Sustained radiosensitization of hypoxic glioma cells after pretreatment with normobaric hyperoxia

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Thesis Abstract

Glioblastoma multiforme (GBM) is the most common and lethal form of brain cancer, with an average patient life expectancy of just 12-15 months beyond diagnosis. These tumors are also highly resistant to chemo- and radiotherapy. Tumor cell resistance to radiotherapy is generally thought to occur due to the low levels of molecular oxygen present in hypoxic tumor regions, which results in reduced DNA damage and enhanced cellular defense mechanisms. Experimental and clinical efforts to counteract tumor hypoxia during radiotherapy are often limited by an attendant increase in the sensitivity of healthy brain tissue to radiation. However, the presence of heightened levels of molecular oxygen during radiotherapy, while conventionally deemed critical for adjuvant oxygen therapy to sensitize hypoxic tumor tissue, might not actually be necessary.

The guiding hypothesis of this thesis is that oxygen pretreatment of hypoxic glioma cells produces a shift in the cellular and molecular underpinnings of hypoxic tumor cell treatment resistance, creating a sustained period of radiation vulnerability in tumor cells, even after these cells have returned to hypoxic conditions. We evaluated this concept using a U87-luciferase xenograft glioma model and *in vitro* models of tumor hypoxia, and were able to test the efficacy of oxygen pretreatment in producing radiosensitization in hypoxic tumor tissue. The results presented herein demonstrate that oxygen-induced radiosensitization of tumor tissue occurs in GBM xenografts, as seen by suppression of tumor growth and increased animal survival. Additionally, rodent and human glioma cells, and human glioma stem cells, exhibit prolonged enhanced vulnerability to radiation after

oxygen pretreatment *in vitro*, even when radiation is delivered under hypoxic conditions. The presence of nuclear HIF-1α protein corresponds closely with cellular resistance to radiotherapy *in vitro* and, interestingly, over-expression of HIF-1α in glioma cells reduces the radiosensitization effects induced by oxygen pretreatment. These results indicate that this sustained oxygen-induced radiosensitization is mediated, in part, via changes in HIF-1-dependent mechanisms. Importantly, this work shows that an identical duration of transient hyperoxic exposure does not sensitize normal human astrocytes to radiation *in vitro*. Taken together, my findings indicate that briefly pre-treating tumors with elevated levels of oxygen prior to radiotherapy may represent a means for selectively targeting radiation-resistant hypoxic cancer cells, and could serve as a safe and effective adjuvant to radiation therapy for patients with GBM.

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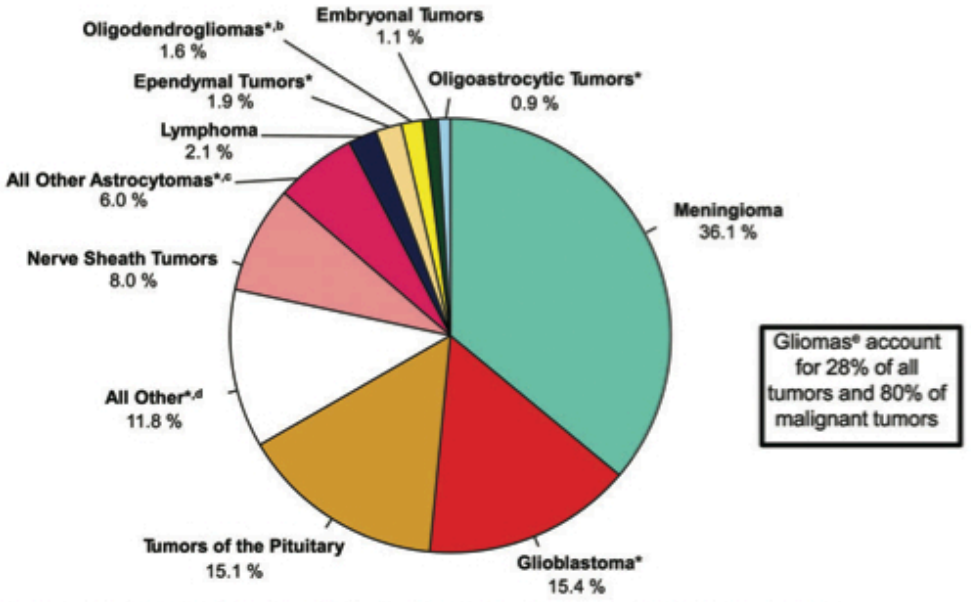
Chapter 1

Introduction

I. Primary tumors of the central nervous system

There are numerous types of tumors of the brain and central nervous system (CNS), which are categorized generally by their location within the CNS. As is the case with all tumors in the body, CNS tumors are classified as primary or secondary depending on their site of origin. Secondary tumors arise from cancers that have metastasized to the CNS from other areas of the body, most often originating as breast, lung, and colon cancers (Barnholtz-Sloan et al., 2004). Primary tumors of the brain and CNS, on the other hand, originate in the CNS and can be further categorized by their cell type of origin, malignancy, and histological characteristics.

Non-malignant meningiomas are the most common type of primary brain tumor occurring in adults, but with surgical resection and follow-up treatment these are typically non-lethal tumors. Of much greater concern clinically are the primary malignant tumors of the CNS, of which primary malignant gliomas are the most common. It is estimated that 70,000 Americans are newly diagnosed with primary brain tumors every year, and more than a third of these patients are diagnosed with primary malignant gliomas. These tumors represent approximately 28% of all primary CNS tumors, but are by far the most common form of CNS malignancy, as they account for 80% of all malignant CNS cancers (Ostrom et al, 2013) (Figure 1-1). Gliomas are broadly defined as tumors that originate from the supporting cells, or glial cells, in the brain. Gliomas can occur almost anywhere in the CNS, but are most often found in the cerebrum, specifically the frontal (23%), temporal (17%) and parietal (11%) lobes (Ostrom et al, 2013). Incidence rates for gliomas are higher in males than in females, and whites are more than twice as likely to develop these



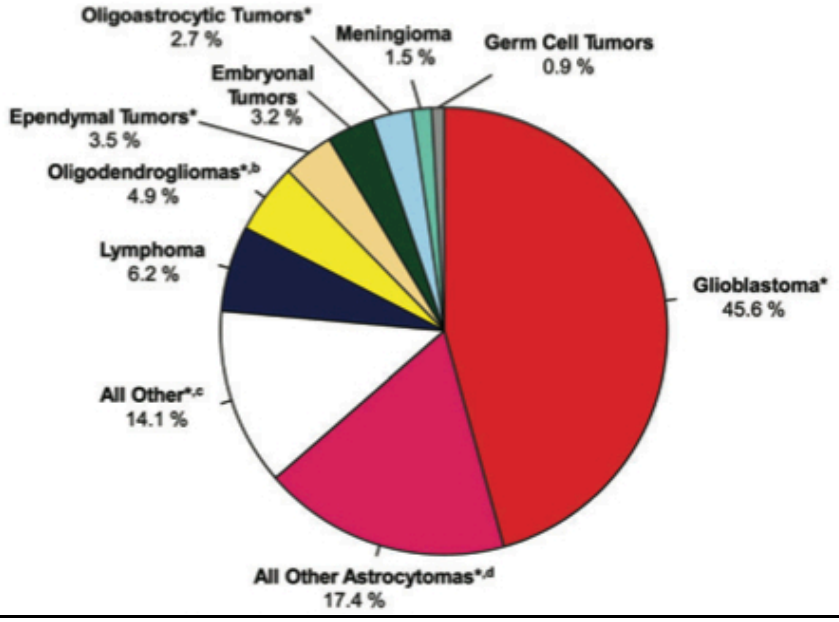


Figure 1-1: Distribution of all Primary Brain and CNS Tumors (A) and Malignant Primary Brain and CNS Tumors (B) by CBTRUS Histology Groupings and Histology (N = 115,799), CBTRUS Statistical Report: NPCR and SEER, 2007–2011. (from Ostrom et al, 2013)

tumors as blacks. And, as is the case with most cancers, the overall incidence rate of gliomas increases with age in the general population.

Among the subsets of glial-derived tumors, astrocytomas, so named because they arise from astrocytes, are the most common type of glioma. The World Health Organization (WHO) uses a four-level system to classify astrocytic gliomas based on clinical diagnosis, patient prognosis, and histopathological characteristics of tumor tissue (Kleihues & Burger, 1993; Louis et al, 2007). WHO Grade I tumors, called pilocytic astrocytomas, are typically benign and patient prognosis is good (10-year survival rate of over 95%) due to the slow rate of tumor growth and a general absence of tumor cell migration into surrounding brain tissue (Burkhard et al, 2003). WHO Grade II, III, and IV astrocytomas are malignant and carry a worsening prognosis with increasing grade. Grade II tumors are also referred to as diffuse astrocytomas. These are relatively slow growing tumors, but are capable of invading healthy brain tissue. Tumor cell invasion can lead to tumor recurrence following resection, and thus Grade II tumors hold the potential to progress to Grades III and/or IV gliomas. Grade III anaplastic astrocytomas are relatively rare when compared with incidence rates of Grade I, II, and IV tumors, and are defined by a number of histological markers that indicate malignancy, including high mitotic index, atypical nuclei, and areas of anaplasia. Anaplastic astrocytomas carry a much worse prognosis for patients in comparison to Grade II tumors, with an average survival time of only 3 years after diagnosis (Ohgaki, 2009). Astrocytic neoplasms that display areas of necrosis and endothelial cell proliferation, in addition to the abnormalities seen in Grade III anaplastic astrocytomas, are classified as Grade IV

astrocytomas, more commonly known as glioblastoma multiforme (GBM). GBM is highly malignant, resistant to even the most current clinical treatment approaches, and universally fatal. Patients with GBM have a median survival time of 12-15 months beyond diagnosis (Louis et al, 2007).

II. Glioblastoma multiforme: incidence and epidemiology

In addition to being the highest grade glioma, GBM is also the most common type of glioma, accounting for 17% of all primary brain tumors and more than half of the approximately 24,000 primary adult brain astrocytomas diagnosed in the United States each year (Ostrom et al, 2013). Incidence rates tend to be higher in more developed, industrialized countries such as those occupying North America, Western Europe and Australia. The overall incidence rate for GBM in the U.S. is 3.19 per 100,000 persons, and as is the case with all gliomas, age, sex, and race play a factor in incidence rates in the general population. While GBM can affect any age or demographic, white males over the age of 65 are at greatest risk for developing this disease (Ohgaki and Kleihues, 2005).

To date, few genetic or environmental risk factors have been identified for developing GBM. However, certain occupations have been linked to an increased overall risk of developing a brain tumor. Notably, exposure to high-dose ionizing radiation, whether for clinical/diagnostic purposes or as a result of living in close proximity to radiation sources, has been associated with an increased risk for development of all brain tumor types, including GBM. Furthermore, a number of studies have examined associations between an increased risk of developing GBM

and exposure to certain chemicals and pesticides, including N-nitrosamines (Ohgaki and Kleihues, 2005). However, any connections that have been made to date have been correlative, at best. More thorough, independent studies are needed in order to properly identify and link environmental factors that significantly increase the risk of developing a brain tumor. The fact remains, for a majority of patients with GBM, that the factors leading to the development of this disease are unknown (Fisher et al, 2006; Ostrom et al, 2013). Interestingly, there have been a number of studies pointing to certain immunological factors that may actually decrease glioma risk. Large group epidemiological studies indicate that individuals with allergic conditions including asthma, eczema, hay fever and food allergies may have a reduced glioma risk of up to 40%. Evidence gathered from these studies indicates that certain genetic polymorphisms that predispose individuals to allergic conditions may also play a role in reducing risk of GBM development (Ostrom, 2013).

