vitro cell culture conditions. Therefore, human tumor cells can be propagated in a less-artificial (though still far from perfect) manner by xenografting human cells into immunocompromised mice and amplifying tumor cells in an in vivo environment [60, 61]. In this way, tumor cell propagation occurs in a way that maintains substantial cellular diversity more accurately than in vitro culture, which exposes cells to the selective pressures of plastic tissue culture dishes and abnormal environmental stresses encountered in standard lab tissue culture environments. Orthotopic xenografting, or implantation of human tumor cells from a particular organ in the corresponding mouse organ, is often considered desirable for the maintenance of tumors in an organ-appropriate environment, but the technical requirements of orthotopic xenograft establishment can be difficult or even prohibitive, depending on the anatomic location and tumor type. As such, non-orthotopic tumor cell implantation (e.g. subcutaneous or under the kidney capsule) can be utilized to capitalize on the maintenance of heterogeneity that in vivo tumor propagation affords if anatomically appropriate implantation is not possible [62, 63].
1.5 Debate Surrounding Cancer Stem Cells

Evidence from a wide range of both human and murine tumor specimens suggests that tumor cells from a number of cancer types possess an augmented capacity for self-renewal as well as an amplified ability to initiate tumors that phenocopy the parental tumor (Table 1). However, the existence of CSCs remains contentious in the field of cancer biology, in part because of the application of inconsistent and inaccurate definitions and disputes over terminology. Below, several of these issues will be addressed.

1.5.1 Theories of tumor evolution: clonal or hierarchical?

Two major theories have been proposed to explain the propagation of, and selective evolution for, neoplastic cells within tumors [36]. The stochastic theory of tumor evolution posits that almost every cell in a tumor maintains tumorigenic potential and Darwinian selection for genotypically distinct clones of tumor cells (generated through stochastic mutation events) determines the ultimate cellular phenotype of the original or secondary tumor lesions.

The second paradigm proposes that the hierarchical organization central to normal stem cell function is also relevant to tumor pathophysiology. This hierarchical theory of tumor organization suggests that cellular tumor initiation and propagation capacity is stratified, with the most tumorigenic cell type (i.e., CSCs) possessing the potential for generating a range of less tumorigenic progeny [36]. Frequently the highly tumorigenic parent CSC displays characteristics reminiscent of stem cells, and its less-tumorigenic progeny often
adopt a variety of morphologies and gene expression profiles that mimic differentiated cells. In this model, the state of a cancer cell and its position in the hierarchy may be controlled by microenvironmental context, transcriptional activation of specific genes, or alterations of the epigenetic regulatory mechanisms [64].

In truth, each model incorporates elements that permit a greater appreciation for cellular heterogeneity within tumors, while also possessing caveats or drawbacks (Table 2). Though often presented as competing and mutually exclusive theories, the actual organization and evolution of heterogeneity within tumors likely derives from elements of both models [64]. One could reasonably envision tumors evolving according to Darwinian selection for cells exhibiting advantageous mutations, while also displaying a wide range of non-genetically determined phenotypes that might also promote the survival of individual cells (or groups of cells) within a tumor. Further, different tumor types likely vary in how rigidly they adhere to either of these models [65]. Some tumors (e.g. leukemias) may primarily be organized hierarchically [66], while other tumors (e.g. metastatic melanomas) may more closely follow the stochastic model of tumor evolution [67, 68].

1.5.2 Terminology

Use of the term “cancer stem cell” has generated substantial disagreement. As previously discussed, the term “cancer stem cell” refers to the stem cell-like characteristics exhibited by cells with the functional characteristics
of secondary tumor initiation capacity and prolonged self-renewal. The use of the term “stem cell” raises many issues amongst researchers, since it is clear that CSCs do not strictly adhere to the rules applied to normal stem cells. This has resulted in much debate over the proper name for this tumor subpopulation. As such, CSCs have also been referred to as tumor-initiating cells, tumor-propagating cells, or stem-like cancer cells; each additional name bears inconsistencies, limitations and insufficiencies [37, 69]. Regardless of the terminology employed, the capacity for self-renewal and potent tumor formation are the definitive characteristics of this tumor subpopulation.

1.5.3 Rarity

Original descriptions reporting the prospective isolation of CSC-enriched populations in human leukemias, brain tumors, and other tissues identified relatively small populations (0.5-5% of the total tumor cell population) that possessed the defining characteristics of CSCs [5, 16-19]. However, further studies have described tumors with a much higher proportion of cells that satisfy the definitive requirements of CSCs, and some genetically driven mouse tumors display very high percentages of cells possessing CSC characteristics [68, 70-72]. Although the original descriptions of CSCs were notable for the prospective isolation of a “rare” population of CSCs within a tumor, the prevalence of CSCs is irrelevant to the designation of cells as CSCs (Figure 1).
1.5.4 **Cellular Origin of Tumors and/or Cancer Stem Cells**

Although they possess stem cell-like properties, it is not necessary that CSCs (or tumors containing cells fulfilling the properties of CSCs) derive directly from normal stem cells. In fact, current evidence suggests that tissue stem cells, transit-amplifying cells, committed progenitors, or even post-mitotic differentiated cells may serve as targets for oncogenic mutations and yield tumors that possess cells that fulfill the criteria required of CSCs, depending on the model, organ, and oncogenic stimulus employed [36, 73, 74]. Though it is clear that each of these cell types are *sufficient* to generate tumors that possess cells fulfilling the CSC-defining characteristics, the identity or even the existence of what cell type is *necessary* for generation of tumors with CSCs remains unclear. Retrospective identification of the cell-of-origin in already established tumors is challenging or even unattainable. Regardless of the identity of the original transformed cell, the eventual presence of cells possessing definitive CSC qualities is compelling to propel research relating to both identification of the cell of origin for tumors and/or CSCs as well as interrogation of critical molecular determinants of CSC properties.

1.6 **Glioblastoma: A Lethal Human Tumor and a Model System for Solid Tumor Cancer Stem Cells**

Malignant gliomas are the most common primary brain tumor in adults and portend dismal prognoses – the median survival for patients with glioblastoma multiforme (World Health Organization grade IV) is a mere 15 months, even with
optimal therapy [75]. Though there have been some recent advances in the standard of care for glioma patients, these interventions have only marginally improved overall survival. Historically, the identification of anti-glioma therapies focused on molecular characteristics shared throughout the tumor bulk or in cell lines, but clinical evaluation of therapeutic approaches identified in this way have been largely disappointing [76], suggesting that more careful evaluation of brain tumor subpopulations may be warranted for the identification of comprehensive and effective anti-tumor therapeutic regimens.

The initial prospective isolation of CSCs from human brain tumors was performed by sorting based on expression of the cell surface marker CD133 (Prominin 1), which had previously been identified as a marker displayed by normal hematopoietic stem cells [18]. The existence of pediatric brain tumor stem cells was subsequently demonstrated [16]; this study also reported overlapping gene expression profiles between normal neural stem cells and brain tumor stem cells.

For many gliomas, CD133+ cells exhibit the required characteristics of CSCs, while they also exhibit other common characteristics of CSCs, including the expression of stem cell markers (CD133, nestin, Sox2, Oct4, etc) and the capacity for multi-lineage differentiation potential [16-18, 77, 78]. CD133 was first described as a marker of hematopoietic stem cells and subsequently reported as a marker of normal human neural stem cells [79-81]. However, CD133 is by no means the exclusive marker of CSCs in gliomas. Additional cell surface markers
have been reported to facilitate the enrichment of CSCs from gliomas depending on the characteristics of the original tumor, including markers like integrin α6, the neuronal surface glycoside A2B5, and SSEA-1 [44, 82-84] (Table 1). Regardless of the specific marker employed for sorting, in defined circumstances these markers have allowed successful enrichment of tumor cell subpopulations exhibiting typical characteristics of CSCs, permitting evaluation of the molecular mechanisms underlying the glioma stem cell phenotype. Over time, as inter-tumoral differences are more thoroughly understood and grade/cytogenetic contributions to CSCs more comprehensively evaluated, it is likely that more established protocols for optimal cell surface marker-based CSC population enrichment will be developed. Further, it is not unlikely that some permutation of the aforementioned markers or unidentified ones may most effectively enrich for brain tumor CSCs.

Taken together, brain tumor-derived CSCs provide an instructive model system for the CSC paradigm. This model not only identifies novel therapeutic targets for a disease with a dismal prognosis, but also concomitantly enriches our understanding of CSC biology.

1.7 Conclusions

Despite the persistent controversy surrounding specific aspects of the CSC paradigm, the theory promotes a greater appreciation of intra-tumoral heterogeneity and the contribution of this cellular diversity to tumor progression
and recurrence. It is not surprising that there are cells within a tumor that are prone to survive therapy or promote tumor maintenance, and it is likely that this heterogeneity of malignant phenotypes derives from some combination of both genetic and non-genetic determinants. In addition to work characterizing the biological effects of genetic alterations in tumors, an increased emphasis should be placed on understanding those epigenetic determinants that permit cancer cells to access the pro-malignant phenotypes afforded by stem cell-related signaling and phenotypes. Elucidating molecular characteristics of CSCs, one subpopulation that appears to play a role in tumor maintenance and recurrence, may help define novel therapeutic targets that may have been overlooked in evaluations of whole tumors. The effectiveness of anti-CSC therapeutic strategies remains to be determined, but it has been suggested that CSC-directed therapies may promise the development of increasingly successful therapeutic options for a variety of tumors [85, 86].
Figure 1. Frequent characteristics and defining qualities of cancer stem cells. CSCs are defined by the capacity for extensive self-renewal and efficient regeneration of the parental tumor in transplantation assays. Other characteristics frequently associated with CSCs, while not defining them, include: expression of stem cell markers, multilineage differentiation, or rarity within a tumor.
Figure 2. Isolation of cancer stem cell-enriched populations. CSC-enriched populations from various tumors are obtained through selection via cell surface marker expression, enzymatic activity, or dye efflux characteristics. The resultant populations continue to evolve: the CSC-enriched population will continue to self-renew and generate more CSCs, but will also regenerate the original tumor cellular diversity, whereas CSC-depleted populations lack the capacity for self-renewal and tumor repopulation. Red cells represent CSCs.
Table 1. Cancer stem cell selection techniques for various human and mouse models of malignant brain, breast, and colon cancer.

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Organism (genetic model)</th>
<th>CSC Selection Marker or Method</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (Glioma)</td>
<td>Human</td>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[17, 18]</td>
</tr>
<tr>
<td>Brain (Glioma)</td>
<td>Human</td>
<td>EGF/bFGF stem cell permissive conditions</td>
<td>[59]</td>
</tr>
<tr>
<td>Brain (Glioma)</td>
<td>Human</td>
<td>SSEA1/CD15&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[44]</td>
</tr>
<tr>
<td>Brain (Glioma)</td>
<td>Human</td>
<td>A2B5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[84]</td>
</tr>
<tr>
<td>Brain (Glioma)</td>
<td>Human</td>
<td>L1 cell adhesion molecule (L1CAM)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[87]</td>
</tr>
<tr>
<td>Brain (Glioma)</td>
<td>Human</td>
<td>Integrin α6&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[82]</td>
</tr>
<tr>
<td>Brain (Glioma) Mouse (PDGF-driven nestin-TVA)</td>
<td>Side population (Hoescht efflux)</td>
<td></td>
<td>[88]</td>
</tr>
<tr>
<td>Brain (Medulloblastoma) Mouse (Patched heterozygote)</td>
<td>SSEA1/CD15&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>[43, 71]</td>
</tr>
<tr>
<td>Breast</td>
<td>Human</td>
<td>CD44&lt;sup&gt;+&lt;/sup&gt;, CD24&lt;sup&gt;-/lo&lt;/sup&gt;, Lineage&lt;sup&gt;-&lt;/sup&gt;</td>
<td>[19]</td>
</tr>
<tr>
<td>Breast</td>
<td>Human</td>
<td>Aldehyde dehydrogenase activity</td>
<td>[56]</td>
</tr>
<tr>
<td>Breast Mouse (p53&lt;sup&gt;+/+&lt;/sup&gt; and MMTV-Wnt1)</td>
<td>CD61/integrin β3&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>[89]</td>
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<td>Breast Mouse (MMTV-Wnt1)</td>
<td>Thy1&lt;sup&gt;+&lt;/sup&gt;, CD24&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>[90]</td>
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<td>Breast Mouse (p53&lt;sup&gt;−/−&lt;/sup&gt;)</td>
<td>Lineage&lt;-, CD29&lt;sup&gt;hi&lt;/sup&gt;, CD24&lt;sup&gt;hi&lt;/sup&gt;</td>
<td></td>
<td>[72]</td>
</tr>
<tr>
<td>Colon</td>
<td>Human</td>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[27, 29]</td>
</tr>
<tr>
<td>Colon</td>
<td>Human</td>
<td>CD44&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[91]</td>
</tr>
<tr>
<td>Colon</td>
<td>Human</td>
<td>CD166&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[91]</td>
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<tr>
<td>Colon</td>
<td>Human</td>
<td>Aldehyde dehydrogenase activity</td>
<td>[57]</td>
</tr>
<tr>
<td>Colon</td>
<td>Human</td>
<td>Wnt&lt;sup&gt;hi&lt;/sup&gt; (TCF/LEF-GFP reporter)</td>
<td>[92]</td>
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Table 2: General characteristics of the stochastic and hierarchical models for tumor organization and the origin of heterogeneity within tumors.

<table>
<thead>
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<th>Model characteristics:</th>
<th>Stochastic Model</th>
<th>Hierarchical Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provides explanation for genetic heterogeneity within tumors</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Provides explanation for non-genetically determined heterogeneity within tumors</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Deterministic, linear evolution of tumor cell phenotypes</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Complex, non-linear, flexible/adaptable adjustment of tumor cell phenotypes</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Predicts that every cell of a defined genotype has the capacity for tumor promotion</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Predicts that the capacity for tumor promotion is hierarchically (and non-genetically) stratified</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Compatible with multi-hit hypothesis of tumor progression</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Provides explanation for post-therapeutic tumor recurrence</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Chapter 2.

Brain Cancer Stem Cells Display Preferential Sensitivity to AKT Inhibition

This chapter is based on a research article concerning a collaborative project completed by myself and Wen-Chi Foo. She initiated the project and I completed experiments needed for the original submission as well as for revised submissions in response to reviewer concerns. It has been published in the journal Stem Cells.


*, indicates that the authors contributed equally
2.1 Summary

Malignant brain tumors are among the most lethal cancers, and conventional therapies are largely limited to palliation. Novel therapies targeted against specific molecular pathways or critical tumor subpopulations may offer improved efficacy and reduced toxicity compared to conventional approaches. Here we demonstrate that glioma populations enriched for CSCs are preferentially sensitive to an inhibitor of AKT, a prominent signaling node involved in cell survival and invasion. Treatment with an AKT inhibitor more potently reduced the numbers of viable brain CSCs relative to matched non-stem cancer cells. This anti-growth effect was associated with a preferential induction of apoptosis and a suppression of neurosphere formation. AKT inhibition also reduced the motility and invasiveness of all tumor cells with a greater impact on CSC behaviors. Furthermore, inhibition of AKT activity in CSCs increased survival of immunocompromised mice bearing human glioma xenografts in vivo. Together, these results suggest that AKT inhibitors may function as effective anti-CSC therapies in malignant brain tumors.
2.2 Introduction

Glioblastomas commonly display hyperactivation of the phosphatidylinositol-3-kinase (PI3K)-AKT (also known as protein kinase B) pathway, a pro-tumorigenic signaling cascade that contributes to the pathogenesis of several human cancers [73, 93-97]. The PI3K-AKT pathway can be activated in tumors through a number of mechanisms, including activation of upstream growth factor receptors, mutations of the PI3K catalytic subunit (PIK3CA), overexpression or amplification of AKT family members, or inactivation of the inhibitory effects of the phosphatase and tensin homolog (PTEN) tumor suppressor [93-97]. Hyperactive AKT signaling promotes tumorigenic cell behaviors by increasing cell survival, proliferation, invasion, and angiogenesis, and it has been directly associated with in vitro conversion of grade III anaplastic astrocytoma to grade IV glioblastoma [73, 93-97].

Due to the association of AKT activity with a wide range of tumorigenic properties, we hypothesized that brain CSCs may exhibit a dependence on the AKT pathway. Indeed, chemoresistance in hepatocarcinoma CSCs may be conferred by activation of AKT [98], and AKT regulates the survival of tumor cells in the perivascular niche bearing stem cell markers in mouse medulloblastoma models [73]. To further investigate the dependence of brain CSCs on AKT signaling, we pharmacologically treated matched populations of glioblastoma CSCs and non-stem glioblastoma cells with a small molecule inhibitor of AKT.
We sought to determine if preferential targeting of brain tumor CSCs could be achieved through inhibition of AKT by decreasing the capacity of these cells to survive, proliferate, and invade, thereby decreasing their malignant potential.

2.3 Results

2.3.1 Brain tumor stem cells exhibit greater sensitivity to Akt inhibition

Cancer stem cells are defined through functional assays to determine the capacity for sustained self-renewal and the ability to recapitulate the full diversity of the parental tumor upon xenotransplantation [16-18, 77, 99, 100]. Several groups, including our own laboratory, have demonstrated that brain tumor CSCs can be enriched prospectively through the use of the CD133 (Prominin 1) cell surface marker [16-18, 77, 99, 100]. The CD133 marker does not absolutely segregate for tumorigenesis as some tumors may contain CD133− cells that form tumors, although this frequently requires transplantation of high numbers of cells [101, 102]. However, we have found that CD133+ tumor cells from patient biopsy specimens display both potent neurosphere formation potential in cell culture and effective tumor generation in immunocompromised animal models while CD133− cells do not form neurospheres and rarely, if ever, form tumors upon xenotransplantation [77]. We, therefore, used models that we have previously characterized in functional assays (sustained neurosphere generation, multi-lineage differentiation, tumor initiation) to define “cancer stemness” in our studies. In addition, CD133+ cells utilized for these novel studies highly
expressed stem cell markers such as Nestin, Oct4, Olig2, and Sox2, whereas CD133− cells did not (Figure 3). Taken together these results suggest that CD133+ cells derived from the models used in the studies described below are enriched in CSCs.

To determine whether CD133+ tumor cells exhibit differential activation of the PI3K-AKT pathway compared to their non-stem counterparts, the activation state of pathway components was examined through immunoblotting. In short term cultures (<5 passages) of T3359 (Figure 4, Figure 5) or D456MG (Figure 5) glioblastoma cells, total levels of AKT protein were similar between CD133+ and CD133− cells. In contrast, basal levels of phosphorylated (activated) AKT were higher in the CD133− populations (Figure 4, Figure 5). Consistent with these data, we found decreased AKT kinase activity in CD133+ cells in comparison to matched CD133− cells (Figure 6). Although we expected CSCs to have a higher basal activation of AKT, the relatively lower activation of AKT in CD133+ cells may be due to differences in cell attachment. Cancer stem cells grow in three-dimensional neurospheres whereas non-stem cancer cells must be cultured in adherent conditions in tissue culture-treated plates. As there may be differences in cell-cell and cell-matrix interactions between these growth conditions, we evaluated AKT phosphorylation in short term cultures of adherent and non-adherent CD133− cells to ensure survival of non-adherent CD133− cells (which is compromised by the lack of serum and cell adhesion). We found adherent CD133− cells had greater AKT phosphorylation than either non-adherent
CD133− or CD133+ cells which had very similar levels of basal AKT phosphorylation (Figure 7).

Several pharmacologic agents have been designed to inhibit AKT function. Phosphotidylinositol ether lipid analogues may target the pleckstrin homology domain of AKT to selectively inhibit cell survival in cancers with high AKT activity [103]. One of these drugs, the AktIII inhibitor (SH-6), reduced AKT activation in CD133+ glioma cells in concentration-dependent manner but had no profound effect on CD133− cells (Figure 4). The temporal course of AktIII inhibitor was also assessed in matched tumor cell populations and demonstrated transient effects in both populations with relatively greater effects on the CD133+ cells when normalized to the basal level of activation (Figure 5). This decrease in activated AKT is not due to a decrease in total AKT levels, nor to improper loading of protein samples, as assessed by tubulin controls (Figure 4, Figure 5). Therefore, these data demonstrate that CD133+ and CD133− cells exhibit differential activation levels of the AKT pathway, and the CD133+ population has a greater sensitivity to the AKT inhibitor.

2.3.2 AKT activity is necessary for cancer stem cell proliferation and survival

To determine if targeting AKT activity would preferentially decrease the pro-tumorigenic behaviors of CD133+ cells, the proliferation and survival of matched CD133+ and CD133− short-term brain tumor cell cultures were interrogated. Despite having identical numbers of cells plated at the initiation of studies, at the end of the experiments CD133+ cultures yielded higher numbers
of viable cells at baseline than the matched CD133− cells, likely due to the long term proliferative potential of these cells. However, the pharmacologic AktIII inhibitor demonstrated a significant concentration dependent effect in reducing the number of viable CD133+ cells with more modest effects on CD133− cells (Figure 8), as determined by trypan blue staining. The preferential decrease in CD133+ cell numbers is not likely to be due to potential differences in basal AKT phosphorylation in this assay with adherent CD133− cells. A similar preferential decrease in CD133+ cell growth was observed when both CD133− and CD133+ cells were cultured in stem cell media under non-adherent conditions with two different AKT inhibitors (Figure 9, Figure 10). Use of the PI3K inhibitor LY290042 to inhibit an upstream component of the AKT pathway also demonstrated greater effects on CD133+ cell growth with increasing concentrations of drug (Figure 9, Figure 10). When mammalian target of rapamycin (mTOR), a downstream target of AKT, was targeted with rapamycin there was a more modest preferential targeting of CD133+ cell growth (Figure 9, Figure 10). Together these results suggest that inhibition of PI3K or AKT more potently regulates the growth of CD133+ cells than CD133− cells.

To evaluate the impact of pharmacologic AKT inhibition on CD133+ cell survival, the killing efficiency was measured over a range of inhibitor concentrations. Although the percentage of dead cells increased in a concentration-dependent manner with AKT inhibition in both the CD133+ and CD133− cells (Figure 8c,d), the CD133+ populations were more significantly
affected than the CD133− cells. After determining that AktIII preferentially targets CD133+ tumor cell survival, we investigated the mechanism of cell death using Annexin V staining to assess apoptosis in CD133+ and CD133− cells. In parallel with our earlier results, each CD133+ tumor cell culture demonstrated a concentration-dependent increase of apoptosis upon treatment with the AKT inhibitor (Figure 11). In contrast, CD133− cells displayed little to no increase in the apoptotic cell fraction in response to AktIII inhibitor treatment (Figure 11). These results indicate that the preferential cell death in CD133+ CSCs is at least partially due to increased apoptosis, consistent with the known effects the AKT pathway has on cellular apoptosis and survival.

2.3.3 Cancer stem cell neurosphere formation requires AKT activity

Both brain tumor stem cells and neural stem cells display the ability to form complex three-dimensional spherical structures (neurospheres) when cultured in serum-free media [18, 104]. In our studies, neurosphere formation potential is restricted to the prospectively enriched CD133+ tumor population, although other labs have generated neurospheres that did not express CD133 [94]. To further investigate the effects of AKT inhibition on the CSC behaviors of CD133+ brain tumor cells, we examined the effects of the inhibitor on neurosphere formation in the presence of increasing concentrations of AKT inhibitor. CD133+ tumor cells displayed a striking concentration-dependent decrease in the ability to generate neurospheres across each time point examined (Figure 12). When neurospheres that did form with AKT inhibitor
treatment were further analyzed, there was a clear qualitative decrease in size and the cells were unable to form secondary neurospheres (Figure 12 and data not shown). These results indicate that CD133+ brain tumor cells require AKT activity for neurosphere formation.

2.3.4 **Targeting AKT decreases cancer stem cell migration and invasion**

The ability for malignant gliomas to invade into normal neural structures leads to the inability for these tumors to be completely surgically resected [105]. Although the mechanisms underlying brain tumor invasion remain incompletely understood, the PI3K-PTEN-AKT axis has been recognized as a contributor to invasion. Therefore, we expected that AKT inhibition might negatively regulate tumor cell invasion [93, 94]. Using Boyden chamber assays, we evaluated the capacity of matched CD133+ and CD133− cells to migrate or invade the migration and invasion capacity of matched CD133+ and CD133− brain tumor cells was evaluated. Interestingly, there was a striking basal difference in the capacity of CD133+ and CD133− cells to migrate through either an uncoated member or a membrane coated with an artificial extracellular matrix (Matrigel). In one model (D456MG), the CD133− cells displayed much greater migratory and invasive potential (Figure 13), perhaps due to the long-term passage of these cells in a xenograft. In contrast, the CD133+ cells in the T3359 model were more invasive than the CD133− cells (Figure 14), consistent with the notion that CD133+ CSCs contribute to invasion and migration. Increasing concentrations of AKT inhibitor significantly attenuated the capacity of both CD133+ and CD133−
cells to both migrate and invade (Figure 13) but in both models the CD133+ cells displayed greater sensitivity to the inhibitory effects of AKT inhibition than the CSC-depleted CD133- population (Figure 13, Figure 14). Thus, migratory and invasive potential of CSCs relative to the non-stem cell population may depend on the tumor model, but CSCs depend on AKT activity for these pro-invasive behaviors.

2.3.5 Inhibition of AKT activity in cancer stem cells increases survival of immunocompromised mice bearing human glioma xenografts

To further determine the potential therapeutic benefit of targeting AKT activity in CSCs, we determine the tumorigenic potential of cells treated with AktIII inhibitor. When $1 \times 10^4$ or $1 \times 10^3$ CD133+ cells were injected into the forebrains of immunocompromised mice, we observed neurologic signs due to the development of brain tumors regardless of AKT inhibitor treatment. These data demonstrate that inhibition of AKT activity alone for the treatment period was not sufficient to significantly reduce the tumor formation potential of cancer stem cells (Table 3). However, the time to the development of neurologic signs was increased with AKT inhibition. The median survival until the development of neurologic signs of animals bearing $1 \times 10^4$ CD133+ tumor cells treated with DMSO control was 24 days while animals bearing identical CD133+ cells treated with the AKT inhibitor survived for a median of 42 days ($p<0.03$). Similarly, the median survival until the development of neurologic signs of animals bearing $1 \times 10^3$ CD133+ cells was 35 for the DMSO control and 66 days for AKT inhibitor
treatment (p<0.03). These data demonstrate that reducing AKT activity in CD133+ cells can increase survival of mice bearing intracranial xenografts.

Prior work in our laboratory has implicated cancer stem cells in promoting tumor angiogenesis via elevated VEGF secretion [99]. However, the increased survival of mice injected with AKT inhibitor treated CD133+ cells is unlikely to be due to changes in VEGF levels as the AKT inhibitor did not reduce VEGF expression (Figure 15) and is more likely to be attributed to induction of apoptosis (Figure 8, Figure 11).

2.4 Discussion

The essential pathways regulating CSC biology remain poorly defined, but molecular targets with defined roles in normal stem cell biology and aberrant activity or expression in cancers [including Notch, Hedgehog, Wnt/β-catenin, Bone Morphogenic Protein, Myc, Epidermal Growth Factor Receptor, Fibroblast Growth Factor Receptor, and PTEN] are likely to be important. Activation of these pathways by autocrine signals from the CSCs themselves or paracrine signals from the CSC niche could be essential for stem cell maintenance [106, 107]. Recognizing that these stem cell maintenance cues regulate cell survival and differentiation, these signals are logical targets for anti-CSC directed therapies.

The potential for inhibition of the PI3K-AKT pathway to target CSCs is supported by the known involvement of AKT signaling in tumorigenesis and
normal stem cell biology as well as the beneficial effects of AKT inhibition on glioma cell growth [108-115]. Multiple pro-tumorigenic behaviors (such as the promotion of cell proliferation, survival, and invasion) now suggested to be driven by CSCs [16-18, 77, 98-100, 116-119] are known to be regulated by AKT signaling [94]. Activation of the PI3K-AKT pathway is common in malignant gliomas and associated with increased tumor grade and decreased glioma patient survival [93-97]. Genetically engineered glioma models also demonstrate that that constitutively activated AKT contributes to tumor initiation [113, 114].

In addition to roles in tumor formation, the PI3K-AKT pathway is implicated in stem cell biology. Loss of the PI3K inhibitor PTEN increases the proliferation and survival of neural and hematopoietic stem cell populations [110, 111]. Recent studies in the hematopoietic system have shown that activation of the PI3K/AKT pathway in normal hematopoietic stem cells can produce leukemia within weeks [108]. However, LSCs are also particularly sensitive to the effects of mTOR antagonism [108], suggesting a potential therapeutic window for targeting the PI3K-AKT pathway in CSCs with minimal effects on normal somatic stem cells. Until recently, this strategy has not been fruitfully applied to the CSCs of solid tumors. Ma et al. demonstrated that the CD133+ cells from two established hepatocellular carcinoma cell lines are less sensitive to chemotherapy and express higher levels of survival proteins involved in the AKT and BCL2 pathway than the CD133− cells, and respond to AKT inhibition by reducing key survival proteins [98]. Hambardzumyan and co-workers used a murine model of
medulloblastoma (a primary brain tumor distinct from gliomas that commonly occurs in children and displays greater radiosensitivity than glioma) to show that a nestin-expressing perivascular tumor cell population survives radiation, activates downstream effectors of AKT, undergoes a p53-dependent cell cycle arrest, and re-enters the cell cycle at 72 hours [116]. In addition, inhibition of AKT signaling sensitizes cells in the perivascular region to radiation-induced apoptosis [116]. We have extended these findings into a cancer type in which the AKT/PTEN axis plays an essential role using models derived from human specimens or maintained in vivo then only briefly cultured to maintain a CSC phenotype. We sought to further determine if inhibition of AKT signaling in glioma stem cells may be a beneficial mechanism for reducing cancer stem cell growth in vitro and increasing survival in vivo.

To evaluate the effects of AKT inhibition in CSCs and non-stem cancer cells, we have built on prior investigations, including our own, that demonstrated that CSCs are enriched through the use of the CD133 (Prominin-1) cell surface marker [16-18, 77, 99, 100]. The use of CD133 must be viewed with caution as CD133 has not been linked to a contributory role in “cancer stemness” and some tumors may have CD133− cells with tumor initiation abilities [101, 102]. However, experiments in our laboratory find a striking enrichment of CSCs in the CD133+ tumor cell population even though CD133 should not be considered a surrogate for a CSC phenotype.
When we compared the effects of AKT inhibition on populations of brain tumor cells, we found a preferential targeting of AKT activity in CD133+ cells in comparison to matched CD133– cells. The AktIII inhibitor that we employed for the majority of experiments is a phosphatidylinositol ether lipid analog that may also activate the stress kinase, p38α. This small molecule inhibitor of AKT effectively reduced the growth, survival, migration, and invasion of glioma cells and did so with greater potency in the CD133+ subpopulation than in matched CD133– cells. Inhibiting the AKT pathway in brain CSCs also increased survival in animal models, suggesting a potential therapeutic benefit. Together these data suggest that many of the malignant characteristics of brain CSCs are dependent on AKT signals.

We were somewhat surprised to find that CD133– tumor cells expressed higher basal levels of activated AKT than the CD133+ cells, considering the recognized role of AKT in survival and prior reports indicating higher levels of phosphorylated AKT in human hepatocellular carcinoma and mouse medulloblastoma CSCs [98, 116]. As our studies were conducted in different tumor types and culture conditions, it is difficult to make direct comparisons. However, we found that differences in attachment between CD133– and CD133+ cells can alter basal AKT phosphorylation, suggesting that differences culture conditions may contribute to the discrepancy in basal AKT activation. All of our primary experiments were performed on short-term cultures in which CD133+ cells formed neurospheres (i.e., were non-adherent) and CD133– cells were...
adherent in the presence of identical media in an effort to make the best possible comparisons. We also used AktII and AktIII inhibitors rather than the previously utilized AktI inhibitor [98] or perifosine [116]. Using these conditions and inhibitors, we found that CD133+ cells exhibited greater sensitivity to AKT inhibition. The precise molecular differences between CD133- and CD133+ cells which contribute to the observed higher potency of AKT inhibition in CD133+ cells remain to be fully elucidated.

The concept of CSCs is still evolving, but data implicating these cells in tumor maintenance and therapeutic resistance indicates the potential benefit of targeting CSCs in combination with conventional therapies. While targeting AKT activation in CD133+ cells increased the survival of mice bearing intracranial human glioblastoma xenografts, AKT inhibition alone is unlikely to directly translate to therapeutic benefit for human patients. Monotherapies against any signaling pathway have been largely ineffective in the clinic. However, data from our laboratory and others suggests the utility of targeting AKT signaling components alone or in combination with other pathways in gliomas [109, 112, 115]. As these prior studies focus on the effects of AKT inhibitors in glioma cell lines that are passaged long term in the presence of serum (a condition which promotes the differentiation of CSCs) future studies determining the effect of combining AKT inhibition with chemo- and radiotherapies on CSC biologies may prove enticing. Ensuring that both glioblastoma stem cells and the more prevalent CD133- cells are targeted may offer the opportunity to eliminate the
last vestiges of the primary tumor after surgical resection, an absolute requirement for preventing recurrence.

In conclusion, CD133+ glioblastoma stem cells were shown to have increased sensitivity to the effects of a small molecule AKT inhibitor, despite exhibiting decreased baseline activation of the AKT pathway compared to the CD133− cells. AKT inhibition produced preferential reduction of CD133+ cell growth, survival, migration, and invasion in comparison to CD133− cells. The AktIII small molecule inhibitor also targeted characteristics unique to CSCs, such as the ability to form neurospheres. Targeting AKT activity in CD133+ cells also increased the survival of immunocompromised mice bearing glioma xenografts, indicating a potential therapeutic benefit. While many pro-tumorigenic behaviors of CD133+ cells were reduced to a greater extent than in the CD133− cells, the secretion of VEGF was not one of them, indicating that not all the unique features of CSCs are dependent on AKT signaling.

2.5 Materials and Methods

2.5.1 Isolation of CD133+ and CD133− tumor cells

T3359 cultures were isolated from primary glioblastoma samples transiently amplified in immunocompromised mice. Tumor specimens were obtained from surgical biopsies of consenting patients under a protocol approved by the Duke University Medical Center Institutional Review Board. D456MG xenografts were originally derived from a pediatric glioblastoma biopsy specimen.
and have been maintained in immunocompromised mice under a Duke Institutional Animal Care and Use approved protocol. Of note, T3359 and D456MG both express wildtype PTEN. Xenografted tumors were dissociated into single cells using an enzyme dissociation kit (Worthington Biochemical, Lakewood, NJ). For fluorescence-activated cell sorting (FACS) into CD133+ and CD133− enriched populations, cells were labeled with an allophycocyanin-conjugated CD133 antibody (Miltenyi Biotec, Auburn, CA) before sorting by FACS. For magnetic bead sorting (MACS) into CD133+ and CD133− enriched cell populations, cells were incubated with CD133 antibodies conjugated with biotin and magnetic beads that bind biotin prior to separation by a magnetic column (Miltenyi Biotec, Auburn, CA). CD133+ cells were maintained in their undifferentiated state using Neurobasal medium (Invitrogen) supplemented with epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF; each at 10 µg/500 mL media), sodium pyruvate, glutamine, B27, non-essential amino acids and penicillin/streptomycin (Gibco, Grand Island, NY). CD133− cells were maintained in their differentiated state with Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and penicillin/streptomycin.

2.5.2 Immunofluorescence

CD133+ cells isolated from an established D456MG pediatric glioblastoma xenograft were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; pH 7.4) for 15 minutes at room temperature and washed 3 times with PBS.
Cells were permeabilized with 0.5% Triton X-100 in PBS for 15 minutes at room temperature before blocking cells in 5% normal goat serum (NGS) in PBS at room temperature for 30 minutes. Cells were incubated for 24 hours at 4°C with the indicated primary antibody (red) diluted in PBS with 5% NGS. Sox2, Olig2, and Oct4 were obtained from R&D Systems and used at a concentration of 1:500, 1:200, and 1:200 respectively. Nestin was obtained from Santa Cruz Biotechnology and used at a concentration of 1:500. After washing 3 times with PBS, cells were incubated with rhodamine-conjugated goat anti-mouse (Abcam) or Alexafluor568-conjugated donkey anti-goat (Invitrogen) secondary antibodies diluted in PBS with 5% NGS for 1 hour at room temperature. After washing 3 times with PBS, cells were mounted in Antifade glycerol mounting medium containing 4’6-diamidino-2-phenylindole (DAPI, blue).