III. GBM: clinical presentation, diagnosis, pathology and treatment

The vast majority of GBMs are primary tumors that arise *de novo*, without any evidence of progression from lower grade tumors. Patients often present with non-specific symptoms including headache, blurred vision, seizures, cognitive deficits, personality changes, and focal cranial nerve deficits. If a brain tumor is suspected based on presentation of these symptoms, MRI is most often employed for diagnostic imaging (Omuro and DeAngelis, 2013). Once diagnosed, the standard clinical approach to treatment of GBM initially involves resection of the tumor mass,

provided that the tumor is located in an area of the brain that makes surgical intervention possible. Surgical resection also provides an opportunity for histological characterization of the tumor in cases where diagnosis of tumor grade is in question. GBMs, by definition, are comprised of a heterogenous collection of cancerous cells and other tissues, which can lead a complex histological presentation. In addition to rapid tumor cell proliferation and invasion into healthy brain tissue, histological hallmarks of GBM include cellular atypia, extensive vascularity resulting from high levels of angiogenesis within the tumor mass, and areas of pseudopalisading necrosis. Pseudopalisading tumor cells can be identified as tight bands of hypoxic tumor cells actively migrating away from a central area of necrosis to form a dense ring-like cell structure within the tumor mass.

Even with surgical resection, significant portions of the tumor remain embedded in the brain tissue due to the highly invasive nature of GBM, making follow-up treatment necessary. In order to treat the tumor tissue remaining in the brain following surgical resection, adjuvant radio- and chemotherapies are employed concomitantly, typically involving a large dose (60Gy) of fractionated radiation delivered during a treatment course of the chemotherapeutic DNA alkylating agent temozolomide. Other biologic and chemotherapeutic agents such as bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), and carboplatin are sometimes used as additional frontline or salvage treatments, but usually provide minimal benefit for patients (Omuro and DeAngelis, 2013).

Tumor recurrence in patients with GBM is universal following these treatment courses, and is normally seen within 7-10 months following initial tumor resection (Omuro et al, 2007). For a variety of reasons, including tumor location, diffuse disease recurrence, and patient fragility, a second tumor resection surgery is typically not recommended. At this point, chemotherapy becomes the primary course of treatment for ongoing tumor progression, with limited use of targeted radiotherapy. However, once these tumors do recur, standard clinical approaches with the available treatment modalities typically fail to extend survival time by more than a few months (Omuro and DeAngelis, 2013).

There are numerous reasons why current approaches to the treatment of GBM ultimately fail to provide any substantial survival benefit or improvement in quality of life. As mentioned previously, the highly invasive nature of GBM makes it impossible to remove all of the tumor mass during surgical resection, and the existence of glioma stem cells (GSCs) within these remaining tumor areas contributes to tumor recurrence and patient death. These GSCs possess some of the same properties of normal stem cells, which provides them greater resistance to current cancer treatments, including radiotherapy, and ultimately drives repopulation of the tumor mass (Bao et al, 2006; Kreso and Dick, 2014). Additionally, although high levels of neovascularization occur in these tumors, allowing for the rapid growth of the GBM tissue, many of the blood vessels within the tumor environment are incompetent, leaky, and/or blunted. This means that the delivery of chemotherapeutic agents, which relies on transport through blood in order to access tumor tissue, is impeded, and the efficacy of these drugs is severely

diminished. Lastly, due to the ineffective network of blood vessels, the high metabolic demand of tumor cells, and ultimately the lack of adequate blood supply within the GBM tumor mass, areas of ischemia and necrosis are present throughout the tumor. Tumor cells within and surrounding these areas can be highly resistant to radio- and chemotherapy and can, in fact, take on a more aggressive phenotype when compared with glioma cells located in areas of normal oxygen tension (Vaupel and Harrison, 2004).

IV. Radiation and oxygen: direct and indirect effects on cellular radiosensitization

The lack of adequate blood supply within areas of rapidly growing tumors, such as GBM, provides for a range of hurdles that researchers and clinicians must overcome when considering novel therapeutic approaches to this disease. In the case of chemotherapy, impaired delivery of therapeutic agents to the tumor tissue as a consequence of reduced blood flow results in the inadequate delivery of drug at concentrations necessary to effectively kill tumor cells. Resistance to radiotherapy in ischemic regions of tumors, on the other hand, is generally attributed to reduced concentrations of dissolved molecular oxygen in the tissue and a subsequent reduction in the production of the reactive oxygen species (ROS) created during radiation exposure that are necessary to kill the cell. ROS directly damage multiple cellular elements and are critical for the inhibition of DNA repair (Coleman, 1990; Shrieve, 2006; Stewart, 1989).

DNA is viewed as the primary target molecule for radiation when considering radiotherapeutic outcomes for the treatment of mammalian cells. Single- and double-strand DNA breaks can activate damage response pathways that ultimately lead to the death of the cell (Shrieve, 2004). Tissue oxygenation at the time of radiation exposure has long been implicated in playing a critical role in modifying the effectiveness of radiation to damage mammalian cells (Gray, 1953). Molecular oxygen acts as a powerful oxidizing agent that reacts with the products of radiation, fixing the DNA damage created by photons passing through the cell (Hornsey, 1977). It follows then that the absence of molecular oxygen in tissue, as is seen in tumor areas of severe hypoxia or anoxia, confers onto cells a resistance to damage to cellular components caused by radiation.

In addition to the direct effects of molecular oxygen on cell sensitivity to radiation, there are numerous cellular and molecular changes that occur in response to low oxygen levels that play a major role in determining cancer cell radioresistance. Although there are a number of factors contributing to the indirect effects of oxygen on radiosensitivity, these changes at the cellular and molecular level are mediated largely by the family of transcription factors known as hypoxia inducible factors (HIFs). The best studied of this family of transcription factors, and seemingly the most influential in terms of cancer cell aggression and resistance to standard therapeutics, is hypoxia inducible factor-1 (HIF-1) (Bertout, 2008; Majmundar, 2010).

v. The role of hypoxia inducible factor-1 (HIF-1) in the cellular response to hypoxia and in human cancers

The HIF-1 transcription factor is a heterodimeric complex consisting of a constitutively present beta subunit (HIF-1ß) and an oxygen-regulated alpha subunit (HIF-1α). Both subunits are constitutively expressed in almost all mammalian cells, but the HIF-1α subunit is tagged for proteosomal degradation in the cytoplasm under normoxic conditions. The normoxic regulation of HIF-1α is carried out via modification of an oxygen-dependent degradation domain (ODDD) contained within the protein. Hydroxylation via prolyl-hydroxylases (PHDs) of two proline residues and acetylation of a lysine residue on the ODDD allows for interaction with the Von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex tumor suppressor protein. The pVHL protein puts an ubiquitin tag on HIF-1α, marking it for degradation by the proteasome. Additional post-translational modification, inhibiting the association of HIF-1α with its coactivator CBP/p300, further prevents any transcriptional activity of HIF-1 under normoxic conditions (Ke and Costa, 2006). However, under physiologically hypoxic conditions, HIF-1α is stabilized in the cytoplasm due to the decreased activity of PHDs and the subsequent reduced affinity of the alpha subunit to pVHL. Once stabilized, the alpha subunit accumulates and is free to dimerize with the HIF-1ß subunit, or aryl hydrocarbon nuclear transporter (ARNT), and bind with the CBP/p300 transcriptional coactivator. The dimerized HIF-1 protein can then translocate to the nucleus where it acts to upregulate the transcriptional activity of a number of genes via binding to target gene hypoxia response elements (HREs), or downregulate transcriptional activity of genes indirectly by facilitating the

upregulation of certain transcriptional repressors. Many of the genes regulated by HIF-1, in certain cancer types including GBM, are crucial for tumor cell survival and metastasis (Ke and Costa, 2006; Semenza, 2010).

Among the hundreds of downstream gene targets for HIF-1 are those that regulate cellular metabolism, angiogenesis, apoptosis, cell motility, growth, and proliferation. The target genes for HIF-1 contribute to the normal physiological response necessary for cells and organs to function at reduced oxygen levels and survive moderate hypoxic challenge. These genes include, among many others, vascular endothelial growth factor (VEGF) (for angiogenesis), matrix metalloproteinase 2 (MMP-2) (for cell motility), glucose transporter 1 (GLUT1) and pyruvate dehydrogenase kinase 1 (for glucose uptake and metabolism), and survivin and BNIP3 (for regulation of apoptosis) (Peng et al, 2006; Bernhardt et al, 2007; Mendez et al, 2010; Li et al, 2011). This natural cellular response to low physiological oxygen levels has been the focus of studies in different organ systems for a number of years (Semenza, 2001; Heyman et al, 2011). The adaptations that occur during, and for some period following, hypoxia allow for cells and tissues to survive stressful, and possibly reoccurring, abnormal physiological conditions. The mechanisms underlying this tissue tolerance to hypoxia have been well studied in the brain, heart and other organ systems in the hopes that understanding the phenomenon will allow researchers to identify and harness those molecular changes that are responsible. In doing so, there is the potential for the development of therapeutics that would protect the body from larger ischemic challenges such as heart attack or stroke (Ratan et al, 2004).

However, in many malignant cancers , including GBM, the cellular response to hypoxia, as regulated by HIF-1, is exploited and aids in promoting tumor aggression and metastasis. Hypoxic and ischemic tumor tissue takes advantage of the natural cellular response to low oxygen levels and display traits of tissue tolerance akin to that seen in normal tissue when tumor cells exhibit resistance to larger challenges such as chemo- and radiotherapy. Indeed, many cancer types favor mutations that lead to constitutive over-expression of HIF-1α in tumor cells (Semenza, 2003). Analyses of human solid tumor tissue samples often reveal elevated levels of HIF-1α in cells, even in normally perfused tissue, and upregulation of HIF-1 transcriptional activity is generally associated with increased patient morbidity and mortality (Zhong et al, 1999; Talks et al, 2000; Semenza, 2010). Evidence to further support the role that HIF-1 plays in tumor progression includes studies demonstrating tumor growth inhibition in mouse xenograft tumors via loss of HIF-1α expression or disruption of HIF-1-CBP/p300 transcriptional coactivator interactions (Kung et al, 2000; Semenza, 2002).

Based on the increasing evidence that implicates HIF-1 as a critical player in tumor development and aggression, a number of therapeutic agents have been developed and tested in recent years that target either the HIF-1α subunit, the signal-transduction pathways involving HIF-1α, or HIF-1 transcriptional activity (Ke and Costa, 2006). The goal of these small molecule inhibitors of HIF-1α or the HIF-1 complex is ultimately to disrupt the expression or suppression of downstream gene products that lead to a survival benefit for cancer cells and tumor progression. While a handful of therapeutics have shown promise in preclinical studies, to date

there has been limited success in translating this success into GBM tumor suppression and patient benefit in the clinical realm.

vi. Preclinical and clinical efforts to counteract tumor hypoxia

While the development of small molecule inhibitors of HIF-1 transcriptional activity remains a worthwhile area of study, the well-established link between tumor hypoxia, HIF-1 activity, and tumor resistance to radiation therapy has prompted a number of experimental and clinical efforts directed at correcting the imbalance observed in therapeutic outcomes of hypoxic versus normoxic tissue. Included in these efforts are normobaric and hyperbaric oxygen treatments, oxygen mimetics, hypoxic cell radiosensitizers, and vascular normalization (Chapman 1979, Fulton, Urtasun et al. 1984, Kohshi, Kinoshita et al. 1999, Kaanders, Bussink et al. 2002, McGee, Hamner et al. 2010, Overgaard 2011). The general focus of these efforts has been to either counteract or exploit hypoxic regions in fast growing tumors such as GBM in an effort to improve therapeutic efficacy.