2.5.3 Small molecule inhibitors

The small molecule inhibitors of AKT (AktIII/SH-6, AktII), PI3K (LY294002), and mTOR (rapamycin) were purchased from Calbiochem (San Diego, CA). For all assays, stock solutions created by dissolving the drug in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) were stored at −80°C. Immediately prior to the experiment, stock solutions were diluted in DMSO to 1000x of the final concentrations indicated. For each experiment, 1 µL/mL of DMSO as a control or inhibitor 1000x stock solutions in DMSO were added to the media of cells to make the indicated final concentrations of inhibitor.
2.5.4 Antibodies and western blotting

CD133+ and CD133− cells were plated in appropriate media in six-well plates at $5 \times 10^5$ cells per well and allowed to recover overnight. CD133− media was changed to CD133+ growth media before each experiment for the indicated times. All cells were harvested together and then lysed in buffer (62.5 mM Tris-HCl, 2% w/v sodium dodecyl sulfate (SDS), 10% glycerol, 40 mM dithiothreitol (DTT) and protease inhibitors). Protein concentrations were quantified (Biorad Protein Assay Reagent, Hercules, CA), and equal amounts of protein were run on polyacrylamide gels (Invitrogen, Carlsbad, CA), followed by transfer to polyvinylidifluoride membranes (Millipore, Billerica, MA) which were then were probed with antibodies. Total AKT and phospho-AKT (Ser473) antibodies were purchased from Cell Signaling (Beverly, MA) while alpha-Tubulin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Proteins were detected with an enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL).

2.5.5 AKT kinase assay

CD133+ and CD133− cells were plated in appropriate media in 10-cm dishes at $10^6$ cells per plate and allowed to recover overnight. CD133− media was changed to CD133+ media before treatment with DMSO or increasing concentrations of the AKT inhibitor. After 24 hours of treatment, cells were changed to Neurobasal media without growth factors for 12 hours. Cells were then stimulated for 20 minutes with EGF and bFGF at a final concentration of 50 ng/mL before harvest and lysis. Equal amounts of total protein (50 µg in 200 µL)
were utilized for the *in vitro* kinase assay, which was performed as per the manufacturer's instructions (R&D Systems).

**2.5.6 Proliferation and survival assay**

CD133+ and CD133− cells were plated in appropriate media in 6-well plates in triplicate at 1×10^5 cells per well and left to recover overnight. CD133− media was changed to CD133+ media before treatment with DMSO or increasing concentrations of the AKT inhibitor. After 48 hours of treatment, cells were harvested, and live and dead cells were quantified via a hemocytometer with trypan blue stain (Gibco, Grand Island, NY).

**2.5.7 Flow cytometric and annexin V analysis**

CD133+ and CD133− cells were plated in appropriate media in 6-well plates in triplicate at 1×10^5 cells per well and were allowed to recover overnight. CD133− media was changed to CD133+ growth media before treatment with DMSO control or increasing concentrations of AKT inhibitor for 24 hours. Cells were then harvested with their conditioned media to ensure collection of floating cells along with adherent cells. Using an Annexin V kit, apoptotic cells were labeled with FITC while necrotic cells were labeled with propidium iodide as per the manufacturer's instructions (EMD Chemicals, San Diego, CA). Proportions of apoptotic cells were then quantified by FACScan gated to exclude cellular debris.

**2.5.8 Neurosphere formation assay**

CD133+ cells were plated in 24-well plates at 20 cells per well with DMSO or AKT inhibitor at increasing concentrations. Wells were inspected every three
days, numbers of neurospheres were quantified at each timepoint, and individual neurospheres were imaged with an Olympus CK40 digital camera mounted to a light microscope.

2.5.9 Migration and invasion assay

Migration/invasion plates were purchased and used according to the manufacturer's instructions (BD Biosciences, San Jose, CA). CD133+ and CD133− cells were pretreated for 1 hour with increasing concentrations of AKT inhibitor, then plated at $5 \times 10^4$ cells per well in upper transwell chambers of inserts uncoated (migration) or coated with Matrigel (invasion) in serum- and growth factor-free media. The bottoms of the chambers were filled with 500 µL media containing 2% fetal bovine serum. After 24 hours, migration inserts were fixed and stained with Diff-Quick Fixative Solutions (Dade Behring, Newark, DE). After 48 hours, Matrigel-coated inserts were fixed and stained. Attached cells were imaged with an Olympus CK40 digital camera mounted to a light microscope and quantified using ImageJ (http://rsb.info.nih.gov/ij/) software.

2.5.10 Statistical analysis

Statistical significance was calculated with GraphPad Prism Software (GraphPad Software Inc.). Data is presented as the mean ± the standard error. Statistical significance in the human xenograft mouse models was calculated using Kaplan-Meier survival curves and statistical analysis with MedCalc Software (www.medcalc.be/).
Figure 3. CD133+ brain tumor cells express stem cell markers. CD133+ and CD133- cells isolated from a D456MG pediatric glioblastoma xenograft were fixed, permeabilized, and stained with antibodies against Sox2, Oct3/4 and Nestin. Nuclei were counterstained with DAPI.
Figure 4. AKT activation is differentially affected by an AKT inhibitor in CD133+ and CD133− brain tumor cells. CD133+ and CD133− cells were isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice and treated with the indicated concentrations of AKT III inhibitor (SH-6, Calbiochem) for 2 hours. Lysates were analyzed by western blotting (A) and the intensities quantified using ImageJ software and normalized to the tubulin loading control (B). The levels of phospho-AKT, normalized to tubulin, decreased in a concentration-dependent manner in the CD133+ population but not the CD133− population.
Figure 5. AKT activation is preferentially targeted by an AKT inhibitor in CD133+ cells. CD133+ and CD133- cells were isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice (A, B) or an established D456MG pediatric glioblastoma xenograft (C, D). Lysates from CD133- and CD133+ cells treated with 50 µM Akt III inhibitor for the indicated times were analyzed by western (A, C) and the relative intensity of phospho-AKT to tubulin calculated using ImageJ (B, D). The levels of phospho-AKT are lower in CD133+ cells compared to CD133- cells at baseline and further decrease with AKT with a maximum decrease in the phospho-AKT in CD133+ cells at 2 hours.
Figure 6. AKT kinase activity is differentially affected by an AKT inhibitor in CD133+ and CD133− brain tumor cells. After treating CD133+ and CD133− cells with AKTIII inhibitor or vehicle control, cells were lysed and immunoprecipitated from lysates and used for an in vitro AKT kinase assay with GSK3α/β fusion protein as the substrate. (C) Western blotting was used to visualize the amount of phosphorylated substrate in the presence or absence of AKT inhibition. (D) Band intensities were quantified using ImageJ software and normalized to total GSK3α/β indicated decreased basal and AKT-mediated phosphorylation of GSK3α/β in CD133+ cells in comparison to matched CD133− cells. AktIII inhibitor decreased the AKT-mediated phosphorylation of GSK3α/β in CD133+ cells, but not matched CD133− cells.
Figure 7. Differential AKT activation in adherent and unattached brain tumor cells. CD133+ and CD133- cells were isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice. Short-term CD133+ and CD133- cultures were both changed to Neurobasal medium supplemented with EGF and bFGF overnight before plating. For unattached cells, CD133+ and CD133- cells were trypsinized and plated in Neurobasal media with EGF and bFGF. For attached cells, CD133- cells were trypsinized and plated in DMEM with 10% FBS. Once the DMEM-plated cells were attached to the plates (6 hours later), media on all cells was changed to fresh Neurobasal medium with EGF/bFGF for 2 hours before cells were harvested and lysed. Total cell lysates were analyzed by western blotting (A) and the relative intensity of phospho-AKT to tubulin calculated using ImageJ (B).
Figure 8. A small molecule inhibitor of AKT targets CD133+ cell growth and survival. CD133+ and CD133− cells were isolated from an established D456MG pediatric glioblastoma xenograft (A, C) or a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice (B, D). Cells were treated with the indicated concentrations of AktIII inhibitor for 48 hours and the numbers of live cells (A, B) and the percentage of dead cells (C, D) as a fraction of the control determined through trypan blue staining. *, p<0.01 with ANOVA comparison of AktIII treated CD133+ cells to the DMSO control treated CD133+ cells; ≈, p<0.01 with ANOVA comparison of AktIII treated CD133− cells to the DMSO control treated CD133− cells; #: p<0.01 with ANOVA comparison of CD133+ cells to identically treated CD133− cells.
Figure 9. Targeting AKT preferentially decreases CD133+ brain tumor cell growth over time. CD133+ and CD133− cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were plated in Neurobasal media with EGF and bFGF, allowed to recover overnight, and then treated with 10 uM AktIII (A), 10 uM AktII (B), 25 uM AktIII (C), 25 uM AktII (D), 50 uM LY290042 (E), and 100 nM rapamycin (F) inhibitors. Cell growth was measured on the indicated days after inhibitor treatment began using the Cell Titer Glo assay (Promega) according to the manufacturer's instructions. The data for each time point were standardized to the DMSO treated controls for the same cell type on each day. *, p<0.05 with t-test comparison of inhibitor treated CD133+ cells to DMSO treated control CD133+ cells on the same day; ≈, p<0.01 with t-test comparison of inhibitor treated CD133− cells to DMSO treated control CD133− cells on the same day; #, p<0.05 with t-test comparison of CD133+ cells to similarly treated CD133− cells on the same day.
Figure 10. Targeting AKT preferentially decreases CD133+ brain tumor cell growth in a concentration dependent manner. CD133+ and CD133- cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were treated with the indicated concentrations of AktIII (A), Akt II (B), Ly290042 (C), or Rapamycin (D) to inhibit different components of the AKT signaling pathway. Cell growth was measured 24 (C) or 48 hours (A, B, D) after inhibitor treatment using the Cell Titer Glo assay (Promega) according to the manufacturer's instructions. The data for each concentration were standardized to the percent of DMSO treated controls for the same cell type. *, p<0.001 with ANOVA comparison of inhibitor treated CD133+ cells to DMSO treated control CD133+ cells; ≈, p<0.001 with ANOVA comparison of inhibitor treated CD133- cells to DMSO treated control CD133- cells; #, p<0.05 with ANOVA comparison of CD133+ cells to similarly treated CD133- cells.
Figure 11. Targeting AKT results in preferential induction of CD133+ cell apoptosis. CD133+ and CD133− cells isolated from an established D456MG pediatric glioblastoma xenograft (A, B) or a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice (C, D) were treated with the indicated concentration of AktIII inhibitor for 24 hours, trypsinized, labeled with an Annexin V kit according to manufacturer's instructions and analyzed by FACS. Apoptosis was induced in CD133+ cells at significantly higher levels than CD133− cells with increasing concentrations of AKT inhibitor. *p<0.001 with ANOVA comparison of AktIII treated CD133+ cells to DMSO control treated CD133+ cells; #, p<0.01 with ANOVA comparison of CD133+ cells to identically treated CD133− cells. Representative FACS gates of CD133+ and CD133− cells from D456MG (B) and T3359 (D) are shown.
Figure 12. Targeting AKT decreases the neurosphere formation efficiency of CD133+ cells. CD133+ cells isolated from an established D456MG pediatric glioblastoma xenograft (A) or a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice (B) were plated at an approximate density of 20 cells per well and treated with the indicated concentration of the AktIII inhibitor. The number of neurospheres per well was quantified over three time points. The number of neurospheres per well significantly decreased in the presence of AKT inhibitor. Representative images of neurospheres photographed at day 9 are shown and demonstrate a clear qualitative difference in size between those grown in the control conditions and those treated with the AKT inhibitor. *p<0.001 with ANOVA comparison of AktIII inhibitor treatment to the DMSO treated control on the same day.
Figure 13. Targeting AKT decreases CD133+ cell migration and invasion. CD133+ cells isolated from an established D456MG pediatric glioblastoma xenograft were plated in serum-free media in the upper chambers of uncoated inserts (A-C) or Matrigel coated inserts (D-F) and were allowed to migrate toward 2% FBS for 48 hours. The migrating or invading cells were then stained and quantified with ImageJ, demonstrating both migration (A) and invasion (D) decrease in CD133+ cells with increasing concentrations of AKT inhibitor. When the percent change from baseline migration (B) or invasion (E) was calculated, CD133+ cells exhibited a greater sensitivity to the effects of AKT inhibitor. Representative images of migrating (C) or invading cells (F) are shown. *, p<0.05 with ANOVA comparison of AktIII inhibitor treated CD133+ cells to DMSO treated control CD133+ cells; ≈, p<0.05 with ANOVA comparison of AktIII inhibitor treated CD133− cells to DMSO treated control CD133−cells; #, p<0.001 with ANOVA comparison of CD133+ cells to similarly treated CD133− cells.
Figure 14. Targeting AKT decreases CD133+ cell migration and invasion. CD133+ cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were plated in serum-free media in the upper chambers of uncoated inserts (A-C) or Matrigel-coated inserts (D-F) and allowed to migrate toward 2% FBS for 48 hours. The migrating or invading cells were then stained and quantified with ImageJ, demonstrating a trend towards decreased migration (B) and a significant decrease in invasion (D) in CD133+ cells with increasing concentrations of AktIII inhibitor. When the percent change from baseline migration (B) or invasion (E) was calculated, CD133+ cells exhibited a greater sensitivity to the effects of AKT inhibitor. Representative images of migrating (C) or invading cells (F) are shown. **p<0.001 with ANOVA comparison to the control of the same cell type. #, p<0.05; ##, p<0.001 with ANOVA comparison to similarly treated CD133- cells.
Table 3. Effect of AKTIII/SH-6 pretreatment of CD133+ xenograft-derived cells on glioma initiation.

Table 1. Effect of AktIII/SH-6 pretreatment of xenograft-derived cells on glioma initiation

<table>
<thead>
<tr>
<th>T3359 CD133+</th>
<th>10,000 cells</th>
<th>1,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO)</td>
<td>Incidence: 3/3</td>
<td>Incidence: 3/3</td>
</tr>
<tr>
<td></td>
<td>Median survival: 24 days</td>
<td>Median survival: 35 days</td>
</tr>
<tr>
<td>25 μM AktIII inhibitor</td>
<td>Incidence: 3/3</td>
<td>Incidence: 3/3</td>
</tr>
<tr>
<td></td>
<td>Median survival: 40 days</td>
<td>Median survival: 66 days</td>
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Abbreviation: DMSO, dimethyl sulfoxide.
Figure 15. AKT inhibition has little impact on VEGF secretion in CD133+ glioma cells. CD133- and CD133+ cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were plated at 250,000 per mL of media. After 24 h all of the media was replaced with fresh Neurobasal media and treated with increasing concentrations of AktIII inhibitor. The conditioned media was harvested after an additional 24 hours and cellular VEGF secretion assayed with ELISA. Although CD133+ cells exhibited significantly more VEGF secretion than CD133- cells, consistent with our prior work (##, p<0.001 with ANOVA comparison to similarly treated CD133- cells), there was no significant decrease in VEGF secretion by either the CD133+ or CD133- cells with increasing concentrations of AktIII inhibitor.
Chapter 3.

The Biochemistry and Cell Biology of Nitric Oxide: A Molecule with Diverse Effects on Tumors and Malignant Cells

3.1 The Basics of Nitric Oxide – Chemistry and Synthesis

The diatomic gaseous radical nitric oxide (NO) plays multiple roles in physiologic and pathophysiologic processes. In mammals, it is synthesized by a family of nitric oxide synthases (NOSs), heme- and flavin-containing proteins that employ NADPH, tetrahydrobiopterin and molecular oxygen to convert L-arginine to L-citrulline and NO [120] (Figure 16). There are three NOS isoforms: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). Though originally named based on apparent tissue expression patterns, it is clear that these three NOS enzymes are expressed in a wide range of cells aside from the respective namesake tissues. Though they all catalyze the reaction of L-arginine to form NO, regulatory mechanisms differ for these enzymes. nNOS and eNOS are both expressed constitutively and are regulated largely at the post-translational level by either calcium availability, association with regulatory proteins, or phosphorylation events. In contrast, iNOS is an inducible isoform that is regulated primarily at the transcriptional level and operates in a calcium-independent fashion. When present, iNOS produces much
higher quantities of NO than the other two isoforms (micromolar versus nanomolar) [121].

Though it is a free radical, electron delocalization along the NO bond affords it reasonable stability in biological systems [122]. Further, the molecule is small, uncharged and non-polar, which permits easy diffusion through lipid bilayers. As a result, NO has the capacity to not only affect the cell in which it is synthesized, but also to have a paracrine effect on neighboring cells within a tissue.

3.2 Impact of Nitric Oxide in Cellular Systems

The identification of NO as endothelium-derived relaxation factor brought great attention to NO as a biologically active molecule, synthesized in mammals and utilized for processes critical to cellular and physiological function. Initial studies completed in the laboratories of Furchgott, Murad, Ignarro and Moncada demonstrated that the endothelium-derived relaxation factor NO induces vascular smooth muscle relaxation [123-126]. Since these original studies, roles for NO have been identified in cell signaling events such as apoptosis regulation, cell cycle control, DNA repair, and metabolic flux (reviewed in [127]). Additionally, NO can impact tissue homeostasis through its effects on angiogenesis and immune cells (reviewed in [128]). Generally speaking, NO impacts cell signaling processes and cellular phenotypes through three major mechanisms: 1) complex formation with transition metals or heme groups, 2) indirect reaction with
cysteines on proteins to form S-nitrosothiols, and 3) participation in to nitrosative and oxidative damage.

**3.2.1 Nitric oxide can bind and affect transition metal-containing enzymes**

The canonical role of NO in cell signaling relates to its ability to complex with transition metals, which are critical cofactors to a variety of enzymes. Pioneering work established that endothelial-derived NO resulted in activation of guanylate cyclase and the resultant generation of cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells, and this occurred via the ability of NO to complex with the ferrous heme in guanylate cyclase (Reviewed in [129]). In the years since this insight, NO has been shown to affect the activity of multiple transition metal-containing enzymes and proteins in mammals. Nitric oxide was reported to decrease the activity of cytochrome c oxidase through formation of an NO-iron complex [130, 131] and heme-containing oxygen transport molecules like hemoglobin or myoglobin efficiently complex with NO. Iron-sulfur clusters also form complexes with NO, which may affect the activity of iron-sulfur containing proteins [132, 133]. The capacity of NO to form complexes with critical transition metal components of proteins is known to drive a substantial portion of microbial responses to nitrosative stress [132-135]. Given the prevalence of transition metal-containing proteins in mammalian cells, it seems likely that NO-metal interactions may dictate some of the unknown biological effects of NO in mammalian cells.
3.2.2 Nitric oxide can directly modify cysteines through S-nitrosylation

Cysteine thiols are relatively nucleophilic and are susceptible to oxidation. Nitrosation, also called nitrosylation, is one type of cysteine modification and involves a cysteine thiolate forming an adduct with a nitroso moiety. Nitrosylation occurs through cysteine thiol reaction with higher N-oxides generated from oxidation of NO (e.g., NO⁺ or N₂O₃), through a free-radical proportionation reaction of NO with an oxidized cysteine thiol radical, or through a cysteine thiol reacting with NO (with a loss of an electron). Due to electron delocalization across the S-NO bond, this modification has some degree of stability and thus can act in a signaling capacity, especially since many enzymatic catalytic centers involve a cysteine thiol. For example, the active site of some apoptotic caspases can be S-nitrosylated to prevent activity, and thus limit apoptosis [136, 137]. Additionally, oncogenic RAS-driven AKT/eNOS activity produces NO, which can feed back to nitrosylate and activate wild type RAS and promote tumor maintenance [138]. Nitrosylation can affect the activity of cellular proteins by affecting homo- or hetero-dimerization (e.g., association of HDM2-p53 is blocked by nitrosylation of HDM2; [139]) cell localization (e.g., nuclear translocation of nitrosylated GAPDH-SIAH1 mediates cell death; [140]) or degradation (e.g., nitrosylation of BCL2 results in protein stabilization; [141]).