The goal of using normobaric (NBO) or hyperbaric (HBO) oxygen treatments as adjuncts to radiotherapy for tumors is to force higher concentrations of oxygen into the blood and, consequently, the tumor tissue via the tumor vasculature. Increasing FiO_2, in the case of NBO, and increasing atmospheric pressure and oxygen, in the case of HBO, allows for elevated concentrations of arterial blood oxygen, which subsequently creates a greater oxygen concentration gradient between the blood and tissues in the body, including hypoxic tumor tissue. In this manner, tumor cells that previously had been at too great a distance from

neighboring blood vessels to receive adequate oxygen supply now see higher oxygen concentrations due to the increased concentration gradient. Previously-hypoxic tissue can then be treated with radiation in the presence of elevated molecular oxygen, which enhances the efficacy of radiotherapy via mechanisms previously discussed. Similarly, the use of hemoglobin- and chemical-based oxygen transport agents, originally developed as blood alternatives for surgery and trauma cases, have been tested in tumor patients as another method of enhancing tissue oxygen levels and hypoxic tumor cell toxicity during radiotherapy. Oxygen-mimetic radiosensitizers, which include the drugs metronidazole, misonidazole, nimorazole, and piminidazole, can selectively sensitize hypoxic glioma cells to radiation. Although less effective than oxygen as a radiosensitizer, these nitroimidazoles provide a means by which radiotherapy may more effectively target hypoxic tumor tissue. Unfortunately, the doses required for adequate radiosensitization of hypoxic tumor tissue, and long half-life of these dugs, means there is a high risk of toxic concentrations of nitroimidazole compunds building up in the patient's system, severely limiting therapeutic efficacy (Rockwell et al, 2009).

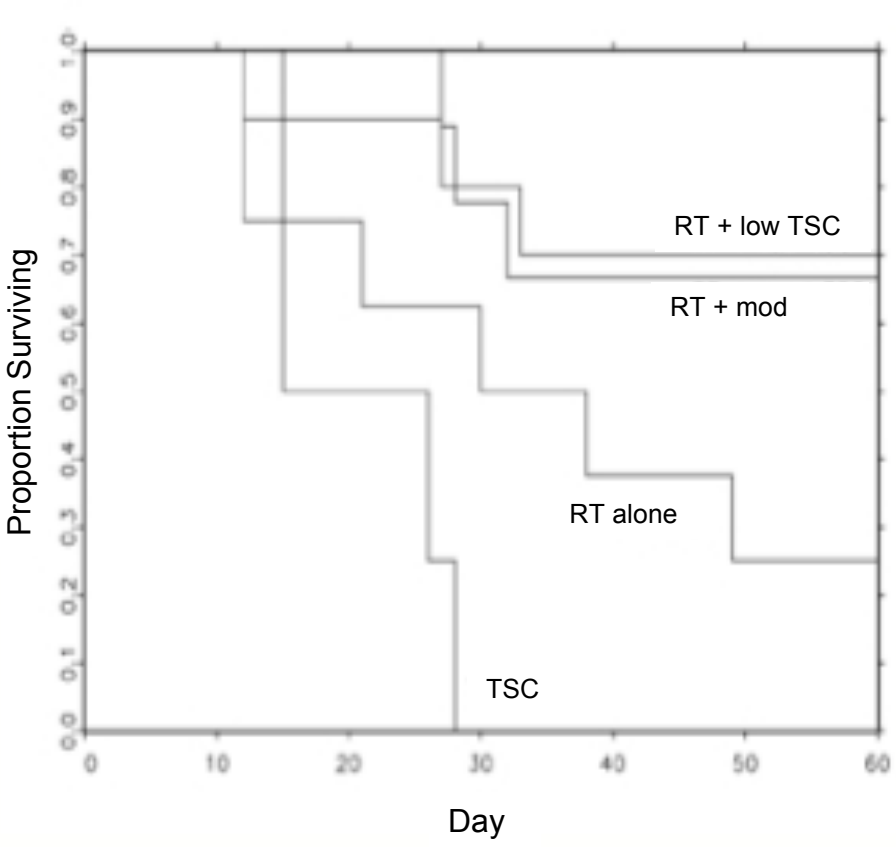
Indeed, one of the serious drawbacks to many of these experimental treatment approaches lies in the increased risk of complications, due to drug toxicity and/or the non-selective nature of the treatments. While these therapeutic strategies can augment the vulnerability of glioma tissue to radiation, they can also generate unintended peripheral tissue toxicity and radiosensitize healthy peritumoral brain tissue, creating a higher risk environment for healthy, non-target cells. Modern clinical imaging and radiotherapeutic approaches continue to evolve,

allowing for a more targeted delivery of radiation to the bulk of the tumor volume in GBM (Grosu, Piert et al, 2005; Grosu, Weber et al, 2005; Whitfield et al, 2014), and this has substantially reduced the risk of collateral tissue damage. Nonetheless, the highly invasive nature of glioma cells means that damage to peritumoral brain tissue during treatment remains a valid concern.

vii. Trans-sodium crocetinate, metabolic reflow, and evidence for oxygen-induced radiosensitization

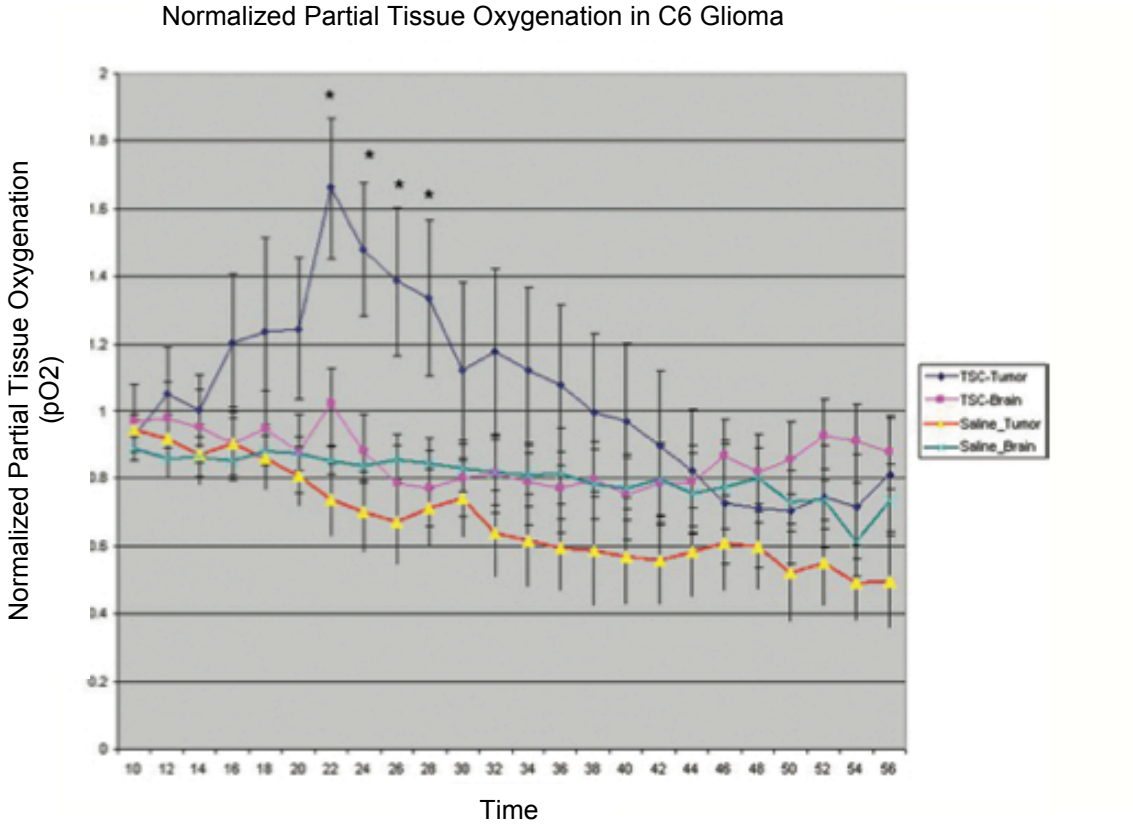
A recent study by Sheehan et al (2008) employed a novel method of enhanced oxygen delivery to hypoxic areas of GBM tissue through the use of a carotenoid compound known as trans-sodium crocetinate (TSC). By altering the aqueous structure of the blood plasma and allowing for small molecules such as oxygen and glucose to diffuse more readily from the blood into surrounding tissue, TSC provides what is termed "metabolic reflow" to areas of ischemia. TSC has been shown to be effective at providing metabolic reflow and increasing tissue oxygenation in a number of organ systems, including the liver, kidney and brain (Okonkwo et al, 2003; Stennett et al, 2007; Manabe et al, 2010). Sheehan et al applied this novel method of enhanced oxygen delivery to a rat model of GBM in an effort to increase the efficacy of radiation treatment of brain tumor tissue. The rationale behind these experiments was that increasing levels of molecular oxygen in hypoxic glioma tissue during radiation therapy would allow for greater free radical production and thusly greater cell death in tumor areas that were previously resistant to radiation treatment. Indeed, when TSC was used as an adjunct to

radiation therapy in this animal model of GBM, a reduction in tumor growth and subsequent increase in survival was observed (Figure 1-2). These studies provide evidence for the use of a novel method of selective oxygen delivery in counteracting tumor hypoxia and increasing efficacy of radiation therapy in GBM.



<u>Figure 1-2</u>: TSC treatment as an adjunct to radiotherapy for glioma improves animal survival. Line graph of the Kaplan-Meier survival plot illustrating proportion of surviving animals per group as a function of days after tumor implantation. (from Sheehan et al, 2008)

Subsequent work by Sheehan et al (2009) verified successful enhancement of delivery of oxygen to hypoxic brain tumor regions using a Licox oxygen probe to measure tissue oxygen levels. Results of this work showed that a bolus injection of TSC created a delayed selective reoxygenation of the hypoxic tumor tissue that peaked around 22 minutes post-injection. This reoxygenation was transient, with tumor tissue returning to baseline hypoxic conditions approximately 45 minutes post-injection (Figure 1-3). It would be expected, based on the original hypothesis of Sheehan's study, that radiation treatment of tumor tissue would be most effective when the maximum amount of molecular oxygen was present, and that the optimal time point at which to irradiate these tumors would be 20-25 minutes post-injection of TSC. However, upon further scrutiny of the timeline of the initial study, it was recognized that, in one of the protocols tested, radiation was administered to the animals 45 minutes post-injection, i.e. after the tumor tissue had returned to baseline hypoxic conditions. Therefore, the vulnerability of the tumor tissue to radiation, evidenced by a reduction in tumor size and increase in survival of the animals, could not be explained by the presence of elevated molecular oxygen. This finding was remarkable because the therapeutic influence of increasing oxygen levels in hypoxic tumor tissue was not dependent on the presence of elevated oxygen during radiotherapy. This suggested that either TSC has additional mechanisms of action beyond enhancing the delivery of oxygen, or that pre-treating hypoxic tumor cells with oxygen was effective in improving radiotherapeutic management of tumors. Either scenario holds potential clinical relevance because the increased risk to eloquent brain tissue during radiotherapy has been removed.