3.2.3 Nitric oxide can participate in nitrosative and oxidative cellular damage

NO-dependent cytotoxicity may be observed when NO is present in excess and/or is present in oxidative environments. High quantities of NO may
initiate apoptotic or necrotic cell death in mammalian cells, which is reminiscent of the cytotoxic effects that immune cell-derived NO can have on invading microbes. Nitric oxide has the capacity to directly react with superoxide to generate the highly reactive intermediate peroxynitrite, which can directly modify DNA, lipids and proteins [142] or can homolyze to form the highly reactive and cytotoxic products HO• and •NO2. It is worth mentioning, however, that several characteristics of the mammalian intracellular environment likely limit NO-derived peroxynitrite cell toxicity. Most importantly, mammalian cells express superoxide dismutase enzymes [143], which rapidly facilitate dismutation of superoxide and thus limit the rate of peroxynitrite formation. Additionally, peroxiredoxin enzymes are a phylogenetically conserved [144] family of proteins expressed in mammalian cells [145]. Members of this family of enzymes have been reported to reduce and detoxify peroxynitrite [144], though they typically function as peroxide reductases and the peroxynitrite reduction function is not well established in mammalian cells. In general, in situations of excessive NO production, cytotoxic effects may dominate, while at more moderate levels the extent of toxicity may be limited.

3.3 Nitric Oxide and Cancer

It is no surprise that a pleiotropic molecule like NO has diverse effects within a disease as heterogeneous as cancer. A substantial body of research has demonstrated that NO plays diverse roles in cancer cells and in tumors, though it
is difficult to generalize these NO-dependent effects, in part because of the variety of different experimental techniques and model systems used (please see section 3.4, “Discrepancies Associated with Nitric Oxide’s Role in Cancer”).

3.3.1 Nitric oxide and tumor cell proliferation and survival

Both pro- and anti-growth effects of NO in tumor cells have been reported. Upregulation of p53 by NO results in cell cycle arrest and apoptosis in wild type cancer cells [146]. NO-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) induce caspase 3 cleavage/activation and resultant apoptosis in human prostate cancer cells [147-149]. NO donors or overexpression of iNOS variably results in tumor cell arrest and apoptosis, and a variety of mechanisms have been proposed for this, including activation of stress kinase pathways like p38 MAPK activation [150], p53 accumulation or direct peroxynitrite-based toxicity on mitochondrial function [151] (reviewed in [152]).

In contrast, more limited NO doses or endogenous NOS expression often results in pro-growth impacts on tumor cells [153-158]. In some cancer cells with mutated p53, NO availability results in increased cell proliferation and tumor growth [146]. Both cGMP-dependent and -independent mechanisms have been proposed for this pro-growth effect, though there is hardly consensus on what consistently occurs in tumor cells. SRC activation by NO-induced cGMP has been reported [159, 160], and signaling via AKT may be influenced by cGMP-activated PKG activity [161]. Expression changes for of several anti-apoptotic NO-induced genes have been reported, though the contribution of cGMP to
induction of these genes is still unclear. NO is known to induce the expression of BCL2 [162], HSP70 [163], thioredoxin interacting protein [164] and hemeoxygenase 1 [165], all of which have the ability to confer cytoprotection. NO-dependent upregulation of survivin, an apoptosis inhibitor and mitotic effector confers chemoresistance and anti-apoptotic effects on several tumor types [166, 167]. NO has been reported to directly nitrosylate several cellular targets, and this direct cGMP-independent modification could provide some pro-survival signals in tumor cells. Caspase 3 and caspase 9 are both susceptible to inactivating nitrosylation events [136, 137], and RAS can be nitrosylated and activated as a result of endogenous eNOS activity [138].

3.3.2 Nitric oxide and angiogenesis

Tumors require nutrients and oxygen for growth, and thus a critical component of tumor progression relates to the ability for a tumor to recruit vascular growth. Aside from the eNOS dependence of endothelial cell VEGF-responsiveness, tumor-derived NO appears to play a role in the vascularity of a variety of tumors (reviewed in [128, 168]). Though a limited number studies have demonstrated an anti-angiogenic effect for tumor-derived NO [169-171], this may be explained by the apparent sensitivity of the angiogenic process to the dose of NO provided by tumor cells [172, 173]. NO availability promotes the expression, stabilization, or activity of a variety of pro-angiogenic factors, such as VEGF [174], bFGF [175], and HIF1alpha [176-180] and decreases the expression of anti-angiogenic factors like thrombospondin 1 [173]. NO has also been reported
to stabilize the vasculature within tumors, which may actually provide a therapeutic opportunity for increased drug delivery or radiosensitivity [181]. Though the precise mechanism by which tumor cell-derived NO contributes to angiogenesis within a tumor is still unclear, and the relative contribution of neoplastic and non-neoplastic sources of NO remain to be examined, levels of NO in tumors appear highly intertwined with angiogenic regulation.

### 3.3.3 Oncogenic role of NO

Some have speculated that NO, and particularly the expression of the high-output isoform iNOS, may play an oncogenic role in the development of some tumor types, colon cancer in particular. Expression of the high output iNOS isoform in aberrant colonic crypt foci and colon adenomas has been described [182, 183]. Some groups have suggested that the expression of iNOS in early colon dysplasias may contribute to accelerate the genetic lesions and resultant malignant phenotype of the tumors, considering the genotoxicity and high rate of mutagenesis that is associated with high NO production in mice [184, 185]. The work completed in the laboratory of Curtis Harris suggests that availability of NO within cells may not only promote mutations in p53 that may permit outgrowth of more malignant cells from low grade or pre-malignant lesions [182], but also that the presence of NO may provide a selective pressure for p53-mutant cells and thus promote malignant conversion in cancer [146]. Inflammatory stress provided by administering heat inactivated C. parvum to p53-null mice resulted in increased spontaneous tumor development in mice homozygous for wild type
iNOS compared with iNOS-null animals, suggesting that inflammation-induced tumor formation may be accelerated by the production of NO [186]. Both iNOS inhibition and genetic disruption of iNOS reduced tumor formation in mice predisposed to intestinal cancers (APC\textsuperscript{min/+} mice)[187] and iNOS null status disrupted tumor formation in murine models of gastric tumors [188] and lung tumors [189].

On the other hand, other work has suggested that iNOS expression is limited throughout malignant progression from aberrant crypt foci to colon carcinoma [190]. Several groups have observed that iNOS activity decreased \textit{in vitro} transformation [191] and tumorigenesis in mice [192, 193]. Clearly, the role for NO in neoplastic transformation and tumor initiation is unclear and requires further investigation.

3.4 Discrepancies Associated with Nitric Oxide’s Role in Cancer

Despite abundant research efforts focusing on the roles of NO in cancer, it is hard to conclusively say that there is one definitive effect of NO on/in cancer cells. Just as the physiological roles for NO are affected by many factors, the role for NO in cancer varies depending on many things, including the tissue examined, the amount of and source for available NO, and the cytogenetic and epigenetic characteristics of the tumor cells. When evaluating work assessing the “role” of NO in cancer, the following issues should be considered. The variability regarding the effect of NO in cancer likely derives in part from the following: 1)
cellular effects may vary due to the diverse biochemical effects of NO (discussed above), 2) cytogenetic tumor cell characteristics can dramatically affect cellular response to NO, 3) different model systems produce and consume NO in different fashions, 4) non-neoplastic microenvironmental producers (and consumers) of NO exist within tumors, and 5) there are variable methods by which researchers manipulate NO.

3.4.1 Cellular p53 status affects responses to nitric oxide

Due to its pivotal role in regulating cell cycle, DNA damage repair, and apoptosis, p53 is one of the most critical tumor suppressors in humans, a fact that is illustrated by the fact that it is also one of the most frequently mutated individual genes in human tumors [194-198]. This highly regulated transcription factor activates the transcription of a variety of genes, including pro-apoptotic and anti-proliferative genes. Inactivating mutations occur throughout the gene and can be either missense mutations leading to nonfunctional protein or nonsense mutations leading to truncated and inactive p53 [199].

Nitric oxide results in the accumulation of p53 [200], possibly through nitrosylation of the p53-regulatory protein human double minute 2 (HDM2, the homologue of the murine mouse double minute MDM) [139] or directly through increased activity or stabilization of p53 [157, 201]. Nitric oxide induces cell cycle arrest and apoptosis in tumor cells with wild type p53, but in transformed cells with mutated or absent p53, NO exposure may provide pro-survival signals and has some role in promoting the synthesis of pro-tumor growth factors such as
This suggests that the role of NO in transformed cell lines or tumors may very much depend on the p53 status of these cells. Interestingly, this may provide strong selective pressure for the outgrowth of p53-mutant or –null populations in tumors with abundant NO, eventually promoting a more malignant tumor phenotype [146, 203] (please see section 3.3.2 on “Oncogenic role of NO”).

There has been some evidence that wild type p53 feeds-back to inhibit the expression of iNOS. p53 mutant mice exhibit higher NO production [204], and work in cell lines have demonstrated a p53-dependent repression of iNOS transcription [200]. Thus, inactivation of p53 might permit higher levels of NO production in iNOS-expressing tumor cells.

3.4.2 Nitric oxide production by iNOS differs between mice and humans

Murine iNOS produces higher levels of NO for a given stimulus. Cytokine-induced iNOS expression within mouse macrophages results in NO levels that are several times higher than those seen in human immune cells exposed to the same stimulus [205]. In terms of mRNA regulation, there also appears to be a differential propensity for human cells to transcribe iNOS, deriving from highly methylated promoter elements [206]. This establishes a situation where it is difficult to directly assume that the role of endogenously-synthesized NO in mice equates with that in human cells. For example, while a few studies of transgenic mice have indicated that endogenous iNOS activity inhibits tumor formation [191-193], it is hard to say that this will definitely translate to the analogous disease in
humans, as the expression of iNOS and thus the production of NO may ultimately be far lower in humans. While mouse tumor models are certainly informative, evidence regarding the role of NO in mouse models of cancer (particularly with regards to iNOS) may not be able to be directly applied to the human condition without further investigation.

3.4.3 Non-neoplastic cells may contribute to NO-dependent tumor biology

Tumor cells, or associated vascular or immune cells, variably express NOS enzymes (reviewed in [128]). Though a free radical, the N-O bond is stabilized by electron delocalization and the molecule is uncharged, allowing it sufficient stability and diffusion characteristics to be able to enter and influence cells at a distance from the original site of production. Thus, NO derived from endothelial cells or from immune cells, such as tumor associated macrophages, has the capacity to reach and influence the biology of physically distant tumor cells. This means that the tumor microenvironment may contribute to determine the ultimate effect(s) of NO on tumor cells and the growth of the overall tumor.

Endothelial-produced NO may be able to modulate the behavior of neighboring tumor cells [88]. Further, the endothelial cells of eNOS -/- mice are known to be defective in VEGF responsiveness [207], and VEGF-induced angiogenesis is known to be critical to tumor growth and progression for a variety of tumor types [208].

Independent of the NOS expression pattern of the tumor cells themselves, stromal fibroblasts or immune cells often display iNOS expression and activity
While some groups have reported that iNOS activity in stromal fibroblasts or tumor-associated macrophages may have anti-tumor effects [209, 210], other groups have demonstrated a pro-tumor effect for stromal-derived NO in tumors [211-214]. As mentioned above, it is difficult to determine the applicability of these studies to the situation in human tumors, since this work involves the more highly inducible iNOS present in the murine stroma; as human levels of iNOS tend to be more moderate than those in mice, the effects of iNOS activity within human tumor-associated stroma may differ substantially from that seen in mice. This said, iNOS expression by tumor-associated non-neoplastic cells is seen in a variety of human tumor types and may contribute to tumor malignancy [128, 215].

3.4.4 Dose and delivery of NO dramatically affects its cellular effects

A substantial body of work describes the biological effects of NO donor administration or iNOS gene transfer on cancer cells and/or tumors (reviewed in [152]). For the most part, these manipulations have anti-tumor effects either through direct cytotoxicity or through an ability to sensitize tumor cells to other therapies (e.g. radiation therapy). Many NO donors have extremely short half-lives and acutely deliver high doses of NO. Similarly, gene transfer of NOS isoforms could result in supra-physiologic levels of NO. When driven by a CMV promoter, iNOS can be expressed at extremely high levels that compare to those seen in LPS-stimulated mouse macrophages (personal observations). It is important to realize that these approaches, while generating anti-tumor effects that may in fact offer useful therapeutic options, do not by themselves give much
insight into the homeostatic role for NO within tumor cells when it is synthesized at more physiological levels. As Thomas et al. point out, the effects of NO within cells are very much related to the levels of available NO, with cytotoxicity occurring with the highest levels [127], so NO-donor based experiments must be interpreted with an appreciation for the concentrations used relative to NO levels that are produced by physiologic levels of NOS activity within cells. One study has found that when tumor cells are transfected with iNOS constructs displaying a range of activity levels, the high levels of iNOS activity resulted in cytotoxicity but lower levels failed to kill cells [216] and other groups have noted that chronic low level NO exposure may promote cytoprotective effects or resistance to otherwise cytotoxic nitrosative stress [217].

3.5 Conclusions

A substantial amount of research has been devoted toward delineating the role of NO in cancer. It is likely that the cell type, tumor type, and NO dose examined contribute substantially to the specific biological role of NO in cancer, and it seems probable that the effect of NOS expression or NO availability in one situation may not be able to be extended to others. Generally speaking, lower levels of NO production associated with endogenous NOS activity in tumor cells promote tumor cell growth, proliferation and metastasis, while exogenous high-dose NO donor administration or overexpression of iNOS may have anti-growth effects.
Pathological analysis of brain tumors has suggested that NOS isoforms are expressed in tumor cells, albeit heterogeneously [218, 219]. Armed with recent findings suggesting that heterogeneity within tumors might relate to the existence of highly tumorigenic CSC subpopulations, and the fact that endogenous NO production can often facilitate the malignant characteristics of some tumor cells, we hypothesized that NOS expression and NO production might be localized to CSC populations within gliomas. This is supported by the fact that several groups have demonstrated that NO can facilitate the expression or activity of stem cell proteins [88, 220] or regulate stem cell populations [221-223]. Chapter 4 will address our efforts to test this hypothesis and describe for the first time a role for CSC-produced NO.
Figure 16. Nitric oxide synthase isoforms catalyze the formation of nitric oxide from L-arginine. The three nitric oxide synthase (NOS) isoforms catalyze the conversion of L-arginine to L-citrulline using oxygen and NADPH, with concimitant release of nitric oxide (NO).
Chapter 4.
Inducible Nitric Oxide Synthase Promotes the Growth and Tumorigenicity of Glioma Stem Cells

This chapter is based off of a manuscript currently under review by Nature Medicine. It describes the work that represents my main focus during my time in the Rich laboratory and is still in progress, though we are currently in the progress of responding to reviewer comments.

4.1 Summary

Malignant human gliomas are aggressive and deadly brain tumors with few effective treatment options. As in other cancers, recent findings support the existence of tumorigenic stem cell-like populations within malignant gliomas called cancer stem cells (CSCs). Though strategies targeting the tumorigenic CSC population have potential to improve the prognosis of malignant glioma patients, anti-CSC agents may also target normal stem cell survival due to shared molecular mechanisms with CSCs. Here we demonstrate that malignant glioma CSCs express high levels of inducible nitric oxide synthase (iNOS) relative to non-CSCs. While iNOS was dispensible for the function of normal neural stem cells, glioma CSC growth and tumorigenic capacity were abrogated by multiple anti-iNOS strategies, including RNA interference, pharmacological inhibition, and heterologous expression of the prokaryotic NO-consuming flavohemoglobin. Our findings identify a novel glioma therapeutic target with minimal role in normal neural stem cells, suggesting clinical utility for selective iNOS inhibitors.
4.2 Introduction

Gene expression profiling has suggested similarities between CSCs and both embryonic stem cells [49] as well as non-malignant stem/precursor cells in the central nervous system [224]. Interruption of many of these stem-cell specific pathways can significantly decrease cell survival and proliferation in vitro as well as tumor growth in vivo. For example, inhibition of the Notch [225] or hedgehog [226, 227] pathways prevents CSC survival and tumor growth in vivo. However, these signaling cascades are critical to many normal human stem or progenitor cells [228, 229], and thus pharmacological inhibition of such pathways may inhibit normal tissue function, especially if administered over a prolonged period of time [230-232]. While these therapeutic strategies are promising additions to tumor treatment regimens, methods that selectively target CSCs without negatively impacting normal stem cell compartments hold great promise, as suggested by comparisons of leukemic and hematopoietic stem cells [66].

The roles of nitric oxide (NO) in cancer have been greatly debated [128], although endogenously synthesized NO can impart various malignant characteristics to cancer cells. Many NO-induced properties – including angiogenic potential [181, 233], chemotherapy resistance [167, 234], resistance to apoptosis [154, 166] and enhanced cell proliferation [138] – are exhibited by CSCs. While pro-inflammatory cytokines have the ability to stimulate “inducible” NO synthase (iNOS) expression in cultured glioma cell lines [235, 236], the
pathophysiological relevance of such stimuli (e.g. lipopolysaccharide) in the context of cancer is unclear. Further, the expression pattern of NOSs within many gliomas appears heterogeneous [218, 219] and could possibly be specific to individual cellular subpopulations. Based on these observations, we hypothesized that NO production might be amplified within glioma CSCs relative to non-stem cancer cells (non-CSCs), thus promoting the former's highly tumorigenic phenotype.

4.3 Results

4.3.1 CD133+ CSCs have elevated NO production, which contributes to CD133+ CSC growth and tumorigenicity

We have previously characterized a variety of human tumor specimens and xenografts for which positive selection for CD133 is useful in segregating CSC-enriched populations from non-CSCs, as demonstrated by measures of self-renewal (e.g., serial neurosphere formation and multilineage differentiation capacity), stem cell marker expression, and tumor initiation potential [77, 87, 178]. To evaluate whether NO production was amplified within glioma CSCs, we compared the cell autonomous NO-production capacity of CD133+ CSC-enriched populations and CD133- CSC-depleted populations (non-CSCs) by measuring the accumulation of nitrite ($\text{NO}_2^-$, a stable aerobic byproduct of NO), in the conditioned culture medium of several matched CD133+ and CD133-xenograft-derived cultures. We observed 3-fold greater nitrite production by CD133+ glioma cells than in matched CD133- non-CSCs (Figure 17), suggesting
that CD133+ CSCs possess an amplified capacity for NO synthesis.

We next wanted to employ an approach that would permit highly efficient intracellular consumption of NO, regardless of the NO source, in order to evaluate the functional role of NO availability within CD133+ glioma CSCs. We designed a strategy to lentivirally introduce into CD133+ CSCs an expression plasmid encoding a highly efficient NO-consuming enzyme normally expressed by microbial enzymes. Bacteria and other microbes employ a highly conserved flavohemoglobin (FlavoHb) to enzymatically consume NO and thus enhance their survival during conditions of nitrosative stress [237]. FlavoHb employs an active site ferrous heme to catalyze the reaction between NO and O₂ to form nitrate (NO₃⁻) and ferric heme, with flavin-based reduction of the heme back to the ferrous state via electrons in NADPH (Figure 18a). Despite its unique specificity and efficiency for NO consumption, we are not aware of the previous use of FlavoHb to manipulate NO levels in human systems (please see chapter 5 for further validation of this technique). To assess the role of NO in the biology of CSCs, a Flag-tagged FlavoHb coding sequence was subcloned into a lentiviral expression vector and introduced to CD133+ CSCs in parallel with a control vector (Figure 18a). Heterologous expression of FlavoHb decreased CSC viability (Figure 18b) and also decreased neurosphere formation (Figure 18c), an in vitro measurement of self-renewal capacity. Importantly, CD133+ glioma CSC stably expressing FlavoHb were less tumorigenic when implanted in the brains of immunocompromised mice than vector control cells (Figure 18d). That FlavoHb-
mediated NO consumption led to a decrease in cell growth, neurosphere formation and tumorigenic capacity suggested that availability of cellular NO contributes to the growth and tumorigenic capacity of CD133+ glioma CSCs.

4.3.2 Glioma CD133+ CSCs express elevated levels of iNOS protein, which is central to the high NO-synthesis of CD133+ CSCs

Cellular NO synthesis occurs via the nitric oxide synthase (NOS) family of enzymes, of which there are three members: neuronal NOS (nNOS, NOSI), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). When cellular lysates were collected and analyzed by western blotting, CD133+ CSCs displayed substantially higher levels of iNOS protein than matched CD133- non-CSCs, though the levels of the other NOS isoforms were generally similar between these two cellular subpopulations (Figure 19a). These expression differences in iNOS protein levels were observed within xenografted gliomas as well (Figure 19b), suggesting that iNOS expression in CSCs is unique to both primary human and xenografted tumors, as well as the likely source of CSC-produced NO. Although CD133 is a useful marker for glioma CSCs in many tumors [18, 77, 100], it is not the only marker that may be informative for enriching for CSCs. The optimal method for determining CSC marker effectiveness likely depends on individual tumor characteristics and is still under intense investigation. To assess the possibility that iNOS co-expression by CSCs is an epiphenomenon of CD133-based selection, we employed CSC selection on two tumors previously demonstrated to be effectively segregated into CSC-enriched and CSC-depleted populations by expression of the cell surface
molecule SSEA1; for these tumors CD133 was not informative as a CSC-specific marker due to low overall expression levels [44]. Western blot analysis of iNOS expression in cell lysates derived from SSEA1+ CSCs and SSEA1- non-CSCs from these previously described tumors (ref [44]) demonstrated similar differences as observed with CD133 (Figure 19c), suggesting that iNOS expression co-segregates with tumorigenic potential even in the absence of CD133.

To examine whether differential iNOS expression is inadvertently driven by cell culture conditions, we assessed the levels of iNOS mRNA in CD133+ and CD133- cells from three primary human brain tumors without any intervening cell culture steps. iNOS mRNA levels were considerably higher in CD133+ cells relative to paired CD133- cells from both primary human gliomas and xenografts (Figure 19d). Cellular co-expression of iNOS and CD133 was confirmed by immunofluorescence in primary human glioma specimens (Figure 19e).

Since iNOS is a highly productive NO-generating enzyme and we observed consistently different levels of iNOS expression between CD133+ CSCs and CD133- non-CSCs, we hypothesized that the differential NO production capacity observed in these two populations (Fig 17) might be explained by iNOS activity. Consistent with this notion, we observed decreased NO$\text{_2}$- accumulation (i.e., NO synthesis) in CD133+ CSCs cultured in the presence of the highly selective iNOS inhibitor 1400W (N-[[3 (aminomethyl)phenyl]methyl]- ethanimidamide) [238] (Fig. 20). These findings
collectively support the notion that iNOS expression is a feature inherent to glioma CSCs, and accounts for the majority of their NO-synthesizing capacity. As we observed a critical role for cellular NO availability for the growth and tumorigenic capacity of CD133+ CSCs (Figure 18), it seemed likely that iNOS activity in glioma CSCs could contribute to the maintenance of the known malignant properties of these cells, including tumorigenic capacity.

### 4.3.3 Genetic or pharmacological blockade of iNOS inhibits glioma CSC growth and tumorigenicity

We next sought to determine the functional role for iNOS within glioma CSC populations. Relative to a scrambled control short hairpin RNA (shRNA), lentiviruses bearing two non-redundant iNOS-directed shRNAs (Figure 21a) induced significant reductions in xenograft-derived CD133+ CSC survival and proliferation (Figures 21b, 22). Such effects were not observed in matched CD133- cells. Further, iNOS-directed shRNA decreased the rate of neurosphere formation in xenograft-derived cells (Figure 22). Neurospheres that managed to survive the iNOS-directed shRNA still expressed iNOS (assessed by quantitative real time polymerase chain reaction; qRT-PCR; data not shown) and thus are likely derived from cells with incomplete efficiency of shRNA-mediated knockdown.

To evaluate the potential therapeutic utility of iNOS inhibition, glioma CSCs were treated with either vehicle or 1400W and functionally examined over time. Similar to iNOS knockdown, daily application of 1400W decreased CSC survival, proliferation and neurosphere formation (Figure 23). Overall these
findings mimicked the results with genetic knockdown of iNOS, and reinforce the potential therapeutic relevance of targeting iNOS in glioma CSCs.

Given our findings with glioma-derived cells in vitro, we sought to evaluate the effects of targeting iNOS in vivo. The effects of iNOS knockdown (Figure 24) were assessed by intracranially implanting lentivirally-transduced CD133+ cells into the forebrains of athymic nude mice. The time until development of neurological signs correlated with the extent of iNOS knockdown in a limiting dilution intracranial transplantation assay (Figure 24); this corresponded to the similar impact on tumorigenicity noted with heterologous expression of the NO-consuming flavohemoglobin in CD133+ glioma CSCs. Consistent with our findings in mice, iNOS expression in human gliomas is inversely correlated with patient survival, irrespective of tumor grade or cytogenetic characteristics (Figure 25; data obtained from National Cancer Institute, 2005 REMBRANDT homepage, http://rembrandt.nci.nih.gov, Accessed 2008 August 21).

4.3.4 iNOS maintains cell cycle rate in CD133+ glioma CSCs

To evaluate the cell biological explanation for the anti-growth effects of iNOS-directed interventions in CD133+ glioma CSCs, we interrogated the role of iNOS in: 1) maintaining stem cell characteristics, 2) modulating apoptosis, and 3) impacting cell cycle rate in CD133+ CSCs. The role of iNOS on cell differentiation appears minimal, as iNOS knockdown did not affect the expression of stem cell-related markers in CSCs (Figure 26). Though iNOS knockdown increased levels of apoptosis in some xenograft-derived CD133+ CSC-enriched cultures, it was
not consistent across all xenografts examined (Figure 27). In contrast, in all xenografts examined iNOS knockdown consistently decreased S-phase flux in CD133+ CSCs, as measured by EdU incorporation (Figure 28).

4.3.5 iNOS activity regulates global gene expression patterns in CD133+ CSCs, including NO-dependent repression of the cell cycle inhibitor Cell Division Autoantigen 1 (CDA1)

As manipulation of iNOS expression by RNA-interference consistently decreased the cell cycle flux of CD133+ glioma CSCs, we employed a microarray-based approach to identify possible molecular determinants of this phenotype. A variety of global gene expression patterns were altered by iNOS-directed shRNA treatment in the two tumors examined. One cell cycle-related gene in particular, the cell cycle inhibitor CDA1, showed higher levels in cells treated with iNOS-directed shRNA (Figure 29a). This iNOS-dependent alteration was validated in several different xenografts by qRT-PCR (Figure 29b) as well as by western blotting (Figure 29c).

We are the first to identify iNOS-dependent repression of the cell cycle inhibitor CDA1, so we wanted to validate whether this is a generalizable phenomenon by evaluating whether this NO-dependent repression occurs in other model cell types. Using HEK293 cells treated with an NO-donor (Figure 29d) or transfected with iNOS (Figure 29e), we verified that NO and iNOS-derived NO can repress the levels of CDA1 in this model system, in addition to the repression observed within glioma CSCs.

CDA1 is a known pan-cell cycle inhibitor and tumor suppressor, likely
working through inhibition of multiple cyclin dependent kinases [239-241]. Although multiple molecular mechanisms likely contribute to the pro-growth effect of iNOS activity in CD133+ CSCs, it seemed that the ability of iNOS to suppress a known cell cycle inhibitor like CDA1 might contribute to its pro-growth effect in CSCs. To more formally assess the contribution of CDA1 repression within CD133+ CSCs, we evaluated whether CDA1 knockdown could rescue the anti-growth phenotype observed with iNOS-directed interventions. Indeed, CDA1 knockdown decreased the extent of the cell proliferation defect observed with iNOS knockdown in CD133+ glioma CSCs, as measured by EdU incorporation (Figure 30).

4.3.6 Normal neural stem cell function is not dependent on iNOS

Although iNOS inhibition or knockdown abrogates the growth of glioma CSCs, iNOS knockout (iNOS-/-) mice appear to undergo normal neural development ([242, 243] and and our unpublished observations) suggesting that pharmacological iNOS inhibition is likely to be minimally toxic on normal neural stem cells. In addition, studies in humans utilizing the iNOS-specific inhibitor 1400W demonstrate its safety [244, 245], suggesting that iNOS-directed anti-glioma therapies would likely spare the remaining normal brain tissue.

To evaluate this hypothesis, we first examined the in vitro characteristics of neural progenitor cells (NPCs) derived from wild type (WT) and iNOS-/- mice. Comparable levels of cell growth and viability were observed in NPC preparations from these two genetic backgrounds (Figure 31a,b), and cell growth
of WT NPCs was unaffected by acute 1400W administration (Figure 31c). Similar markers of proliferation (i.e., phospho-histone H3) were observed in the subependymal zone of WT and iNOS-/− mouse brains (Figures 31d). Long-term retention of bromodeoxyuridine (BrdU) within the subependymal neural stem cell niche was also similar in WT vs. iNOS-/− mice, suggesting that the slow-cycling long-term adult neural stem cells are not dependent on iNOS (Figure 31e). Further, WT mice do not appear to express iNOS within this subependymal stem cell niche (data not shown).

Though the aforementioned studies of murine NPCs do not suggest a role for iNOS in the maintenance of the normal neural stem cell population, we also sought to evaluate iNOS expression and functional importance in analogous human populations. We examined iNOS expression in normal human fetal NPCs vs. CD133+ glioma CSCs and observed expressed markedly less iNOS expression in the NPCs than was present in glioma CSCs (Figure 32a,b). The iNOS inhibitor 1400W did not impact the growth of two different NPC preparations (Figures 32c), or an embryonic stem cell-derived neural progenitor preparation (Figures 32d). Together, these data suggest that targeting iNOS in glioma CSCs would likely spare normal neural growth and function.

4.3.7 iNOS inhibitors have potential to effectively target glioma CSCs in vivo

Our data demonstrate that genetic manipulation of iNOS via shRNA-based knockdown negatively impacts CSC survival and tumorigenicity in vivo. Despite the efficiency of these genetic approaches, most medical treatments rely on
pharmacological manipulations of enzymatic activity due mostly to pharmacokinetic and toxicological considerations. We first examined the anti-tumor effect of iNOS inhibitors on mice bearing subcutaneous human glioma xenografts. Mice bearing subcutaneous human glioma xenografts were intraperitoneally injected daily with 50 mg/kg of the iNOS inhibitor 1400W or vehicle control for 2 weeks. Treatment with 1400W was initiated 10 days after the implantation of tumor cells (depicted schematically in Figure 33a). Tumor volume was measured daily, with final harvesting of tumors on day 17. Overall tumor burden was decreased by treatment with 1400W relative to vehicle (Figure 33b,c). At the end of this study, tumors from 1400W-treated mice displayed decreased cell proliferation as measured anti-Ki67 immunofluorescence (Figure 33d,e) as well as decreased tumor vascularity (Figure 33d,f).

We sought to evaluate the ability of iNOS inhibitors to affect the growth of glioma xenografts implanted intracranially. Since the blood brain barrier provides an obstacle to effective intracranial delivery, we utilized two different iNOS-selective inhibitors (1400W [238] and BYK191023 [246]) with different chemical structures (Figure 34a,b) that both effectively inhibited CD133+ CSC growth in vitro (Figure 34b). Glioma xenografts stably expressing firefly luciferase were inoculated into the brains of immunocompromised mice. After a 10 day engraftment period, treatment with vehicle, 1400W or BYK191023 was initiated and tumor size was periodically measured by bioluminescence imaging. Treatment with both inhibitors demonstrated anti-tumor effects, particularly at
early time points in tumor growth (Figure 35). No toxicity was noted in the mice treated with either inhibitor (data not shown). These data suggest that iNOS-directed therapeutics may represent a non-toxic and effective CSC-specific anti-tumor strategy.

4.4 Discussion

Patients diagnosed with malignant gliomas face a grave prognosis with no effective treatment options upon inevitable post-therapy tumor recurrence. Current treatments are generally aimed at the entire tumor (e.g., radiation, systemic chemotherapy, resection). Recent studies suggest that CSC subpopulations may contribute to tumor progression/recurrence in some cancers. It may therefore be important to identify therapies specifically able to target CSCs. Indeed, such strategies have demonstrated highly effective anti-tumor activity [225-227], but most stem cell-directed therapies are also likely to inhibit normal stem cell function. Development of effective therapies with low potential for toxicity depends on identification of critical mediators of CSC growth and survival that are unique to CSCs and not critical for normal stem cells.

Here we demonstrate that NO production is elevated in glioma CSCs relative to glioma non-CSCs and specific NO consumption using our novel FlavoHb-based technique results in decreased CSC growth and tumorigenic capacity, suggesting an integral role for NO in maintaining glioma CSC proliferation. Further, we demonstrate that this elevated NO production derives
from elevated iNOS expression and activity in glioma CSCs relative to glioma non-CSCs and that targeting iNOS via pharmacologic or genetic means also decreases the cell growth and tumorigenicity of CSCs. We are the first to demonstrate NO-mediated repression of the cell cycle inhibitor protein CDA1, and demonstrate that this repression accounts for some portion of the pro-proliferative effects of endogenous iNOS activity in glioma CSCs. We observe that iNOS is dispensible for the function of normal mouse and human neural progenitor cells, likely through the low level of iNOS expression in these cells. In vivo administration of iNOS inhibitors to mice bearing glioma xenografts decreased tumor growth, suggesting that iNOS inhibition may function as a CSC-directed therapy with limited toxicity. These data also suggest that utilization of our novel FlavoHb-based technique may prove a useful investigative and possibly therapeutic tool.

Despite many reports that employ supraphysiological levels of exogenous NO (i.e., µM to mM NO) to argue for its toxicity, evidence from iNOS knockout mice suggest that endogenous NO is frequently cytoprotective [247, 248]. Importantly, iNOS has an established cytoprotective role in chronic lymphocytic leukemia [154, 155], although whether this is attributable to a stem cell subpopulation has hitherto remained unexplored. The molecular mechanisms by which iNOS may facilitate cell survival are broad, as NO regulates a plethora of signaling pathways via both cGMP- and S-nitrosylation-based mechanisms. Current efforts in our laboratory are aimed at further elucidating these
downstream effectors of iNOS in glioma CSCs. We suspect that a multitude of pathways and molecules are involved, based on the wide range of gene expression changes we observed in our microarray analysis of global gene expression changes in glioma CSCs after iNOS knockdown. Though we validate that iNOS-derived NO can repress the known cell cycle inhibitor CDA1 and this may account for some portion of the pro-proliferative effect of iNOS activity in glioma CSCs, we anticipate that a wide range of cellular effects contribute to this phenotype and this is an active area of investigation in our laboratory.

Several iNOS-specific inhibitors, including 1400W, aminoguanidine, and GW274150, have been investigated in clinical trials for asthma, cirrhosis, chronic obstructive pulmonary disease, and heart disease [155, 244, 245, 249, 250]. Although iNOS inhibition demonstrated limited efficacy for the pathologies investigated thus far, these studies demonstrated very few toxic effects [155, 244, 245, 249, 250]. However, L-NAME, a pan-NOS inhibitor with activity against all three NOS isoforms, decreased survival in patients experiencing septic shock, likely due to the hemodynamic effects of inhibiting eNOS in such clinical situations [251]. These data suggest it would be critical to utilize iNOS-specific inhibitors for brain tumor treatment in initial clinical investigations. We observe effective anti-tumor growth characteristics of 1400W against subcutaneous glioma xenografts, and our initial assessments suggest that both 1400W and another potent and iNOS-selective inhibitor, BYK191023, can impair the growth of intracranial xenografts.
Though most of of the in vitro small molecule inhibitor treatments were completed with 1400W in this study, we also incorporated the inhibitor BYK191023 [246, 252] in our assessments of iNOS inhibition in intracranial tumors because its structure suggested a high capacity for blood brain barrier penetration. It is worth mentioning that BYK191023 did demonstrate iNOS inhibition in vitro, mimicking the effects of 1400W (Figure 34c). Both inhibitors appear to have some anti-tumor activity against intracranial xenografts (Figure 35), which supports the claim that this anti-tumor activity is due to an iNOS-specific effect. We believe that either 1400W or BYK191023 would be strong candidates for clinical trial as they both: 1) exhibits at least 1000 fold selectivity of iNOS over eNOS and nNOS [242, 246], 2) adhere to “Lipinski’s rules of five” for optimal pharmacokinetics and bioavailability [253], 3) are unlikely to exhibit unwanted toxicity [244, 245, 252], 4) decrease glioma CSC growth and survival in vitro, and 5) decrease tumor growth in animals bearing human glioma xenografts. Considering all of these properties, we strongly advocate assessment of 1400W or BYK191023 (or other low toxicity, iNOS-specific inhibitors with the capacity for blood brain barrier penetration) in human clinical trials for malignant glioma therapy. The efficiency of these CSC-directed therapeutics may be synergize with strategies targeting the remainder of the tumor bulk as well (e.g., cytotoxic agents and/or radiation therapy), so assessments of appropriate multi-modal and multi-therapeutic approaches incorporating iNOS-directed therapies would be promising areas for future studies.
We now identify iNOS expression in malignant glioma CSCs as a potentially effective therapeutic target. As the prognosis for patients with malignant brain tumors has improved little despite extensive research efforts, investigation of alternative strategies is warranted and anti-CSC therapies hold promise to potentially synergize with other more traditional therapies directed against the tumor bulk. iNOS inhibitors have already been evaluated in clinical trials, so the importance of iNOS to glioma CSCs and tumor growth represents a solid foundation for the initiation of clinical trials investigating the effect of iNOS inhibitors against human gliomas.