<u>Figure 1-3</u>: TSC treatment enhances hypoxic brain tumor oxygenation. Graph showing normalized partial tissue oxygenation following administration of TSC in a rat model of C6 glioma. The TSC was administered 20 minutes after the start of Licox monitoring. Statistically significant differences (denoted by asterisks) were noted at 22-, 24-, 26, and 28-minute time points in the tumor tissue of the animals receiving TSC. (from Sheehan et al, 2009)

Prior strategies to elevate molecular oxygen in hypoxic tumor tissue have been non-selective, and ultimately place healthy tissue at increased risk of radiation damage. It is therefore important to develop methods of selectively sensitizing glioma cells during radiation therapy in order to better slow tumor progression and limit collateral damage to brain tissue.

viii. Rationale for research and experimental design

The established link between low molecular oxygen in hypoxic regions of fast-growing solid tumors such as GBM, and the aggression and treatment resistance of tumor cells within these hypoxic environments, drew the focus of my research upon investigating the role of oxygen in tumor pretreatment-induced vulnerability. Inasmuch as the presence of elevated molecular oxygen in glioma tissue at the time of radiation does not explain the increased tumor cell vulnerability, it is reasonable to assume that some underlying cellular or molecular modifications provide the basis for this shift in therapeutic tolerance. It was thus hypothesized that molecular oxygen, and not TSC, was the agent responsible for directly modifying the vulnerability of hypoxic glioma cells. I postulated that pretreatment with transiently elevated oxygen creates a window of vulnerability in hypoxic tumor tissue that renders glioma cells more susceptible to subsequent radiotherapeutic challenge. This can be differentiated from oxygen-based sensitization because of the fact that oxygen is at very low levels during this extended period of vulnerability, and can therefore not be acting directly as the primary radiosensitizing agent. The enhanced radiosensitivity, therefore, must result from a change in cellular defense

mechanisms that are normally present in these tumor cells under sustained hypoxic conditions. Based on the critical role that the transcription factor HIF-1 is known to play in GBM progression and treatment resistance, I evaluated HIF-1α expression levels at multiple time points following hypoxic conditions and reoxygenation exposures in several different glioma cell lines, in an effort to begin to define the molecular mechanisms behind this enhanced radiosensitivity.

The possible clinical relevance of this oxygen-induced vulnerability phenomenon in hypoxic glioma cells is that it holds the potential to be effective by selectively enhancing the vulnerability of treatment-resistant tumor cells to radiation, while not placing non-target, healthy brain tissue at an increased risk of treatment damage. In fact, based on evidence from previous work on the effects of HBO and NBO on the brain, it is conceivable that oxygen preconditioning could actually lower the vulnerability of healthy tissue (Peng et al, 2008; Liu et al, 2012). A key assumption of these vulnerability shifts is that the phenomenon of oxygen-pretreatment-induced radiosensitivity resides in the glioma cells themselves. I tested this assumption by designing and utilizing *in vitro* models of tumor hypoxia, in which the hypoxic environment of the glioma cells can be manipulated in isolation from the rest of the tumor microenvironment.

The broader goal of this thesis work is to characterize and evaluate oxygen pretreatment of hypoxic tumor tissue, in conjunction with radiotherapy, as a potential therapeutic tool for treatment of GBM. It is also anticipated that, by developing an understanding of the molecular underpinnings of the tumor cell response to hypoxia and reoxygenation, we may be able to identify novel

therapeutic strategies that will help to improve treatment outcomes for this disease. Thus, the studies presented herein provide, to my knowledge, the first evidence of using oxygen pretreatment as a therapeutic adjunct to radiation as a means to sensitize treatment resistant tumor cells. This work also begins to outline the molecular mechanisms behind this phenomenon, defining a role for the transcription factor subunit HIF-1α in the sustained radiosensitivity of hypoxic tumor cells following oxygen pretreatment.

Chapter 2

Effects of oxygen pretreatment on radiosensitization of hypoxic glioma cells and mechanisms of vulnerability

Abstract

Glioblastoma multiforme (GBM) is the most common and lethal form of brain cancer and these tumors are highly resistant to chemo- and radiotherapy. Radioresistance is thought to result from a paucity of molecular oxygen in hypoxic tumor regions, resulting in reduced DNA damage and enhanced cellular defense mechanisms. Efforts to counteract tumor hypoxia during radiotherapy are limited by an attendant increase in the sensitivity of healthy brain tissue to radiation. However, the presence of heightened levels of molecular oxygen during radiotherapy, while conventionally deemed critical for adjuvant oxygen therapy to sensitize hypoxic tumor tissue, might not actually be necessary. We evaluated the concept that pre-treating tumor tissue by transiently elevating tissue oxygenation prior to radiation exposure could increase the efficacy of radiotherapy, even when radiotherapy is administered after the return of tumor tissue oxygen to hypoxic baseline levels. Using nude mice bearing intracranial U87-luciferase xenografts, and *in vitro* models of tumor hypoxia, the efficacy of oxygen pretreatment for producing radiosensitization was tested. Oxygen-induced radiosensitization of tumor tissue was observed in GBM xenografts, as seen by suppression of tumor growth and increased survival. Additionally, rodent and human glioma cells, and human glioma stem cells, exhibited prolonged enhanced vulnerability to radiation after oxygen pretreatment *in vitro*, even when radiation was delivered under hypoxic conditions. Over-expression of HIF-1α reduced this radiosensitization, indicating that this effect is mediated, in part, via a change in HIF-1-dependent mechanisms. Importantly, an identical duration of transient hyperoxic exposure does not sensitize normal human

astrocytes to radiation *in vitro*. Taken together, these results indicate that briefly pre-treating tumors with elevated levels of oxygen prior to radiotherapy may represent a means for selectively targeting radiation-resistant hypoxic cancer cells, and could serve as a safe and effective adjuvant to radiation therapy for patients with GBM.

Introduction

Primary malignant gliomas are the most common type of brain cancer in adults, with an estimated 23,000 people newly diagnosed each year in the U.S. (Ostrom, Gittleman et al. 2013). Glioblastoma multiforme (GBM) - the most common and deadly form of these tumors - remains an incurable disease, and even with the most aggressive treatment protocols available the average life expectancy for patients diagnosed with GBM is 12-15 months (Kanu et al, 2009). The World Health Organization (WHO) classifies GBM as a Grade IV primary brain tumor, characterized by its rapid cell proliferation, cellular atypia, angiogenesis, and aggressive invasion of tumor cells into healthy brain tissue (Louis et al, 2007). The GBM tumor mass also displays areas of ischemia and necrosis. The rapid rate of tumor growth can outstrip neovascularization, creating diffusion distances too great to provide sufficient blood flow, and poorly orchestrated angiogenesis can lead to incompetent, leaky, and blunted blood vessels (Rampling et al, 1994; Lakka et al, 2004; Kaur et al; 2005). Tumor hypoxia in GBM is an important factor in tumor aggression and progression, but also represents a significant impediment for the

success of chemo- and radiotherapies (Knisely and Rockwell, 2002; Evans et al, 2004).

Resistance to radiotherapy in ischemic regions of tumors is generally attributed to reduced concentrations of dissolved molecular oxygen in the tissue and a subsequent reduction in the production of reactive oxygen species (ROS) during radiation exposure. ROS directly damage multiple cellular elements and are critical for the inhibition of DNA repair (Stewart, 1989; Coleman and Turrisi, 1990; Shrieve, 2006). Additionally, many cellular and molecular changes occur in response to decreased oxygen levels, mediated largely by the transcription factor hypoxia inducible factor-1 (HIF-1) (Majmundar et al, 2010). Among the hundreds of gene targets for HIF-1 are those that regulate cellular metabolism, angiogenesis, apoptosis, cell motility, growth and proliferation, all of which may provide cancer cells with a treatment-resistant and aggressive phenotype during periods of hypoxic stress (Zagzag et al, 2000; Dery et al, 2005,; Kaur et al, 2005; Bernhardt et al, 2007; Bertout et al, 2008; Benita et al, 2009).

The well-established link between tumor hypoxia, HIF-1 expression and tumor resistance to radiation therapy has prompted a number of experimental and clinical efforts to either counteract or exploit hypoxic regions in fast growing tumors such as GBM. Included in these efforts are normobaric and hyperbaric oxygen treatments, oxygen mimetics, hypoxic cell radiosensitizers, and vascular normalization (Chapman, 1979; Fulton et al, 1984; Kohshi et al, 1999; Kaanders et al, 2002; McGee et al, 2010; Overgaard, 2011). However, a serious drawback to many of these experimental approaches lies in the non-selective nature of the

treatments. While these strategies can increase the vulnerability of glioma tissue to radiation, they can also radiosensitize healthy peritumoral brain tissue, creating a higher risk to healthy, non-target cells. Although modern clinical imaging and radiotherapeutic approaches continue to evolve, allowing for a more targeted delivery of radiation to the bulk of the tumor volume in GBM (Grosu, Piert et al, 2005; Grosu, Weber et al, 2005; Whitfield et al, 2014), the highly invasive nature of glioma cells means that damage to peritumoral brain tissue during treatment remains a valid concern. It is therefore important to develop methods of selectively sensitizing glioma cells during radiation therapy in order to better slow tumor progression and limit collateral damage to healthy brain tissue.

In the present study, we investigated a means by which hypoxic glioma cells and human glioma stem cells (GSCs) can be sensitized to ionizing radiation following transient exposure to normoxic conditions. Using nude mice bearing orthotopic U87-luciferase xenografts, and *in vitro* models of tumor hypoxia, our findings indicate that briefly exposing hypoxic cancer cells to normoxic or near-normoxic levels of oxygen, and then returning these cells to baseline hypoxic conditions, provides a window of vulnerability to ionizing radiation in the absence of elevated levels of molecular oxygen. This enhanced glioma cell vulnerability appears to be mediated, at least in part, by a change in the activity of HIF-1α. Notably, exposing normoxic human astrocytes to transient hyperoxia *in vitro* before radiation exposure does not sensitize these cells to treatment, further indicating the selective vulnerability of hypoxic tumor cells afforded by this type of oxygen therapy.

Methods

Ethics statement. Primary human GBM cultures enriched for glioma stem cells (GSCs) termed 0308 were initially obtained from surgical resections from GBM patients ("Following informed consent, human tumor samples classified as GBM based on World Health Organization (WHO) criteria were obtained from patients undergoing surgical treatment at the National Institutes of Health in accordance with the appropriate Institutional Review Boards.") as previously described (Lee et al, 2006). The University of Virginia Institutional Animal Care and Use Committee (IACUC) approved all procedures involving animals in this study (protocol #3819). All surgeries were carried out under ketamine/dexmedetomidine, and all efforts were made to minimize suffering.