4.5 Materials and Methods

4.5.1 Materials and transgenic mice

All materials were from Sigma unless otherwise indicated. N-(3-(aminomethyl)benzyl)acetamidine (1400W) was obtained from Cayman Chemical and 2-[2-(4-methoxy-2-pyridinyl)ethyl]-1H-imidazo[4,5-b]pyridine dihydrochloride (BYK191023) was obtained from Tocris Bioscience. All mice were housed in the Biological Resources Unit at the Cleveland Clinic or the Cancer Center Isolation Facility at Duke University with a 12 hour day-night cycle, filtered air and water and a normal diet. iNOS+- mice were derived by crossing wild type (B6129PF2J, Jackson Laboratories) and iNOS-/- (B6.129PF2-NOS2tm1/lau/J) mice. The heterozygotes then were bred to produce littermates, which (following genotyping), were used for all experiments.
4.5.2 Primary human specimen acquisition and xenograft maintenance

All human tissues were acquired in accordance with a Duke University Institutional Review Board (IRB) protocol (specimens with a T prefix), a Cleveland Clinic IRB protocol (specimens with a CCF prefix), or a Case Western Reserve University IRB protocol (specimens with a CW prefix). Where indicated, tumor specimens were xenografted by subcutaneous injection into the flanks of athymic/nude mice in accordance with Institutional Animal Care and Use Committee-approved protocols at either Duke University or the Cleveland Clinic. For maintenance, xenografts were excised, dissociated with papain and unsorted tumor cells were re-injected into fresh mice.

4.5.3 Isolation and culture of glioma CSCs and non-CSCs from xenografts and primary human specimens

All cells were cultured at 37 °C in an atmosphere containing 5% CO2. Glioma CSCs and non-CSCs were isolated essentially as described [77, 78, 99]. In brief, tumor tissue was isolated and immediately enzymatically dissociated with a Papain Dissociation System (Worthington Biochemical). Dissociated cells were separated from undigested tissue by straining through a 70 µm filter per the manufacturer’s instructions, and pelleted by gentle centrifugation. To lyse contaminating red blood cells, the pellet was rapidly resuspended in 20 ml of a hypotonic solution (PBS diluted 1:3 with water), then were gently pelleted by centrifugation. Single dissociated glioma cells were resuspended and allowed to recover overnight in supplemented Neurobasal growth medium.
CD133+ and CD133- cells were obtained either by FACS or magnetic activated cell sorting (MACS). All flow cytometry was performed on a FACS Aria II Cell Sorter (BD Biosciences). By FACS, single cells were labeled with an allophycocyanin-conjugated monoclonal antibody against CD133 (CD133/2; Miltenyi Biotec). Viable cells were sorted into CD133+ and CD133- populations with gating parameters derived from the analysis of cells labeled with an isotype control antibody. For MACS, cells were incubated with microbead-conjugated anti-CD133 monoclonal antibodies (Miltenyi) for 1 h at room temperature, loaded onto magnetic columns, washed and mechanically eluted per the manufacturer’s instructions. CD133- flowthrough from the first column was run through a second column and the resultant flow through collected to ensure thorough depletion of CD133-expressing cells.

Glioma-derived CD133+ CSCs were cultured in Neurobasal media (Invitrogen), supplemented with the following: B27 lacking vitamin A (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 ng/mL bFGF (R&D Systems), and 10 ng/mL EGF (R&D Systems). CD133- non-CSCs were plated in DMEM with 10% fetal bovine serum for at least 12 hours to permit cell survival. Prior to performing experiments, DMEM was replaced with supplemented Neurobasal media as described above for at least 2 hours.

4.5.4 Nitrite level determination by 4,5-diaminofluorescein (DAF-2)

Cells were cultured for 96 hours with daily administration of PBS (vehicle) or 100 µM 1400W in PBS. Fifty µl of conditioned supernatant or nitrite standards
were introduced into a 96-well plate in triplicate for each condition. Twenty-five µl of 30 µM 4,5-diaminofluorescein (DAF-2; Cayman Chemical) in PBS was added and the plate was incubated for 15 minutes at room temperature in the dark. The samples were then acidified with 50 µl 1M HCl and incubated for 15 minutes in the dark at room temperature. Samples were neutralized with 50 µl 1.5M NaOH and fluorescence was measured with λex = 488 nm and λem = 525 nm. A standard curve was created using the fluorescence from the nitrite standards and samples were normalized to cellular protein content (measured by Bradford assay; Bio-Rad).

### 4.5.5 Analysis of glioma and normal specimens by immunofluorescence

Primary human glioma specimens or subcutaneous xenografted human tumor specimens were cryosectioned, fixed with 4% PFA in PBS for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS containing 10% normal goat serum for 30 min. Samples were then washed with PBS and incubated with the indicated antibodies in PBS containing 10% normal goat serum as described below.

For intracranial xenografts or normal mouse brain specimens, tissues were fixed by intracardiac perfusion of anaesthetized mice with both PBS, then PBS containing 4% PFA (10 ml of each per mouse). Tissues were excised and incubated overnight in 4% PFA, and stored in 70% ethanol for up to 3 weeks. Prior to sectioning, samples were cryoprotected by incubation overnight at 4 °C in PBS containing 30% sucrose, then were embedded in optimal cutting
temperature polymer (OCT; Sakura). Cryosectioning was performed with a Leica CM1900 Cryostat, and tissue sections were fixed/permeabilized as described above.

The following antibodies were used for immunofluorescence: monoclonal mouse anti-human CD133 (Miltenyi Biotec clone WSB3C1, cat# M0823; 1:20), monoclonal mouse anti-human CD31 (Dako Clone JC7C0A; 1:250), polyclonal rabbit anti-mouse collagen IV (Millipore, 1:200); polyclonal rabbit anti-iNOS (Upstate/Millipore, cat# 06-573; 1:250), monoclonal mouse anti-Ki67 (Dako clone M7240; 1:50); monoclonal mouse anti-polysialated neural cell adhesion molecule (PSNCAM, clone 5A5 developed by Thomas M. Jessell and Jane Dodd, obtained from the developmental studies hybridoma bank maintained under the auspices of the NICHO and maintained by the University of Iowa Department of Biology, Iowa City, IA 52242; 1:20); polyclonal rabbit anti-phosphorylated histone H3 (Millipore, cat# 06-570; 1:300). Sections were incubated with primary antibody for 16 hours at 4 °C, and visualized with Alexa568 or Alexa488-conjugated secondary antibodies per the manufacturer's instructions (Invitrogen). Sections were counterstained for 10 minutes at room temperature with 5 µg/ml Hoescht in PBS. Images were acquired with a 40X or 63X oil immersion objective lens on a Leica SP5 confocal microscope using the sequential scanning technique to eliminate fluorescent bleed-through and were processed with Adobe Photoshop.

To assess long-term BrdU retention within the subependymal stem cell niche, the indicated mice were pulsed intraperitoneally with 100 mg/kg BrdU
(Fisher, cat# BP2508-250) every day for 5 days. After 30 days, mice were subjected to intracardiac perfusion and fixation as described above, brains harvested and embedded/sectioned as described. BrdU-antigen retrieval was completed as follows: sections were boiled in citrate buffer (0.01 M citrate, pH 6.0) twice for 5 min each, incubated in 3M HCl at 37 °C for 20 min, then washed and neutralized with borate buffer (0.1M BO3-, pH 8.5). Sections were blocked as above and were stained with mouse monoclonal anti-BrdU antibody (BD Biosciences, cat# 347880; 1:200).

4.5.6 Western blotting and antibodies

Cells were collected and lysed in hypotonic buffer with nonionic detergent (50mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% NP-40, 50 mM NaF with protease inhibitors), incubated on ice for 15 min and cleared by centrifugation at 10,000 g at 4 °C for 10 min. Protein concentration was determined with the Bradford assay (Bio-rad). Equal amounts of protein were mixed with reducing Laemmli loading buffer, boiled and electrophoresed on NuPAGE Gels (Invitrogen), then transferred to PVDF membranes (Millipore). Blocking was performed for 30 min with 5% nonfat dry milk in TBST and blotting performed with primary antibodies for 16 hours at 4 °C. Antibodies included: mouse monoclonal anti-iNOS (BD Biosciences, cat# 610328, 1:1000), rabbit polyclonal anti-iNOS (Millipore, cat# 06-573; 1:5000), rabbit polyclonal anti-eNOS (Santa Cruz, cat# sc-654; 1:1000), rabbit polyclonal anti-nNOS (Santa Cruz, cat# SC-648; 1:1000), mouse monoclonal anti-FLAG (Sigma clone M2, cat# F1804; 1:1000), rabbit polyclonal
anti-CDA1 (Proteintech cat# 12087-2-AP). HRP-conjugated donkey anti-rabbit or anti-mouse secondary antibodies (Zymed) were incubated with the membrane, and signal detected by enhanced chemiluminescence substrate (Pierce).

4.5.7 Analysis of mRNA by qRT-PCR

Total cellular RNA was isolated with the RNeasy kit (Qiagen) and reverse transcribed into cDNA using the Superscript III Reverse Transcription Kit (Invitrogen). Real time PCR was performed on an Applied Biosystems 7900HT cycler using SYBR-Green Mastermix (SA Biosciences) and intron-spanning, gene-specific primers as follows:

_ -actin forward: 5’-AGAAAATCTGGCACCACACC-3’
_ -actin reverse: 5’-AGAGGCGTACAGGGATAGCA-3’
iNOS forward: 5’-TCGGCAGAATCTACAAAGTCC-3’
iNOS reverse: 5’-TGGGCATCCTCACAGGAG-3’

Differences in expression were calculated using the Ct method.

4.5.8 Lentivirus-mediated knockdown of iNOS and heterologous expression of E. coli flavohemoglobin

Lentiviral-compatible shRNA clones against iNOS (TRCN0000003764 targeting the 3’ untranslated region and TRCN0000003765 targeting the coding sequence) and scramble control (SHC002) were obtained from Sigma. shRNA-bearing lentiviral particles were generated by co-transfecting 293FT cells (using Lipofectamine 2000) with shRNA plasmid DNA, and the packaging and enveloping constructs, pCI-VSVG and ps-PAX2, respectively (Addgene). Media was changed 8-12 hours after transfection to supplemented Neurobasal, and the
virus-laden supernatant was collected at 24 and 48 hours. The supernatant was filtered and employed for experiments or frozen at -80 °C in aliquots. Puromycin selection was not employed, as iNOS knockdown resulted in complete cell death in the targeted groups prior to the selection timeframe (data not shown).

Flavohemoglobin (FlavoHb) overexpression vectors were generated using the SystemBio lentiviral expression system. An N-terminal Flag-tagged cDNA encoding the *E. coli* FlavoHb (gene *hmpA*) was amplified by PCR from *E. coli* genomic DNA with the following primers: 5'-AATAGAATTCCATGGGACTACAAAGACGACAAAGCTTGACGCTCAAACCATCGCTAC-3' and 5'-AAATGGATCCTTACAGCACCTTATGCGG-3'. The 1.2 kb PCR product was purified by gel extraction, digested with EcoRI and BamHI, and ligated into pCDH-CMV-MCS-EF1-copGFP. Resultant plasmids containing *E. coli* hmpA were fully sequenced at the Duke DNA Sequencing Facility. Lentiviral particles were generated as above, and expression verified with anti-Flag western blotting (Supplementary Fig. 4b).

4.5.9 **Cell counting and viability assessment with trypan blue exclusion**

Cells in Neurobasal media were either treated daily with 100 μM 1400W or BYK191023, or twice (24 hours between treatments) with lentiviral particles bearing either iNOS-directed shRNA, scramble control shRNA, a FlavoHb overexpression construct, or the vector control overexpression construct. After 72 hours (for lentiviral experiments) or 96 hours (for 1400W experiments), cells were harvested, dissociated by trypsinization, and mixed for 2 minutes with trypan blue
(1:1 volumes); live (white) and dead (blue) cells were immediately counted on a hemacytometer.

4.5.10 Assessment of cellular proliferation with the $^3$H-thymidine incorporation assay

Fifteen thousand cells were plated in 1 ml media, prepared, and treated as described above. 48 hours (for lentiviral experiments) or 72 hours (for 1400W experiments) after treatment, cells were incubated for 5 h with 4 $\mu$Ci of $^3$H thymidine (Perkin Elmer). Cells were washed once with PBS, fixed for 1 hour in 10% trichloroacetic acid (TCA), and incubated overnight in 0.01 M NaOH before assessing radioactivity incorporation of the lysed cellular contents by scintillation counting in a Beckman scintillation counter (Beckman 156000).

4.5.11 Neurosphere formation assay

Twenty four hours after the treatment with the indicated lentivirus, cells were trypsinized, incubated with propidium iodide (PI), and viable PI-negative cells were single-cell sorted by FACS into 96 well plates containing supplemented Neurobasal medium. For neurosphere formation of cells treated with 1400W, freshly isolated xenograft cells were labeled with APC-conjugated anti-CD133 antibody for 1 h with agitation at room temperature, and sorted into single cells in 96 well plates using FACS based on CD133 level into wells containing supplemented Neurobasal medium. Either vehicle control or 100 $\mu$M 1400W were then added daily. Ten days after sorting, neurosphere formation was quantified by counting the percent of wells containing neurospheres with a
Leica DMI3000B microscope.

**4.5.12 Isolation and characterization of normal neural stem cells**

Neural stem cells were isolated from 8 to 10 week-old WT mice and iNOS-/- mice by microdissection of the peri-ventricular region, digestion by papain and trypsin, and lysis of red blood cells by incubation with a hypotonic solution. Cells were plated in suspension culture in supplemented Neurobasal and were allowed to grow through 3 passages before plating them for assessment by $^3$H thymidine incorporation or trypan exclusion (assays were performed 2 or 4 days after plating, respectively).

Examination of brain differentiation, stem, or proliferation markers was completed by harvesting, sectioning and staining brains from wild type or iNOS-/- littermates, and these mice were also used for assessing long-term BrdU incorporation (see above).

Three different human fetal neural progenitor cell line preparations were obtained from Cambrex (now Lonza, cat# PT-2599) and was maintained in suspension culture with supplemented Neurobasal media. The H9-ESC-derived neural stem cell culture was obtained from Invitrogen and cultured on CellStart according to the directions in the kit accompanying the cells (Invitrogen cat# N7800-200)

**4.5.13 Assessment of in vivo (intracranial) glioma progression**

Xenograft-derived CD133+ cells were treated twice with lentivirus bearing either non-targetted scrambled shRNA or iNOS-directed shRNAs; alternatively,
cells were treated with lentivirus bearing *E. coli* FlavHb or a vector control. Twenty-four hours after infection, cells were harvested, counted and injected into the right forebrains of 4-6 week old athymic nude mice. Mice were monitored daily until the development of neurological or constitutional signs (e.g., ataxia, lethargy, seizures), at which point the mice were sacrificed and harvested as described above.

### 4.5.14 Microarray analysis

CD133+ CSCs derived from two different xenografts were treated with the following for 72 h, in duplicate: non-targeted scramble control shRNA, iNOS-directed shRNA-1 or iNOS-directed shRNA-2. Total cellular RNA was harvested by the RNeasy kit (Qiagen) and submitted to the Cleveland Clinic Genomics Core facility where mRNA quality was verified by Bioanalyzer analysis (Agilent) before RNA amplification, hybridization to Human-6 Expression Beadchips (Illumina), washing, and finally imaging and generation of raw output files. Data were processed by ExpressionAnalysis (www.expressionanalysis.com). Probes meeting standard quality control criteria that had differential regulation of more than 3-fold in one of the iNOS shRNA treatments (relative to the corresponding scramble control) were retained. These probes were filtered for those that had consistent gene expression changes in the remaining treatments (i.e. if xenograft 1 had a 6 fold upregulation of gene x for iNOS shRNA1, it would be kept in the analysis if it were over 1.4 fold upregulated in all other shRNA treatments). The resultant list was hand-examined for genes known to be involved in cell cycle
regulation.

4.5.15 Evaluation of CDA1 expression changes in HEK293 cells

HEK293T cells were subjected to one of the following for 18 h before total cellular protein was harvested and analyzed by western blotting: 1) Vehicle or 0.1 or 0.2 mM DETA-NO, or 2) transient transfection with pCDH-EF1-copGFP vector or pCDH-CMV-iNOS-EF1-copGFP.

4.5.16 Generation of stable firefly luciferase-expressing glioma cells and performance of real-time tumor measurements by bioluminescent imaging with systemic iNOS inhibitor treatment

Firefly luciferase was subcloned into the multiple coding site of pCDH-CMV-MCS-EF1-Puro and lentivirus with this construct was generated as described in section 4.5.8. Unsorted xenograft-derived glioma cells were infected twice over 72 hours with this virus, and were selected for 72 hours with 5 µg/mL puromycin, at which point cells were trypsinized, allowed to recover for 18 hours, and then sorted by MACS as in section 4.5.3 and CD133+ cells were plated again in puromycin-containing media. Luciferase activity was verified by bioluminescent imaging before 5,000 CD133+ luciferase-expressing cells (T3691-Lucif) were inoculated into the brains of immunocompromised mice.

After a 10 day engraftment period, baseline bioluminescent images were taken with the Xenogen bioluminescence imaging system 5 minutes after intraperitoneally administering (per animal) 100 µL of 30 mg/mL potassium D-luciferin salt dissolved in PBS. Twice daily intraperitoneal treatment was initiated with 75 mg/kg BYK191023 or 50 mg/kg 1400W or vehicle control. Images were
taken periodically as described above.

**4.5.17 Caspase 3/7 activity assays**

One thousand CD133+ cells were dissociated and plated per well of a 96 well plate in 100 uL of medium, were allowed to recover for 12 hours, and were treated twice with 33 uL of virus-laden supernatant (generated as described in section 4.5.8). Forty-eight hours after the second shRNA treatment, cells were analyzed for caspase 3/7 activity using the Promega Caspase-Glo 3/7 Assay (cat# 8090).
Figure 17. CD133+ glioma cancer stem cells produce more nitric oxide (NO) than matched CD133- cultures. Matched CD133+ and CD133- cells were isolated from four different xenografted brain tumors (T3359, T3691, T4121, T3832). Identical cell numbers were plated in triplicate, allowed to recover, and were then incubated in identical media for 96 h at which point conditioned supernatant was collected and analyzed for nitrite with the fluorophore DAF-2 and normalized to total cellular protein. **, p<0.01.
Figure 18. Heterologous expression of the NO-consuming E. coli flavohemoglobin inhibits cancer stem cell growth, neurosphere formation, and tumorigenic capacity. (a) FlavoHb consumes NO by catalyzing the aerobic oxidation of NO with \( O_2 \) to form the nonreactive product nitrate (\( NO_3^- \)). The reduced ferrous heme center is regenerated with electrons from NADPH. N-terminal Flag-tagged E. coli FlavoHb was cloned into a lentiviral expression vector. Lentivirus was created by cotransfecting 293FT cells with plasmid with packaging/enveloping vectors and infected CD133+ glioma stem cells were assessed for the presence of Flag-FlavoHb by western blotting. (b) CD133+ cells from brain tumor xenografts were infected with the indicated lentiviruses for 48 h and viable cells were identified by trypan blue exclusion and were counted 96 h later. (c) Cells were infected as in c except GFP+ cells (i.e., virally infected) were individually sorted into wells after 48 hours of infection, and neurosphere formation was assessed after 10 days. Representative images of neurospheres formed are shown; scale bar = 50 µm. (d) CD133+ cells infected with lentivirus encoding FlavoHb or empty vector for 48 hours were harvested by trypsinization and viable cells were intracranially injected into mice (5 mice per treatment, 5000 cells per mouse). Mice were monitored daily and the time until development of neurologic signs of tumor growth were plotted on a Kaplan-Meier plot. *, p<0.05; **, p<0.01
Figure 19. Glioma cancer stem cells express high levels of iNOS relative to non-stem cancer cells. Expression levels of NOS isoforms in CD133+ and CD133- cells isolated from (a) primary human specimens and (b) xenografted brain tumors were analyzed by western blotting. (c) In gliomas where SSEA1 was demonstrated to be most effective for segregating tumorigenic potential [44], expression of iNOS in matched SSEA1+ and SSEA1- cells was analyzed by western blotting. (d) Expression of iNOS mRNA in CD133+ and CD133- from primary human and xenografted tumors was assessed without intervening cell culture steps. Following FACS, cellular RNA was immediately isolated and assessed by qRT-PCR with normalization to β-actin. (e) Immunofluorescence of iNOS and CD133 in primary human brain tumor specimens; arrows indicate cells that express both CD133 and iNOS. Written beside or above each sample is an identification number corresponding to an individual patient-derived primary specimen or xenograft; scale bars = 25 μm. *, p<0.05; **, p<0.01.
Figure 20. iNOS activity accounts for the high NO production in glioma cancer stem cells. Matched CD133+ and CD133- cells were isolated and sorted from three different xenografted brain tumors. Identical cell numbers were plated in triplicate and treated daily with either vehicle control or 100 µM 1400W. After 96 h, conditioned supernatant was collected and analyzed for NO$_2^-$ with the fluorophore DAF-2 and normalized to total cellular protein.
Figure 21. iNOS-directed shRNA treatment effectively and specifically decreases iNOS expression in CD133+ glioma cancer stem cells. (a) CD133+ cells were infected for 48 h with lentivirus bearing either a scrambled or two non-redundant iNOS-directed shRNAs and analyzed for iNOS knockdown efficiency and specificity relative to eNOS and nNOS. (b) Representative images of shRNA-infected CD133+ cells; scale bar = 50 µm.
Figure 22. shRNA-mediated iNOS knockdown decreases glioma CD133+ cancer stem cell viability, proliferation and neurosphere formation. CD133+ cells were infected for 48 h with lentivirus bearing either a scrambled or two non-redundant iNOS-directed shRNAs. (a) CD133+ and CD133- cells were grown for 72 hours after infection and cell number assessed by trypan blue staining with cell counting. (b) To examine rates of cellular proliferation, cells were analyzed for 3H-thymidine incorporation 48 hours after infection with the indicated shRNA-bearing lentiviruses. (c) CD133+ and CD133- cells from xenografted tumors were infected for 48 h with the indicated lentivirus and then viable cells were individually sorted into single wells. After 10 days, neurosphere formation was assessed as the percentage of wells with neurospheres. Written above each sample is an identification number corresponding to an individual patient-derived primary specimen or xenograft; *, p<0.05; **, p<0.01.
Figure 23. iNOS inhibition decreases glioma CD133+ cancer stem cell viability, proliferation and neurosphere formation. CD133+ and CD133- cells were treated with either vehicle control or 100 µM 1400W for 96 h and the number of cells per well assessed by trypan blue staining with cell counting. (g) T4302 CD133+ and CD133- cells were treated with either vehicle control or 100 µM 1400W for 72 h and assessed for proliferation by 3H-thymidine incorporation. (h) T3691 CD133+ and CD133- cells FACS sorted to one cell per well were treated with either vehicle control or 100 µM 1400W and neurosphere formation assessed after 10 days. Representative images of neurospheres formed are displayed; scale bar = 50 µm. *, p<0.05; **, p<0.01.
Figure 24. shRNA-mediated iNOS knockdown decreases glioma CD133+ cancer stem cell tumorigenicity. Xenograft-derived T3691 CD133+ cells were treated with lentivirus encoding scrambled or iNOS-directed shRNAs for 48 h and iNOS expression was assessed by qRT-PCR. The indicated number of viable lentivirus-treated CD133+ T3691 cells were intracranially injected into athymic mice and monitored for neurologic or constitutional signs as an indication of tumor growth. Indicated in the table are the number of mice that developed tumors, as well as median time to neurologic signs. Kaplan-Meier curves demonstrate targeting iNOS increased the survival of immunocompromised mice injected with 10,000 CD133+ CSCs. **, p<0.01.
Figure 25. iNOS expression in human gliomas is associated with poor prognosis. The relationship between relative iNOS expression and survival in patients with malignant glioma was retrospectively examined via the Repository of Molecular Brain Neoplasia Data (REMBRANDT) developed at the NCI. Patients were stratified by relative iNOS expression (***, p < 0.0001 for decreased patient survival with iNOS expression > 2.0 fold above mean relative to all glioma patients). All other survival differences were not statistically significant (p>0.1).
Figure 26. iNOS knockdown does not affect stem cell gene expression in CD133+ glioma cancer stem cells. CD133+ cells derived from xenografts were treated for 48 h with lentivirus bearing shRNA before they were harvested. Total cellular RNA was immediately isolated and assessed by qRT-PCR with normalization to β-actin.
Figure 27. iNOS knockdown induces caspase 3 cleavage in some, but not all, CD133+ glioma cancer stem cells. CD133+ cells derived from xenografts were treated for 96 h with lentivirus bearing shRNA before cells were harvested and caspase 3/7 assayed with a luminescent DEVD reporter substrate. **, p<0.01.
Figure 28. shRNA-mediated iNOS knockdown decreases cell cycle rate in CD133+ cancer stem cells. CD133+ glioma cells were treated for 72 h with control or iNOS shRNA by lentiviral infection. After trypsinization and a recovery period, cells were pulsed with 1 μM EdU for 1 h. After cell fixation, permeabilization, antigen recovery and reaction with the 488Alexafluor-conjugated detection reagent. Nucleated cells were determined by 7-AAD positivity and EdU incorporation based off of control cells that had never been exposed to EdU.
Figure 29. The cell cycle inhibitor cell division autoantigen 1 (CDA1) is repressed by iNOS-derived NO. (a) CD133+ glioma cells were treated for 48 hours with lentivirus bearing either control or iNOS shRNA and total cellular RNA was isolated, processed for hybridization, hybridized to Illumina microarray chips and hybridization signals determined as in the Methods. Probes meeting quality control criteria were retained for analysis. iNOS-dependent repression of CDA1 was validated in glioma CD133+ cells by (b) qRT-PCR and (c) western blotting. (d) HEK293 cells were treated with the indicated concentrations of DETA-NO donor compound for 18 h, then were harvested and analyzed by western blotting. (e) For 14 h, HEK293 cells were transiently transfected with an iNOS overexpression construct or vector control in the presence of either vehicle control or the iNOS inhibitor 1400W, then were harvested and analyzed by western blotting.
Figure 30. shRNA-mediated repression of CDA1 attenuates the cell cycle decrease observed with iNOS knockdown in CD133+ glioma CSCs. CD133+ glioma cells were treated for 72 h with control or CDA1-directed shRNA by lentivirus. Cells were dissociated by trypsin, allowed to recover, then were treated for 72 h with lentivirus bearing either control or iNOS-directed shRNA. After trypsinization and a recovery period, cells were pulsed with 1 µM EdU for 1 h. After cell fixation, permeabilization, antigen recovery and reaction with the 488Alexafluor-conjugated detection reagent. Nucleated cells were determined by 7-AAD positivity and EdU incorporation based off of control cells that had never been exposed to EdU.
Figure 31. Normal mouse neural stem/progenitor cells are not dependent on iNOS. (a-c) Periventricular neural stem cells were isolated from adult wild type (WT) and iNOS-/- mice and were passaged 3 times in vitro to ensure a self-renewing population. (a) Equal numbers of cells were plated and cultured for 96 h before total cell numbers were assessed by trypan blue staining with cell counting. (b) Equal numbers of cells were plated and cultured for 48 h before proliferation was assessed by ³H-thymidine uptake assay. (c) Equal numbers WT and CD133+ glioma CSCs were plated, treated daily for 96 h with 1400W or vehicle control before total cell numbers were assessed by trypan blue staining with cell counting. (d) Brains from WT and iNOS-/- littermates were assessed for the presence of the proliferation marker phospho-histone H3 (PH3) and the number of positive cells per field quantified using by blinded counting; scale bar = 50 µm. (e) WT and iNOS-/- littermates received daily intraperitoneal injections of 100mg/kg BrdU for 5 days, then were sacrificed and their perfused brains were analyzed for cellular proliferation by immunofluorescence/BrdU-uptake, which was quantified by blinded counting; scale bar = 50 µm. *, p<0.05.
Figure 32. Human neural stem/progenitor cells exhibit low levels of iNOS expression and are not dependent on iNOS. (a) Protein expression levels of iNOS in normal fetal neural progenitor cells (preparation 167, Lonza) were compared to glioma CSCs and non-CSCs by western blotting. (b) mRNA levels of iNOS in preparation 167 normal fetal neural progenitor cells were compared to glioma stem cells and non-stem glioma cells by qRT-PCR with normalization to β-actin. (c) Equal numbers of CD133+ glioma CSCs were plated alongside (c) fetal neural progenitor cells (preparations 893 and 231) and (d) embryonic stem cell-derived neural progenitor cells (H9 NSC; Invitrogen) and were treated daily for 96 h with 1400W or vehicle control before total cell numbers were assessed by trypan blue staining with cell counting.
Figure 33. Treatment of subcutaneous human glioma xenografts with the iNOS inhibitor 1400W decreases tumor growth. (a) Schematic overview of the experiment examining 1400W-mediated inhibition of glioma tumor growth in vivo. (b-f) CD133+ tumor cells (500,000 total) were injected subcutaneously into mice and were allowed to engraft for 10 days prior to initiating daily intraperitoneal (IP) injections of 1400W (7 mice) or vehicle (6 mice). These were continued for 14 days, and mice were then sacrificed 3 days later. (b) Tumor dimensions were measured daily, followed by tumor excision on day 17. (c) Images of dissectable tumors at time of excision. (d) Tumor proliferation was assessed by immunofluorescence for Ki67 and vascular density assessed by immunofluorescence for collagen IV. Bar= 50µm. Arrows indicate positive cells. Cells positive for (e) Ki67 and (f) collagen IV were quantified for two fields of view per tumor in each of the treatment groups. *, p<0.05.
Figure 34. 1400W and BYK191023 are two potent, iNOS-selective inhibitors that inhibit glioma CD133+ cancer stem cell growth *in vitro*. (a) 1400W [238] and (b) BYK191023 [246, 254] are irreversible competitive inhibitors of iNOS with over 1000-fold selectivity over eNOS and nNOS. (c) CD133+ CSCs were plated in equal numbers and treated daily with either 100µM 1400W or 100µM BYK191023. After 96 h, cells were dissociated, harvested and live cells quantified using trypan blue. *, p<0.05.
Figure 35. Treatment of intracranial human glioma xenografts with iNOS inhibitors delays intracranial tumor growth. Glioma cells were engineered to stably express firefly luciferase (Lucif-T3691) and 5000 CD133+ Lucif-T3691 cells were inoculated into the brains of immunocompromised mice. Ten days after engraftment, baseline bioluminescence images were obtained and twice daily intraperitoneal administration of 1400W, BYK191023 or vehicle control was initiated, with periodic assessment of tumor size by bioluminescence imaging. Shown are the first three mice to develop tumors out of each group of 5.
Chapter 5.

Studying Mammalian Nitric Oxide Biology via Heterologous Expression of Bacterial Flavohemoglobin

In order to interrogate the cell-autonomous function of nitric oxide within glioma stem cells, I wanted to employ a technique that would efficiently and specifically consume nitric oxide within mammalian cells. To do this, I worked with a colleague, Michael T. Forrester, to validate the ability of lentiviral-delivered *E. Coli* flavohemoglobin (an enzyme expressed by *E. Coli* and other microbes in response to nitrosative stress) to consume nitric oxide in mammalian cells and manipulate NO-dependent cell biology. This technique was useful as part of my focus on the role for iNOS in glioma stem cells (Figure 18), but we wished to more comprehensively validate this technique as useful for interrogating NO biology in conditions of both physiologic NO levels and supraphysiologic conditions of nitrosative stress. Though Michael spearheaded this validation effort, I have made major contributions to the project through evaluations by immunofluorescence, viability assays, generation of stable flavohemoglobin-expressing cells as well as replicating some of the western blotting and nitrate/nitrite determinations. This chapter is based on a manuscript we have submitted as a brief report to *Nature Methods*, on which I am second author.

5.1 Summary

The prokaryotic flavohemoglobins – highly efficient nitric oxide-consuming enzymes – evolved nearly 2 billion years ago to protect microbes from nitrosative stress. Here we demonstrate the utility of *E. coli* flavohemoglobin to heterologously manipulate nitric oxide-dependent stress and signaling in mammalian cells. This approach should facilitate future studies of nitric oxide biology, particularly in situations where manipulations of known nitric oxide sources (e.g. nitric oxide synthases) are either problematic or ineffective.
5.2 Introduction

The diatomic gas nitric oxide (NO) is a unique molecule that plays a wide range of roles in mammalian, plant and microbial biology. In mammalian models of infection, host-derived NO facilitates microbial killing [255, 256]. In return, bacteria possess two major systems to metabolize NO: the flavorubredoxin NO-reductase [257] and flavohemoglobin (FlavoHb) NO-dioxygenase [237, 258, 259] systems, which operate under strictly anaerobic and aerobic/microaerophilic conditions, respectively. The highly conserved FlavoHb family is believed to have evolved nearly 2 billion years ago [260], suggesting that microbes have been coping with nitrosative stress long before mammals existed.

A wealth of studies over the past decade has elucidated the mechanism and function of FlavoHb in bacteria and fungi. Under aerobic/microaerophilic conditions, FlavoHb has been shown to convert NO and O$_2$ into nontoxic nitrate (NO$_3^-$) with concomitant oxidation of the ferrous (Fe$^{2+}$) to ferric (Fe$^{3+}$) heme[237]. The active site flavin adenine dinucleotide (FAD) supports one-electron reduction back to the ferrous state, driven by electrons from NADH or NADPH (Figure 36a). Importantly, FlavoHb is transcriptionally induced by NO in many bacterial and fungal pathogens, and deletion of the FlavoHb gene has consistently been shown to result in NO-hypersensitivity and decreased pathogenicity in vivo.

In contrast to microbes, the FlavoHb gene is not present in metazoans. On the contrary, mammals synthesize NO via three conserved NOS isoforms: iNOS,
eNOS and nNOS, each of which play distinct biological roles [120]. Though the overall importance of NO is widely appreciated, techniques to determine the cellular roles of NO have relied predominantly on manipulating NOS expression or activity, most frequently via arginine-based NOS inhibitors. While this approach is undoubtedly powerful, there are several drawbacks to NOS inhibitors: 1) they rarely exhibit strong isoform selectivity (with the exception of some iNOS inhibitors), 2) they are typically arginine analogues, and several studies have suggested they may perturb arginine uptake or metabolism [261-263] and 3) NOS-independent sources of NO are unaffected. Vice versa, NOS overexpression or administration of NO-donor compounds may result in supraphysiologic levels of NO leading to aberrant cellular effects. A technique to selectively deplete NO – independently of NO synthesis – might therefore be of significant utility in studies of NO biology.

5.3 E. Coli Flavohemoglobin is Cytoplasmic and Enzymatically Active when Expressed in Mammalian Cells

Given the remarkable specificity and catalytic efficiency of E. coli FlavoHb compared to even the most robust mammalian NOS isoform, iNOS (Figure 36b), we surmised that heterologous expression of FlavoHb in mammalian cells might be a useful tool to interrogate the role of NO in cells. Following the cloning of E. coli FlavoHb (gene hmpA) into a mammalian expression vector in a lentiviral delivery system, the cellular localization of FlavoHb was determined in HEK293 cells via confocal immunofluorescence (Figure 37a), as well as subcellular
fractionation (Figure 37b). These experiments suggest that *E. coli* FlavoHb exhibits diffuse subcellular localization with a cytosolic predominance, which is expected since FlavoHb lacks any known human localization signal or sequence. To examine whether *E. coli* FlavoHb is enzymatically active in human cells, cytosolic extracts from transfected HEK293 cells were assayed for enzymatic activity by measuring NO-dependent NADPH consumption at 340 nm (Figure 38a). As anticipated, extracts from cells expressing FlavoHb exhibited markedly increased NADPH consumption, which was dependent on the addition of exogenous NO. Importantly, expression of FlavoHb did not exhibit any noticeable toxicity or growth suppression in several tested mammalian cell types.