Cell culture. The U87 cell line (American Type Culture Collection) and a U87 cell line genetically engineered to expresses the firefly luciferase gene (U87-luc) (a generous gift from Hong Zhong at Parabon Nanolabs) were maintained in Dulbecco's minimal essential medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The GL261 cell line (NCI, Frederick Repositories) was maintained in RPMI medium (Life Technologies) supplemented with 10% FBS and 1% penicillin-streptomycin. The previously validated 0308 glioma stem cell line [27] was obtained from Dr. Jeongwu Lee and was maintained in non-treated culture flasks in Neurobasal media supplemented with 1% B27, 0.5% N2, 0.01% FGF, 0.1% EGF, 0.3% L-Glutamine, and 1% penicillin-streptomycin. The normal human astrocyte cell line (National Institutes of Health) was maintained in

F12/MEM supplemented with 10% FBS and 1% penicillin-streptomycin. All cell lines were maintained at 37˚C in a humidified incubator containing 5% CO_2 and 21% O_2.

Human tumor xenografts in nude mice. All experiments with animals were performed in accordance with protocols approved by the University of Virginia Institutional Animal Care and Use Committee (IACUC). 14-16 week old nude female mice (National Cancer Institute) were anesthetized with ketamine (50mg/kg) and dexmedetomidine (0.5mg/kg) administered i.p. and placed in a stereotactic frame. A midline scalp incision was made and a burr hole was drilled in the right-side of the skull 2.3 mm lateral to the midline and 0.1 mm posterior to the bregma. A 10µl Hamilton syringe, containing 250,000 U87-luc cells in 4µl, was positioned in the brain at a depth of 2.35mm from the cortical surface and cells were injected into the striatum at a rate of 0.3µl/min. After cell injection, the syringe was slowly removed, the burr hole was sealed with bone wax, and the scalp was sutured together. Post-operatively, animals were administered s.c. buprenorphine (0.1mg/kg) and antisedan. Tumors were allowed to grow for 14 days, at which time imaging and treatments were initiated.

The nude-U87 glioma model is a well-established, well-characterized orthotopic xenograft model (Candolfi et al, 2007; Radaelli et al, 2009; Seshadri and Ciesielski, 2009). The benefits of this model include the fact that the U87 cell line is a well-defined, widely-recognized human glioma cell line that exhibits certain tumor characteristics in nude mouse glioma models that mimic glioma cell behavior in

patient tumors, particularly when pertaining to the tumor vasculature (Seshadri and Ciesielski, 2009). While this tumor model displays less infiltrative growth properties and greater tumor encapsulation than certain other established glioma cell lines, the more predictable tumor vascularity that accompanies the U87 model was well suited for the current studies.

Tissue oxygen measurements. Tumor-bearing animals (n=3) at day 14 post-tumor implant (PTI) were sedated using a mixture of ketamine (30mg/kg) and dexmedetomidine (0.3mg/kg) and placed in a stereotactic frame. For tumor tissue monitoring, the same burr hole drilled for tumor implantation was used to position a Licox oxygen probe (Integra Neurosciences) at a depth of 2.35mm from the cortical surface - the same coordinates at which glioma cells were injected. For monitoring of healthy striatal brain tissue in the same animal, a new burr hole was drilled in the contralateral skull 2.3 mm lateral to the midline and 0.1 mm posterior to the bregma, and the oxygen probe was positioned at a depth of 2.35mm from the cortical surface. The same oxygen probe was used in a given animal for monitoring the contralateral brain and the tumor. Tissue pO_2 (tpO_2) readings were taken every minute for 20 minutes under ambient air (21% O_2) respiration in order to establish a baseline. Fraction of inspired oxygen (FiO_2) was then increased to 100% O_2 for 25 minutes, after which it was returned to 21% O_2 for the remainder of the observation period. All recorded tpO_2 values were converted to a percentage of the baseline value obtained from the normal neural tissue in the contralateral brain. Animals

used for tissue oxygen monitoring were not included in the subsequent survival study.

Hyperoxic treatment and in vivo irradiation. Based on *in vivo* tumor measurements (described below), animals implanted with U87-luc cells were assigned to experimental groups on day 14 PTI in order to achieve an equivalent distribution of tumor sizes among groups. On the day of radiation treatment (day 14 PTI), tumor-bearing animals were sedated using a mixture of ketamine (30mg/kg) and dexmedetomidine (0.3mg/kg). Animals receiving 100% FiO_2 pretreatment were placed in a modified gas mask apparatus that covered the entire snout of the animal and administered 100% oxygen for 25 minutes. Oxygen treated animals were administered a whole-head dose of 8Gy radiation at a rate of 3.3Gy/min starting 25 minutes after the cessation of hyperoxic pretreatment. The 8Gy dose selected for the *in vivo* experiments represents a slightly lower dosage compared with similar work using radiation treatment in the same animal model (Lund et al, 2000; Hadjipanayis et al, 2008). We selected a dosage in the lower range in order to assess the postulated enhanced sensitivity to radiation produced by oxygen pretreatment. Animals that did not receive hyperoxic pretreatment instead breathed ambient air, and the same sedation and irradiation procedures were used. Radiation was administered using a SARRP research platform (Xstrahl Life Sciences) with a lead body shield. The *in vivo* radiation treatment study was done in two stages. Stage 1 was a preliminary study of 8 animals per group. Data from stage 1 were then used to

estimate the number of animals needed to have 80% power under different assumptions about effect sizes consistent with the stage 1 data.

In vivo tumor measurements. Orthotopic U87-luc tumors were visualized using an In Vivo Imaging System (IVIS; Xenogen Corp.) at day 14, 18, 22, 27, 31, 36, 41, and 46 PTI, or up until day of death. Twelve minutes prior to imaging, mice were given an i.p. injection of D-luciferin (Gold Biotechnology) (150 mg/kg). Five minutes prior to imaging, mice were initially anesthetized by placement in a chamber containing 5% Isofluorane, and anesthesia was maintained at 1-1.5% Isofluorane inside the IVIS to obviate movement during imaging. The light emitted from the luciferase-expressing U87 tumor cells of the nude mice was detected with the IVIS camera. Quantification and normalization of light intensity and photon counts were performed using Living Image software (Xenogen Corp.).

Determination of Humane Endpoints. Tumor-bearing mice were housed in a barrier vivarium facility and evaluated twice daily for signs of deteriorating physical and behavioral health with the assistance of veterinary technicians. Humane endpoints were determined according to a clinical scoring system based on that outlined in the University of Virginia Guidelines and Policy for Determination of Humane Endpoints for Vertebrate Animals Used in Research. Animals were checked for their health during their entire survival period using a scoring scale consisting of 4 categories: Body Weight Changes, General Activity, Eyes and Nose Discharge, and Posture. Total scores of 7 or greater, or maximum scores in at least 2 categories warranted

immediate euthanasia. Any animal with a weight loss of 20% or more was euthanized. Individual records were maintained for the life span of each animal. When criteria were met for euthanasia, mice were anesthetized with ketamine (50mg/kg) and dexmedetomidine (0.5mg/kg) and perfused intracardially, first with PBS and then with a 4% paraformaldehyde solution. Brains were saved for further analysis.

In vitro hypoxic/hyperoxic exposure and radiation treatment. Cells were subjected to varying levels of oxygen in a Galaxy 14S incubator with oxygen control (New Brunswick Scientific). For hypoxic conditions, the chamber was charged with N_2 and CO_2 to produce gas concentrations of 1-5%% O_2 and 5% CO_2 at 37˚C during graded chronic hypoxia (GCH) or rapid acute hypoxia (RAH). The GCH and RAH protocols are described in greater detail in the Results section. For transient reoxygenation, cells were transferred to another incubator containing 5% CO_2 and 21% O_2 for 25 minutes at 37˚C. Return to 1% O_2 for radiation exposure was achieved by placing cells in a modular incubator chamber (Billups-Rothenberg) and flushing with a 95% N_2/5% CO_2 gas mixture for 4 minutes at 5psi. For hyperoxic conditions, cells were placed in a Galaxy 14S incubator and the chamber was charged with a 95%O_2/5%CO_2 gas mixture until oxygen levels within the incubator reached 50%. Cells were maintained at this oxygen level for 25 minutes and then returned to an incubator containing 5% CO_2 and 21% O_2 for 25 minutes at 37˚C prior to radiation. For radiation treatment, all cells were γ-irradiated at ~80% confluency using a single 5Gy dose administered at 2.2Gy/min in a Mark I irradiator (J. L. Shepherd).

This radiation dose was chosen based on previously established dosing ranges in studies using similar glioma cell lines (Mukherjee et al, 2009; Hsieh et al, 2010; Strofer et al, 2011).

Colony formation assays. Anchorage-independent growth for the U87, U87-luc, GL261 and 0308 cell lines was assayed by the ability to form colonies in soft agar. Bottom layer agar was prepared in the appropriate medium for the cell line being assayed plus 0.6% Seakem GTG agarose (Lonza) in 6-well tissue culture plates. 3000-6000 cells (depending on cell type) were resuspended in appropriate medium containing 0.3% agar and added to each well to create the top soft agar layer. Cells were then incubated at 37°C for 3-4 weeks to allow for growth of colonies. Colonies were subsequently washed with PBS, fixed for 30min with a 4% PFA solution, and visualized using an inverted light microscope. Anchorage-dependent growth of normal human astrocytes was assayed by the ability to form colonies on a tissue cultured treated 6-well plate. 500 cells were resuspended in appropriate base medium, added to each well, and incubated at 37°C for 7 days to allow for growth of colonies. Colonies were subsequently washed with PBS and then fixed/stained with a 4% PFA/0.05% crystal violet solution prior to visualization by microscopy. All experiments were repeated using at least three independent cell samples plated in triplicate. Statistical analyses of clonogenic survival results for all cell lines and conditions were conducted on raw data values, but are displayed as a percentage of the corresponding negative control group to allow for ease of comparison among cell lines.

Plasmid transfection. U87 cells were transiently transfected with either an empty vector (pcDNA3) or a HA-tagged HIF-1α coding construct (HA-HIF1α-pcDNA3) (Addgene, plasmid 18949) using the FuGENE6 transfection reagent (Promega) according to the manufacturer's protocol. Transfections were timed to allow for maximal transcriptional activity during *in vitro* reoxygenation and radiation treatments.

Western blotting. Cells were harvested at the appropriate times to match *in vitro* radiation treatment time points. Nuclear lysates were prepared in buffer containing 20mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.4M KCl, 1.5mM $MgCl_2$, 0.2M EDTA, supplemented with 10mM Na_3VO_4, 5mM NaFl, 5mM DTT, 0.2mM PMSF, and Complete Protease Inhibitor Cocktail (Santa Cruz). Nuclear protein was obtained from cells after centrifugation at 25,000 x g for 30 min at 4˚C. Protein concentrations were measured using Coomassie Plus Reagent (Thermo Scientific). Nuclear lysates were run on a 10% SDS-PAGE gel and electrophoretically transferred to a PVDF Immobilon membrane (Millipore). Blots were then blocked for 1hr in blocking buffer (5% milk, 5% BSA, 2.5% normal goat serum, 0.05% Tween20, 0.02% sodium azide in TBS) and probed for nuclear proteins of interest using rabbit primary antibodies against HIF-1α (Novus Biologicals, 1:4000) and lamin A/C (Cell Signaling Technologies, 1:500) in 0.1x blocking buffer overnight at 4˚C. After primary antibody incubation, blots were washed 4x10min in TBS-Tween20 and incubated with HRP-conjugated goat anti-rabbit secondary antibody (Vector Labs, 1:25,000) in

0.1x blocking buffer at room temperature for two hours. Blots were then washed 4x10min in TBS-Tween20 and bands were detected using SuperSignal West Dura ECL substrate (Thermo Scientific) according to the manufacturer's protocol. Blots were imaged with a Chemidoc XRS+ Imaging System (Bio-Rad). Image analysis was carried out using Image Lab software (Bio-Rad).

Brain Tissue Immunohistochemistry. Two groups of tumor-bearing animals (n=4 per group) were assessed for radiation-induced apoptosis using caspase-3 immunohistochemistry. Both groups received a single dose of whole head 8Gy radiation on Day 14 post-implantation. Prior to irradiation, one group was pretreated with 100% O_2, while the other continued to breathe 21% O_2. 24 hours post-irradation, mice were perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were then removed, post-fixed in paraformaldehyde, and then cryo-protected in 30% sucrose solution prior to freeezing. Coronal sections were cut at a thickness of 30μm using a cryostat and floated in PBS with 0.02% sodium azide. Slices were washed 3 x 10 minutes in 0.1M phosphate buffer (PB) and then blocked for 2 hours in PB-based blocking buffer containing 0.05% bovine albumin, 5% normal goat serum, 0.3% triton X-100, and 0.02% sodium azide. Blocked slices were then incubated with rabbit monoclonal anti-cleaved-caspase-3 antibody (1:400, Cell Signaling Technologies, #9664) at 4˚C O/N. The next day, slices were washed 4 x 10 minutes in 0.1M PB and incubated in goat anti-rabbit Alexafluor 488 secondary antibody (1:500, Life Technologies, #A-110008) in blocking buffer. Slices were washed 4 x 10 minutes in 0.1M PB,

counterstained with diaminobenzidine (DAB), again washed 4 x 10 minutes, then slide mounted, and cover slipped with Prolong Gold anti-fade mounting medium (Life Technologies, #P36934). Fluorescence imaging was done on a Leica inverted microscope (DMI-6000B) using Metamorph imaging software (Molecular Devices).

Cell Culture Immunohistochemistry. U87 glioma cells and normal human astrocytes were assessed for HIF-1α immunoreactivity after hyperoxic treatment. Cells were grown in monolayers on poly-L-lysine-treated coverslips and maintained in culture media, as described earlier, until ~80% confluency was reached. One group of each type of cells was then exposed to 50% O_2 for 25 minutes before being returned to normoxic conditions. Cells were fixed immediately after hyperoxic exposure or 25 minutes after returning to normoxia. Negative control cells were maintained under constant normoxic conditions prior to fixation, while positive control cells were exposed to 1% O_2 conditions for 1 hour before fixation. All cells were fixed by first washing 3x 1 minute in PBS and then 4% paraformaldehyde for 20 minutes. Cells were blocked for 2 hours in PB-based blocking buffer containing 0.05% bovine albumin, 5% normal goat serum, 0.3% triton X-100, and 0.02% sodium azide, and then incubated with rabbit monoclonal anti-HIF-1α antibody (1:200, Novus Biologicals, NB100-479) at 4˚C overnight. The following day, cells were washed 4 x 10 minutes in 0.1M PB and then incubated in goat anti-rabbit Alexafluor 488 secondary antibody (Life Technologies, #A-110008) 1:1000 in blocking buffer. Next, cells were washed 4 x 10 minutes in 0.1M PB, counterstained with diaminobenzidine (DAB), again washed 4 x 10 minutes in 0.1M PB, slide mounted,

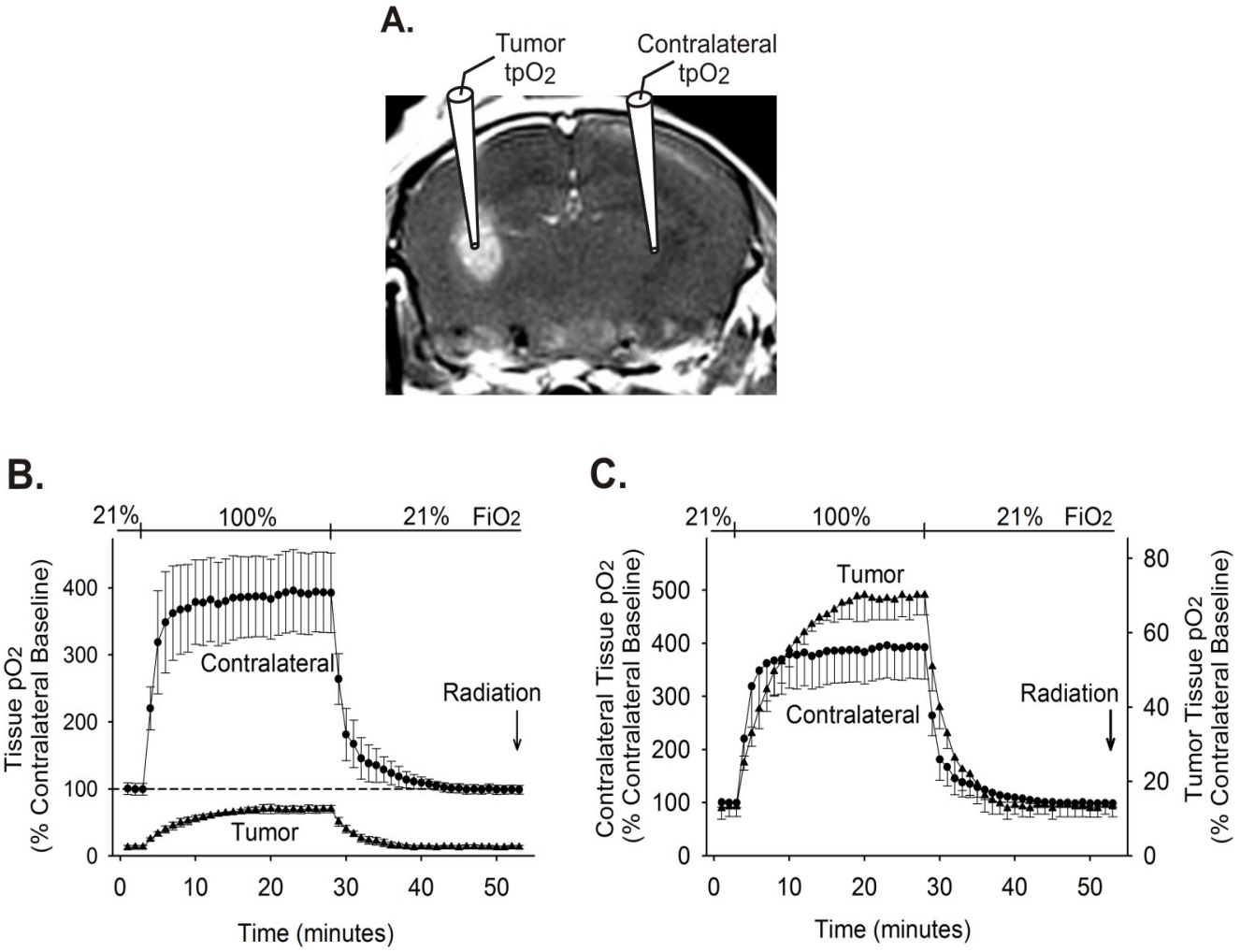
and cover slipped with Prolong Gold anti-fade mounting medium (Life Technologies, #P36934). Fluorescence imaging was done on a Leica inverted microscope (DMI-6000B) using Metamorph imaging software (Molecular Devices).

Statistical analysis. Statistical analyses were performed using SigmaStat software. Specific statistical testing used for a given experimental design is described in the figure legends.

Results

Hyperoxic FiO_2 elevates dissolved oxygen levels in brain and tumor tissue. The *in vivo* oxygen pretreatment protocol consisted of a brief (25 min) exposure to 100% FiO_2, followed by a return to ambient air (21% FiO_2) for 25 min. In order to assess the impact of this pretreatment protocol on tissue oxygen levels, a Licox oxygen probe was used to measure levels of dissolved oxygen in both tumor, and contralateral healthy brain tissue of the same brain, in tumor-bearing mice at day 14 post-tumor implant (PTI) (Figure 2-1A). Baseline tpO_2 was substantially higher in contralateral healthy striatal tissue as compared with tumor tissue, consistent with hypoxic conditions in the tumor environment (Figure 2-1B). Elevation of FiO_2 from 21% to 100% effectively augmented tissue oxygenation in both healthy brain and tumor tissues. Though there was a delay to plateau in tumor tissue, both tissue types had reached asymptotic levels of oxygen enhancement ~15 min after the onset of elevated FiO_2 exposure (Figure 2-1B). The magnitude of the oxygen increase was substantially greater in the healthy brain tissue than in tumor

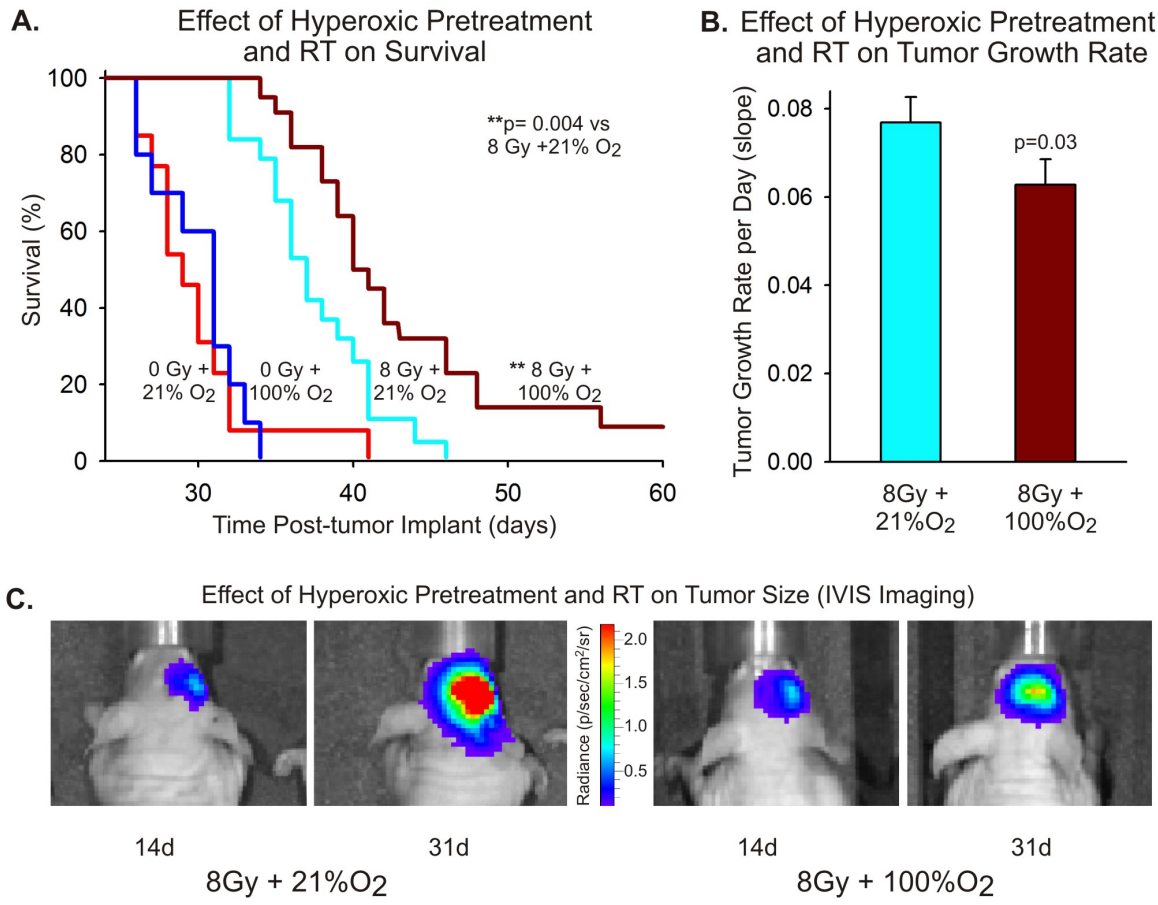
tissue. Although the tumor tissue responded more slowly to hyperoxic FiO_2, as compared with healthy brain, the percentage change in dissolved oxygen levels was somewhat greater in tumor tissue (Figure 2-1C). Upon cessation of 100% FiO_2 treatment, both contralateral healthy brain tissue and hypoxic tumor tissue quickly returned to pretreatment oxygen levels (Figure 2-1B, C). These results indicate that the oxygen treatment paradigm effectively elevates dissolved oxygen levels in hypoxic tumor tissue to near-normoxic levels, and then allows this elevated oxygen to dissipate prior to radiation treatment. These findings also demonstrate that normal brain tissue reaches hyperoxic conditions during 100% FiO_2 treatment and returns to baseline prior to radiation.