Endogenously synthesized NO is normally oxidized to NO$_2^-$ (nitrite) and NO$_3^-$ in an approximately 60:40 ratio via auto-oxidation. Since FlavoHb converts NO to NO$_3^-$ (not NO$_2^-$), the efficacy of FlavoHb to metabolize endogenous NO was examined via measuring NO$_2^-$ and NO$_3^-$ in the media of FlavoHb-transfected HEK293 cells. As shown in Figure 38b, the iNOS inhibitor 1400W led to decreased NO$_2^-$ and NO$_3^-$ due to inhibition of NO synthesis, whereas FlavoHb heavily shifted the ratio towards NO$_3^-$.

Similar results were obtained in LPS-stimulated RAW264.7 macrophages stably expressing FlavoHb (Figure 38c). Importantly, these data demonstrate that *E. coli* FlavoHb indeed metabolizes endogenously synthesized NO to NO$_3^-$ in mammalian cells without affecting NO synthesis itself.
5.4 Mammalian Cells Expressing E. Coli Flavohemoglobin are Protected from Nitrosative Stress-Induced Toxicity

FlavoHb is known to play a major role in protection from nitrosative stress in bacteria and fungi. As high levels of NO are known to cause nitrosative stress and toxicity in mammalian cells as well, we therefore evaluated the ability of FlavoHb to protect mammalian cells from nitrosative stress. In HEK293 cells, FlavoHb blocked the growth suppressive effects of both exogenously-delivered NO (Figure 39a) and iNOS transfection (Figure 39b), as assayed by $^3$H-thymidine uptake. Similar data were obtained via cell counting (Figure 39c) and colony formation assays (Figure 39d). Collectively, these experiments demonstrate that FlavoHb – the predominant NO-protective system in microbial systems – exhibits a similar ability to protect mammalian cells from both exogenous and endogenous nitrosative stresses. Further, they emphasize that FlavoHb is enzymatically active in human cells, which is remarkable given the disparities between these two cellular environments.

5.5 Heterologous Expression of Flavohemoglobin can Modulate Known Nitric Oxide-Dependent Cell Signaling Events

As NO is frequently studied in the context of cell signaling, the utility of FlavoHb for interrogating NO-dependent signaling pathways was examined. In HEK293 cells, FlavoHb blunted both NO-dependent Txnip suppression [164] and VASP phosphorylation [264] (Figure 40a), two established downstream mediators of NO in epithelial cells. FlavoHb also inhibited COX-2 induction [265]
and Txnip suppression [164] in LPS-stimulated RAW264.7 macrophages (two established NO-dependent events) despite actually facilitating iNOS expression (Figure 40b). The enhanced iNOS expression in the presence of FlavoHb may be due to loss of NO-feedback control on iNOS expression or decreased iNOS-dependent toxicity.

5.6 Conclusions

Here we provide evidence that *E. coli* FlavoHb is enzymatically active in mammalian cells, thereby protecting them from nitrosative stress and inhibiting NO-signaling pathways. Importantly, the FlavoHb protein does not exhibit any apparent toxicities in the tested mammalian cell types. Heterologous expression of FlavoHb is therefore a novel and useful strategy to probe the effects of NO in mammalian cells independently of NO synthesis. This tool will likely find application in a range of NO-related studies and may provide a novel route to therapeutic depletions of NO *in vivo*.

5.7 Materials and methods

5.7.1 Materials

All materials were from Sigma unless otherwise indicated. LPS was from *E. coli* strain 026:B6. Protease inhibitor (PI) cocktail was from Roche. “NONOate” NO donors and 1400W were from Cayman Chemical. Sources of antibodies were: α-Txnip/Vdup1 mouse mAb (MBL International, #K0205-3), α-GAPDH
mouse mAb (Millipore, #MAB374), α-COX2 rabbit pAb (Cell Signaling, #4842), α-VASP rabbit pAb (Cell Signaling, #3112), α-phospho-VASP rabbit pAb (Cell Signaling, #3111), α-iNOS mouse mAb (BD Biosciences, #610328), α-Flag M2 mouse mAb for western blotting (Sigma, #F1804), α-Flag rabbit pAb for immunofluorescence (Cell Signaling, #2368), α-catalase mouse mAb (Sigma, #C0979), α-PCNA mouse mAb (Cell Signaling, #2586).

5.7.2  Cell culture

All cell lines were obtained from ATCC. The RAW264.7 and HEK293 cell lines were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For transient transfection experiments, HEK293 cells were transfected in 6 well dishes with 5 µg total DNA and 20 µl superfect (1:4 DNA:Superfect ratio).

5.7.3  Cloning and DNA manipulation

All PCRs were performed with Advantage DNA polymerase (Clontech). The E. coli gene for FlavoHb (hmpA) was amplified from genomic DNA (Strain BW25113) via PCR with the following primers: 5'-AATAGAATTCCATGGACTAAAGACGATGACGACAAGCTT
GACGCTCAAACCATCGCTAC-3' and 5'-AAATGGATCCTACAGCAGTACCTATGCGG-3'. The 1.2 kb PCR product was digested with EcoRI and BamHI, and ligated into the lentiviral expression plasmid pCDH-CMV-MCS-EF1-copGFP (System Biosciences) digested at the same sites. All products were verified by DNA sequencing (Duke DNA Sequencing
Facility). Human iNOS was cloned into pCDH-CMV-MCS-EF1-copGFP at the XbaI and NotI sites using the following PCR primers: 5’-CTTATATCTAGAACCATGGCCTGTCCTTGG-3’ and 5’-ATTATAGCGGGCCTTAGAGCGCTGCTGACA-3’.

5.7.4 Lentiviral production

Lentiviral particles were generated by co-transfecting subconfluent (60-70%) 293FT cells with pCDH-CMV-FlavoHb-EF1-copGFP plasmid DNA and the packaging and enveloping constructs, pCI-VSVG and ps-PAX2, respectively (Addgene). For a 10 cm dish, 6 µg plasmid DNA was co-transfected with 2 µg pCI-VSVG and 3 µg ps-PAX2 using 33 µL Lipofectamine 2000. Media was changed 8-12 hours after transfection to fresh DMEM with 10% serum, and the virus-laden supernatant was collected at 24 and 48 hours. The supernatant was filtered and employed for experiments or frozen at -80 °C in aliquots.

5.7.5 Generation and isolation of cells stably infected with empty vs. FlavoHb lentivirus

Stable FlavoHb-expressing or vector control cells were generated by treating 293FT or RAW264.7 macrophages twice with virus prepared as described above. Twenty-four hours after the second infection, 293FT or RAW264.7 cells were dissociated using trypsin or cell scraping, respectively, and viable GFP-positive cells were single-cell sorted into 96-well cell culture plates containing DMEM using a FACS AriaII Cell Sorter (BD Biosciences). Single cell sorting was verified 24 hours after sorting and media changed on amplifying
clones every 3 days until cell growth warranted splitting cells to a larger culture vessel. Amplified stable GFP-expressing cultures were evaluated for FlavoHb-expression by anti-Flag immunoblotting.

5.7.6 Measurement of FlavoHb activity in cellular extracts via NO-dependent NADPH consumption

A 15 cm dish of HEK293 cells stably infected with either empty lentivirus or FlavoHb was lysed via repeated passage through a 28 g needle in 50 mM PO₄, 0.5 mM EDTA, 2 μM FAD, pH 7.0 containing 1 mM DTT and PI cocktail. A cytosolic-enriched sample was obtained via clarification at 20,000 g x 30 min. Protein was measured via BCA assay. For measurement of NADPH consumption, 94 μl of lysate was mixed with either 2 μl of buffer or 10 mM NADPH, and absorbance at 340 nm was measured on a Beckman DU650 UV-Vis Spectrophotometer. Spermine-NONOate (final 4 mM, stock solution in 10 mM NaOH) was added to initiate the reaction, and absorbance at 340 nm was followed over time.

5.7.7 Subcellular fractionation for determination of FlavoHb localization in cells

Basic fractionation was achieved via differential centrifugation as briefly described. HEK293 cells were lysed via repeated passage through a 28 g needle in 25 mM Tris, 25 mM NaCl, 0.1 mM EDTA, pH 7.4 containing PI cocktail. Lysis efficiency was verified > 99% by trypan blue staining. The lysates were centrifuged at 500 g x 10 min to obtain a nuclei-enriched pellet. The supernatant was removed and centrifuged at 20,000 g x 30 min to obtain a
membrane/organelle-enriched pellet and cytosol-enriched supernatant. To facilitate solubilization, Triton X-100 (and lysis buffer) was added to each fraction to a final concentration of 0.2% (v/v). Protein was measured by BCA assay, and equal amounts of material (40 µg each) were employed for western blotting analysis.

5.7.8 Measurement of NO$_2^-$ and NO$_3^-$ in culture media

Following the indicated iNOS transfection(s) or LPS-stimulations, culture media was collected and clarified by centrifugation at 5,000 g x 5 min. The supernatant was saved, and NO$_2^-$ was measured by the standard Griess reaction. In brief, 40 µl of media or NO$_2^-$ standard in culture media was transferred to a 96 well dish and 100 µl of 1% sulfanilamide (w/v) in 1 M HCl was added. The mixture was incubated at room temperature for 5 min, and 50 µl 0.1% N-1-napthylethylenediamine dihydrochloride (NEDD) in 0.5 M HCl was added to each well. Absorbance at 540 nm was measured via spectrophotometry and all samples were normalized to standard NO$_2^-$ solutions. Measurement of NO$_3^-$ was performed essentially as described, with minor modifications. In brief, 40 µl of media or NO$_3^-$ standard in culture media was transferred to two wells of a 96 well dish (labeled “A” and “B”). To all wells was added 40 µl of PBS containing 4 µM FAD, then 10 µl of 4 mM NADPH. To “A” and “B” wells was added 10 µl PBS or NO$_3^-$ reductase (2 mU/µl in PBS), respectively. Plates were incubated at 37 °C for 1 h, and 100 µl of 1% sulfanilamide (w/v) in 1 M HCl was added. The mixture was incubated at room temperature for 5 min, and 50 µl 0.1% N-1-
napthylethylenediamine dihydrochloride (NEDD) in 0.5 M HCl was added to each well. Absorbance at 540 nm was measured via spectrophotometry. The value of “B” minus “A” (i.e., NO$_3^-$-derived signal) was calculated for all samples/standards, and normalized accordingly.

5.7.9 Immunofluorescence of FlavoHb in mammalian cells

For confocal imaging, HEK293 cells were plated onto tissue-culture treated chamber slides (Lab-Tek). Twenty-four hours after plating, cells were washed with PBS x 2 and fixed in paraformaldehyde (4% in PBS) for 15 minutes at room temperature. Cells were washed three times with PBS, and were permeabilized with 0.1% Triton X-100 in PBS and blocked with normal goat serum (10% in PBS) for 1 hour at room temperature. Slides were stained overnight at 4 °C with anti-Flag rabbit pAb (1:100), washed three times with PBS and incubated with Alexafluor-568 conjugate (Invitrogen) at 1:250 dilution for 20 min at room temperature. Cells were washed three times with PBS, were stained with Hoechst 33258 at 5 µg/mL for 10 min at room temperature before washing three times with PBS. Cells were coverslipped using FluorSave mounting media (Calbiochem) and imaged on a Leica SP5 confocal microscope, using sequential scanning technique with a 63x oil immersion objective.

5.7.10 Assessment of nitrosative stress by $^3$H-thymidine uptake and cell counting

For evaluation of $^3$H-thymidine uptake, HEK293 cells were stably infected with either empty lentivirus or FlavoHb and viable cells were plated in equal
numbers in 12-well plates (30,000 cells per well). Cells were treated with: 1) 0, 200, 500 or 1000 µM DETA-NO for 24 hours, or 2) were transfected with pCDH-CMV-iNOS-EF1-copGFP or vector control (pCDH-CMV-MCS-EF1-copGFP) for 12 hours before the media was changed to fresh DMEM + 10% FBS for 48 hours. At this point, cells were incubated for 5 h with 4 µCi of ³H-thymidine (Perkin Elmer), washed once with PBS, fixed for 1 hour in 10% trichloroacetic acid, and incubated overnight in 0.01 M NaOH before assessing radioactivity incorporation of the lysed cellular contents by scintillation counting (Beckman 156000).

For cell counting, HEK293 cells were stably infected with either empty lentivirus or FlavoHb and viable cells were plated in equal numbers in 6-well plates (100,000 cells per well). Cells were treated with 0, 200, 500 or 1000 µM DETA-NO for 48 hours, at which point cells were dissociated by trypsin, resuspended in a defined volume, and were mixed 1:1 with trypan blue for two minutes before viable cells were counted on a hemacytometer.
Figure 36. Flavohemoglobin From *E. Coli* (gene hmpA) Is an Efficient NO-dioxygenase Enzyme. (a) Schematic of the reaction catalyzed by FlavoHb under aerobic conditions. (b) A kinetic comparison of FlavoHb and iNOS. The latter was chosen for comparison because it is the most active NOS isoform (i.e., highest $V_{max}$).
Figure 37. *E. Coli* Flavohemoglobin is Cytoplasmic When Expressed in Mammalian Cells. (a) Confocal immunofluorescence reveals that Flag-FlavoHb exhibits primarily cytoplasmic cellular localization in transfected HEK293 cells. (b) Subcellular fractionation demonstrates that Flag-FlavoHb partitions with cytosolic markers (e.g. GAPDH), and is therefore likely localized to the cytosol.
Figure 38. E. Coli Flavohemoglobin is Catalytically Active when Expressed in Mammalian Cells. (a) HEK293 cells were transfected for 24 h with either empty pCDH or pCDH-Flag-FlavoHb, and cytosolic extracts were analyzed for NADPH consumption via spectrophotometry at 340 nm. Reactions were initiated by adding 5 mM spermine-NONOate. The presence of FlavoHb shifts the balance of NO oxidation from uncatalyzed auto-oxidation (NO$_2^-$) to FlavoHb-catalyzed NO dioxygenation (NO$_3^-$), as measured by the Griess reaction in the media of HEK293 cells transfected -/+ iNOS and FlavoHb (b) and in RAW 264.7 mouse macrophage cells stimulated with lipopolysaccharide (c).
Figure 39. Flavohemoglobin can Protect Mammalian Cells from Nitrosative Stress-Induced Toxicity. HEK293T cells into which either pCDH or pCDH-Flag-FlavoHb was stably integrated were plated in quadruplicate and (a, c) treated with the indicated concentrations of the NO donor DETA-NONOate for 24 hours, or (b) transfected with either pCDH or pCDH-iNOS for 48 hours. Total cellular proliferation was measured by pulsing each well with 2 microcuries/mL of tritiated thymidine for 4 hours. Cells were harvested, and (a, b) tritiated thymidine incorporation was measured by scintillation counting or (c) total viable cell number determined by cell counting using trypan blue. Data are expressed as a percentage of untreated control (i.e. no DETA-NONOate or iNOS exposure). (d) Stable pCDH or pCDH-Flag-FlavoHb expressing HEK293T cells were plated at a density of 500 cells per well and exposed for 14 days to the indicated concentrations of the DETA-NONOate before cells were fixed and stained for visualization with methylene blue. Figure is representative of three independent experiments.
Figure 40. Flavohemoglobin can Modulate Nitric Oxide-Induced Cellular Signaling Events. (a) HEK293 cells were transfected with either pCDH or pCDH-Flag-FlavoHb and were then exposed for 18 h to the indicated concentrations of DETA-NONOate before cell lysates were collected and analyzed by western blotting. (b) RAW264.7 mouse macrophages stably transduced with either pCDH or pCDH-Flag-FlavoHb were stimulated with 500 ng/ml LPS for 18 h before cell lysates were harvested and analyzed by western blotting.
Recent appreciation for heterogeneity within brain tumors has led to the identification and characterization of distinct neoplastic CSC subpopulations. These cells display a panoply of malignant characteristics, including therapeutic resistance, sustained proliferation, resistance to apoptosis, persistent self-renewal, a capacity for migration and invasion, and an ability to potently initiate tumors in transplantation assays. As these pivotal behaviors can be perpetuated by activation of the PI3K/AKT pathway in some cell systems, we investigated whether AKT activity might critically support some of these pivotal CSC-specific behaviors. Indeed, we found that AKT activity was critical to the proliferation, survival, and tumorigenicity of CSCs, while it had a relatively minimal effect against non-stem cancer cells. Though inhibition of AKT has demonstrated reasonable toxicity in pre-clinical and early clinical studies, there have been dose-limiting gastrointestinal toxicities reported from human trials [266]. Considering this and the central role for this pathway in multiple non-neoplastic cell types prompted us to extend our predictive strategy to attempt the identification of other critical molecular determinants for CSC behaviors.

Endogenous NO synthesis can promote malignant characteristics that are also exhibited by CSCs, so we hypothesized that intracellular NO production or availability may maintain critical glioma CSC characteristics. After our original
observation of increased endogenous NO synthesis within CSCs, we employed
heterologous expression of bacterial flavohemoglobin (a novel NO-consuming
approach) to evaluate the functional role of NO availability in CSCs and
determined that it supports CSC proliferation and tumorigenicity. Further
investigation established that the iNOS isoform is specifically expressed within
glioma CSCs relative to non-CSC tumor cells, while there is low expression and
little discernible role for iNOS in non-neoplastic normal neural stem cells. Anti-
iNOS interventions decreased the proliferation and growth of glioma CSCs in
vitro and decreased tumorigenicity in vivo. Systemically-delivered iNOS inhibitors
decreased tumor growth in mice with no associated toxicity against normal
tissues, ultimately suggesting potential for anti-iNOS therapies as non-toxic,
CSC-directed modalities for the treatment of malignant gliomas in humans.

Cancer stem cells are one of many neoplastic subpopulations that exist
within tumors. My thesis research focused on two specific molecules (AKT and
iNOS) critical to the biological phenotypes exhibited by glioma CSCs, and
demonstrated that anti-AKT or anti-iNOS strategies may represent effective anti-
CSC therapies. However, tumors might be better considered as systems of
diverse neoplastic and non-neoplastic cell types; it has been suggested that
tumors are, in fact, a type of “aberrant organ system” [36]. Thus, while anti-CSC
therapies might ultimately become a critical component of therapeutie regimens
for malignant brain tumors, it is perhaps overly reductionist to expect that mono-
therapies targeting CSCs will effectively treat the tumor as a whole. Successful
therapeutic regimens will likely incorporate anti-CSC strategies in addition to approaches that target the tumor bulk and also the tumor microenvironment. My future investigations of anti-AKT and/or –iNOS modalities in the treatment of CSC populations will focus on combining these CSC-specific strategies with other modalities that might potentially provide additive or synergistic therapeutic benefits.
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