<u>Figure 2-1</u>: 100% FiO_2 elevates tpO_2 in tumor and healthy brain tissue. (A) Contrast-enhanced MRI of a mouse brain that was implanted 14 days prior with U87-luc glioma cells is shown. The drawings on the MRI depict the placements of Licox probes used for measuring oxygen levels in the tumor and contralateral brain (striatum). (B and C) The time courses of partial tissue oxygen levels (tpO_2) in response to modified FiO_2 are shown for tumor (triangles) and striatal (circles) tissue (n=3). Oxygen measurements are displayed as a percentage of the baseline tpO_2 levels recorded in the contralateral brain. In B, the time courses are plotted using a common ordinate, in order to show the relative levels and changes in tpO_2 levels observed in tumor and contralateral brain. In C, the time courses for tumor

and contralateral brain are shown on individual ordinates in order to highlight the difference in the rate of change of oxygen levels between the two tissues. Values shown are means and SEMs. Although radiation was not administered in this experiment, the arrow denoting time of radiation delivery provides a reference for subsequent experiments.

Hyperoxic pretreatment of tumor tissue improves survival and slows intracranial tumor growth. In order to evaluate the efficacy of hyperoxic pretreatment as an adjuvant to radiation therapy for GBM, nude mice with U87-luc tumors were assigned to one of four experimental groups after preliminary tumor imaging on day 14 PTI: untreated (0Gy+21% O_2, n=13), hyperoxic pretreatment without radiation (0Gy+100% O_2, n=10), radiation alone (8Gy+21% O_2, n=19), and hyperoxic pretreatment with radiation (8Gy+100% O_2, n=22). Tumor growth was monitored using IVIS imaging every four to five days during the survival period of each animal. Animals that received transient hyperoxic pretreatment prior to radiotherapy showed a significant improvement in survival as compared with animals that received radiation alone (Figure 2-2A). As measured by IVIS, tumor growth was slowed significantly in animals receiving hyperoxic pretreatment prior to radiation (Figure 2-2B; Table S1; Figure S1). Representative IVIS images from animals in the 8Gy+21%O_2 group and 8Gy+100%O_2 group are shown in Figure 2-2C. The animal that received hyperoxic pretreatment exhibited less luciferase activity at day 31 PTI compared with the animal that did not receive pretreatment. It should be noted that while IVIS measurements provide an index of tumor size that is useful for monitoring tumor growth, IVIS luminescence does not reflect precisely the anatomical volume of the tumor. Luminescence signal appears widespread in the Day 31 PTI images because of the low signal threshold utilized for comparison among early and late time points.



<u>Figure 2-2</u>: Hyperoxic pretreatment improves survival and slows tumor growth. (A) Surviving fractions of nude mice implanted with U87-luc cells are shown in a Kaplan-Meier plot for animals receiving: no treatment (0Gy+21%O_2, n=13), hyperoxic pretreatment without radiation (0Gy+100%O_2, n=10), radiation alone (8Gy+21%O_2, n=19), and hyperoxic pretreatment with radiation (8Gy+100%O_2, n=22). Statistical significance was calculated using a Log-Rank (Mantel-Cox) test. (B) A bar graph shows tumor growth rates for animals in the 8Gy+21%O_2 group and the 8Gy+100%O_2 group. Tumor size was assessed by IVIS imaging *in vivo* performed every 4-5 days. The log of the radiance from up to eight IVIS time points (day 14 to day 46 PTI, depending on individual survival) for each animal was used to perform linear regression analyses of tumor growth. Calculated slopes from these linear regressions were then used to determine average tumor

growth rate per day for each animal from each treatment group. Statistical significance was calculated using t-test with Welch's correction for unequal variance. Error bars are SEMs. (C) IVIS images taken at day 14 and day 31 PTI for two animals, one from the 8Gy+21%O_2 (left) and one from the 8Gy+100%O_2 (right) groups. The scale bar for IVIS radiance is shown between the images for the two animals. Note that the IVIS signal in these images does not directly correspond to tumor size. A low luminescence signal threshold was used to permit comparison between time points.

Transient reoxygenation sensitizes glioma cells to radiation and temporarily reduces nuclear HIF-1α accumulation in *in vitro* models of tumor hypoxia. In order to further assess the role of transient oxygen elevation in the enhanced radiotherapeutic vulnerability seen in tumors *in vivo*, we utilized two simplified *in vitro* models of tumor hypoxia. The glioma microenvironment represents a heterogeneous mixture of oxygen conditions for tumor cells that arise from several intra-tumor vascular factors, many of which stem from immature and incompetent blood vessels. Consequently, glioma cells can be exposed to highly variable durations and severities of hypoxic stress, which has important implications regarding how these cells respond to various anti-tumor therapies, including radiation. In an effort to mimic two different hypoxic conditions in a controlled manner, a graded chronic hypoxia (GCH) protocol and a rapid acute hypoxia (RAH) protocol were used (see Figures 2-3A and 2-4A). U87, U87-luc, and GL261 glioma cells and 0308 GSCs exposed to the GCH protocol (Figure 2-3A) showed a significant resistance to a 5Gy dose of radiation under continuous hypoxic conditions compared with normoxic controls, as demonstrated by anchorage-independent colony forming assays (Figure 2-3B). When these cells were transiently exposed to normoxic levels of oxygen for 25 minutes and then returned to severe hypoxia (1% O_2) for 25 minutes prior to radiation exposure, clonogenicity was significantly impaired in all assayed cell lines and was below the clonogenic ability of cells irradiated under normoxic conditions in the U87 and U87-luc cell lines (Figure 2-3B). Nuclear fractions from all cell lines and conditions at the time of radiation exposure were assessed using Western blot analysis with an anti-HIF-1α antibody.

Results from these experiments demonstrated dramatically elevated accumulation of HIF-1α protein in the nuclei of these cells under the GCH protocol. Moreover, following brief reoxygenation and return to hypoxia for 25 minutes, this nuclear accumulation of HIF-1α protein was reduced substantially (Figure 2-3B). Similar clonogenic results were seen in cells exposed to the RAH protocol (Figure 2-4A) and treated with a 5Gy dose of radiation. Transient reoxygenation prior to radiation produced a significant radiosensitization of each cell line, although the clonogenic abilities of the U87 and U87-luc cells were not impaired to the extent that they were following the GCH protocol (Figure 2-4B). As seen in cells exposed to GCH, there was significant accumulation of HIF-1α protein in the nuclei of cells under the RAH protocol, and this nuclear accumulation was reduced substantially following reoxygenation and return to hypoxia.

Transient reoxygenation-induced glioma cell sensitization persists for up to 3 hours after return to hypoxia. The time frame over which glioma cells sustain this increased radiosensitivity following transient oxygenation was investigated by irradiating cells at one of three post-oxygenation time points. Glioma cells subjected to the GCH protocol and then returned to 1% O_2 for 1, 3, or 6 hours prior to 5Gy radiation treatment (see Figure 2-5A) demonstrated oxygen-induced radiosensitization persisting for at least one hour after returning to hypoxic conditions. U87 and U87-luc cell clonogenic capacities remained impaired at 3 hours post-reoxygenation before these cells began to recover resistance at 6 hours post-reoxygenation. GL261 clonogenicity recovered more quickly, remaining impaired at

1 hour, but recovering by 3 hours post-reoxygenation. 0308 GSCs proved more refractory to the effects of transient reoxygenation, reestablishing significant radiation resistance at 1 hour post-reoxygenation (Figure 2-5B). Western analysis of nuclear lysates revealed a recovery of HIF-1α accumulation that begins prior to the recovery of cellular resistance to radiation in U87, U87-luc, and GL261 cells, but corresponds temporally with recovery of radiation resistance in 0308 GSCs (Figure 2-5B). The same post-reoxygenation time points for radiation (1, 3, 6 hours) were also tested using the RAH protocol (see Figure 2-6A). In general, each of the cell lines appears to recover resistance to radiation exposure more quickly as compared with cells that were subjected to the GCH protocol. U87, U87-luc, and GL261 clonogenic capacity improved progressively from 1 hour to 6 hours post-reoxygenation; 0308 GSCs almost fully recover radiation resistance by 1 hour post-reoxygenation (Figure 2-6B). The timing of recovery of nuclear HIF-1α accumulation following the RAH protocol, as shown by Western blot, closely corresponds with recovery of cellular resistance to radiation in all cell lines (Figure 2-6B).

Overexpression of HIF-1α rescues radiation resistance in cells exposed to RAH, but not GCH. In order to examine the effect of nuclear accumulation of HIF-1α in the radiation resistance of glioma cells following transient reoxygenation *in vitro*, U87 cells were transfected with either a HIF-1α-expressing construct (HA-HIF1α-pcDNA3) or an empty vector (pcDNA3). Cells were then exposed to the GCH or RAH protocol (see Figures 2-3A and 2-4A), without or with reoxygenation, and assayed for clonogenicity following 5Gy radiation exposure. Cells transfected with

an empty vector and exposed to GCH(+) prior to radiation demonstrate enhanced radiation vulnerability comparable to that of non-transfected cells, when assessed with the colony forming assay. While HIF-1α overexpression in GCH(+) treated cells provided for a trend towards increased clonogenicity compared with over-expressing normoxic controls; however, this change did not achieve statistical significance. Similarly, RAH(+) cells transfected with an empty vector mirrored the enhanced vulnerability of non-transfected cells. However, overexpression of HIF-1α in RAH(+) treated cells rescued radiation resistance and abolished the effects of transient reoxygenation (Figure 2-7). Western blot analyses showed substantial nuclear accumulation of HIF-1α in cells transfected with the HIF-1α over-expression construct under all experimental conditions. Most notably, high levels of nuclear HIF-1α protein were maintained in U87 cells following transient reoxygenation in both the GCH and RAH protocols, and were present at the time of radiation exposure (Figure 2-7). These results suggest that HIF-1α may play a more critical role in the resistance to hyperoxic pretreatment of glioma cells exposed to acute levels of hypoxia than it does in cells maintained under more chronic hypoxic conditions.

Transient hyperoxia does not sensitize normal human astrocytes to radiation *in vitro*. As demonstrated by the tissue oxygen probe results presented in Figure 1, the administration of 100% FiO_2 dramatically elevates oxygen levels in healthy brain tissue. In order to investigate the possibility that this transient hyperoxic treatment could sensitize previously normoxic brain tissue, we exposed normal human astrocytes to 25 minutes of hyperoxia (50% O_2) before returning

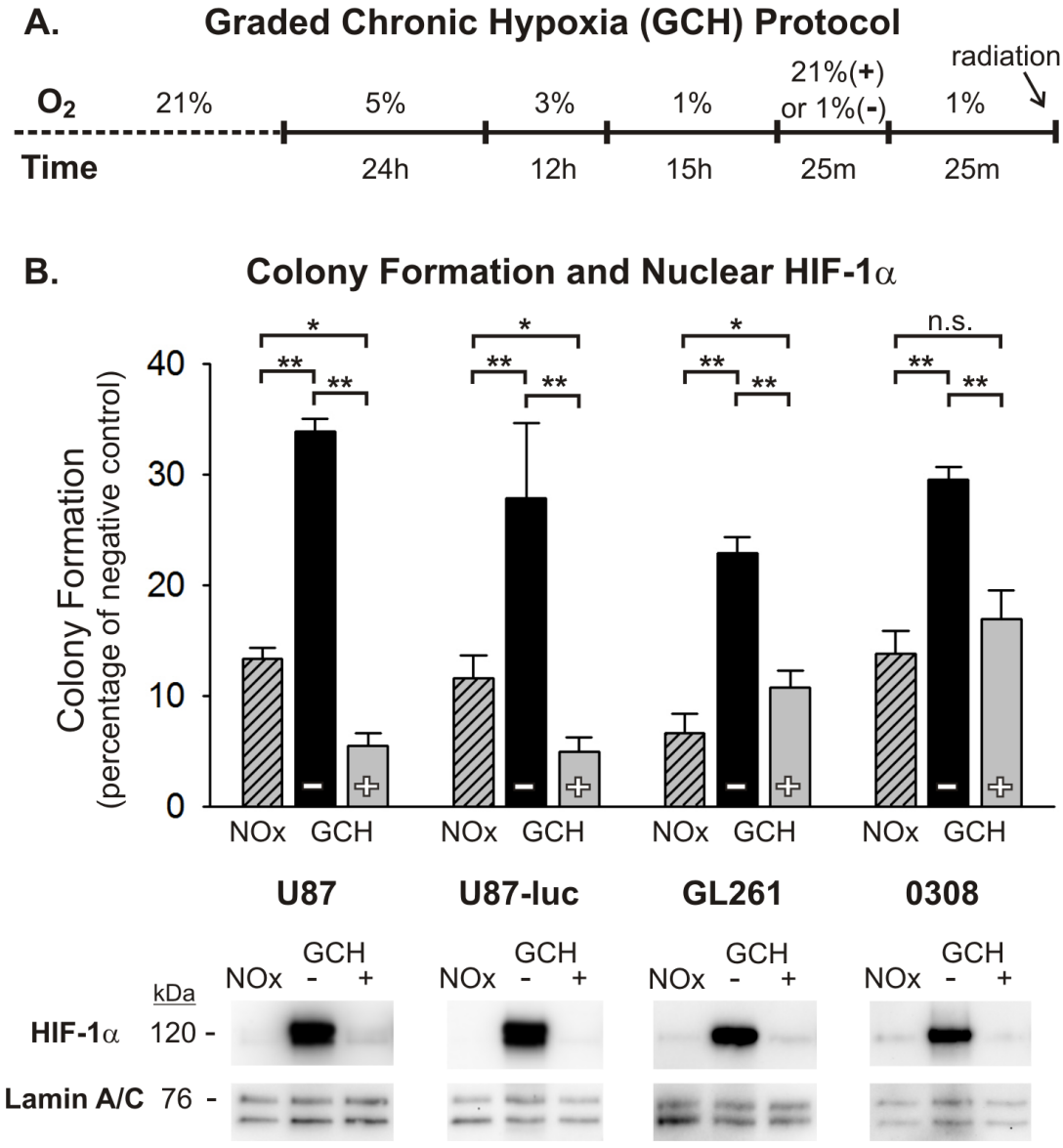
them to normoxic (21% O_2) conditions for 25 minutes and treating them with a 5Gy dose of radiation. Results from clonogenic assays demonstrate that hyperoxic pretreatment does not sensitize normal human astrocytes and, in fact, trended towards protecting these cells from the effects of radiation, although this effect did not achieve statistical significance (Figure 2-8). Identical hyperoxic pretreatment of the glioma cell lines and GSCs generally showed no effect on clonogenicity compared with normoxic controls in the U87, U87-luc and CSC lines (Figure 2-8). GL261 cells, however, exhibited enhanced radiosensitivity following transient hyperoxic exposure that was statistically significant, indicating a potential divergence in vulnerabilities of normal astrocytes and glioma cells following pretreatment with hyperoxia, even when baseline conditions are normoxic. Nuclear fractions from all cell lines at the time of radiation following both normoxic and hyperoxic exposure were assessed using Western blot analysis with an anti-HIF-1α antibody. No discernable differences in nuclear HIF-1α accumulation were observed in any of the cell lines following hyperoxic exposure, when compared with cells maintained under normoxia.

Transient hyperoxia impacts HIF-1α expression differentially in normal human astrocytes and U87 glioma cells. U87 glioma cells and normal human astrocytes were exposed to transient hyperoxia *in vitro*, as described in the previous experiment. Cultured cell monolayers were fixed before, during, and after transient hyperoxia, and HIF-1α expression and localization were assessed immunohistochemically. Under normoxic conditions (21% O_2), very low levels of

cytoplasmic and nuclear HIF-1α are evident in normal human astrocytes, while U87 glioma cells exhibit moderate diffuse cytoplasmic expression and low nuclear HIF-1α accumulation (Figure 2-9). During hyperoxia (at the 25th minute of 50% O_2), both cell lines show diffuse cytoplasmic HIF-1α protein staining, and nuclear staining in some cells. After the cells have returned to normoxia from hyperoxia for 25 minutes, normal human astrocytes show HIF-1α staining close to normoxic baseline levels. Under the same conditions, U87 cells appear to have high cytoplasmic HIF-1α expression and a general absence of staining in the nucleus. Interestingly, the cytoplasmic HIF-1α staining in U87 cells appears to be localized to the endoplasmic reticulum, which is in contrast to the diffuse cytoplasmic staining seen under other conditions. As expected, under positive control conditions (1 hour at 1% O_2), both cell lines exhibited increased nuclear HIF-1α immunoreactivity, with some cells exhibiting very high levels of nuclear expression. Cytoplasmic expression of HIF-1α was low in astrocytes but moderate in U87 cells, with some localization to endoplasmic reticulum. These results imply a fundamental difference between these two cell types in terms of the regulation and expression of HIF-1α after hyperoxic exposure.

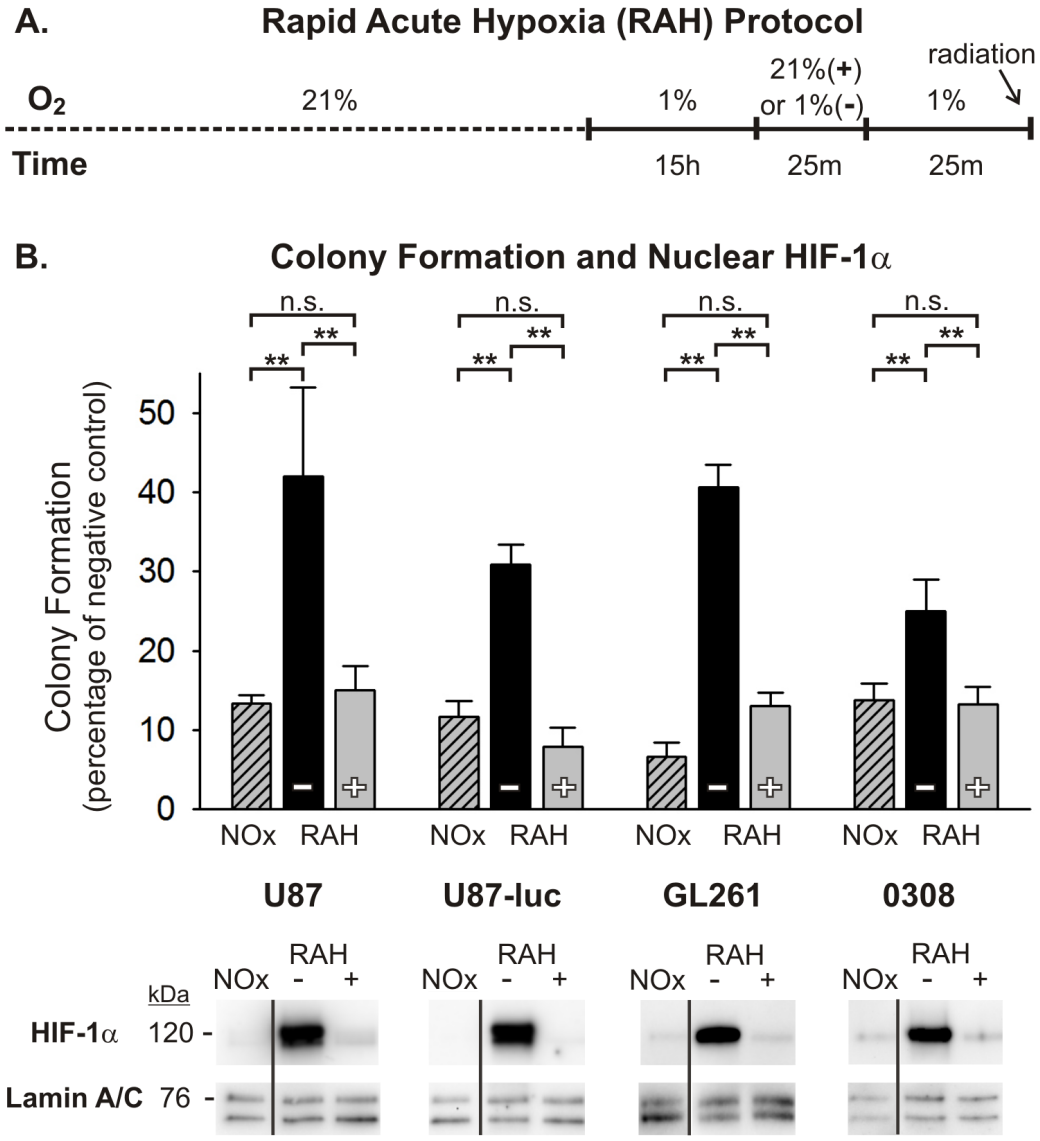
Transient hyperoxia does not appear to sensitize normal brain tissue to radiation. In order to further investigate the impact of transient hyperoxia on the radiosensitivity of normal brain cells located outside of the tumor, brains were examined for cleaved-caspase3 immunoreactivity in mice from both the 8Gy+21% O_2 group and the 8Gy+100% O_2 group 24 hours after radiation exposure. Tissue

sections were stained with cleaved-caspase-3 antibody to assess for apoptosis in the hippocampus. Results from this staining show no detectable apoptotic activity of hippocampal cells in animals from either treatment group (Figure 2-10). These results indicate that a single 8Gy dose of radiation is not sufficient to induce apoptosis at the 24 hour post-radiation time point in the post-mitotic cell populations analyzed in this experiment. It also provides initial evidence that oxygen pretreatment does not sensitize normal brain tissue to radiation at this dose.



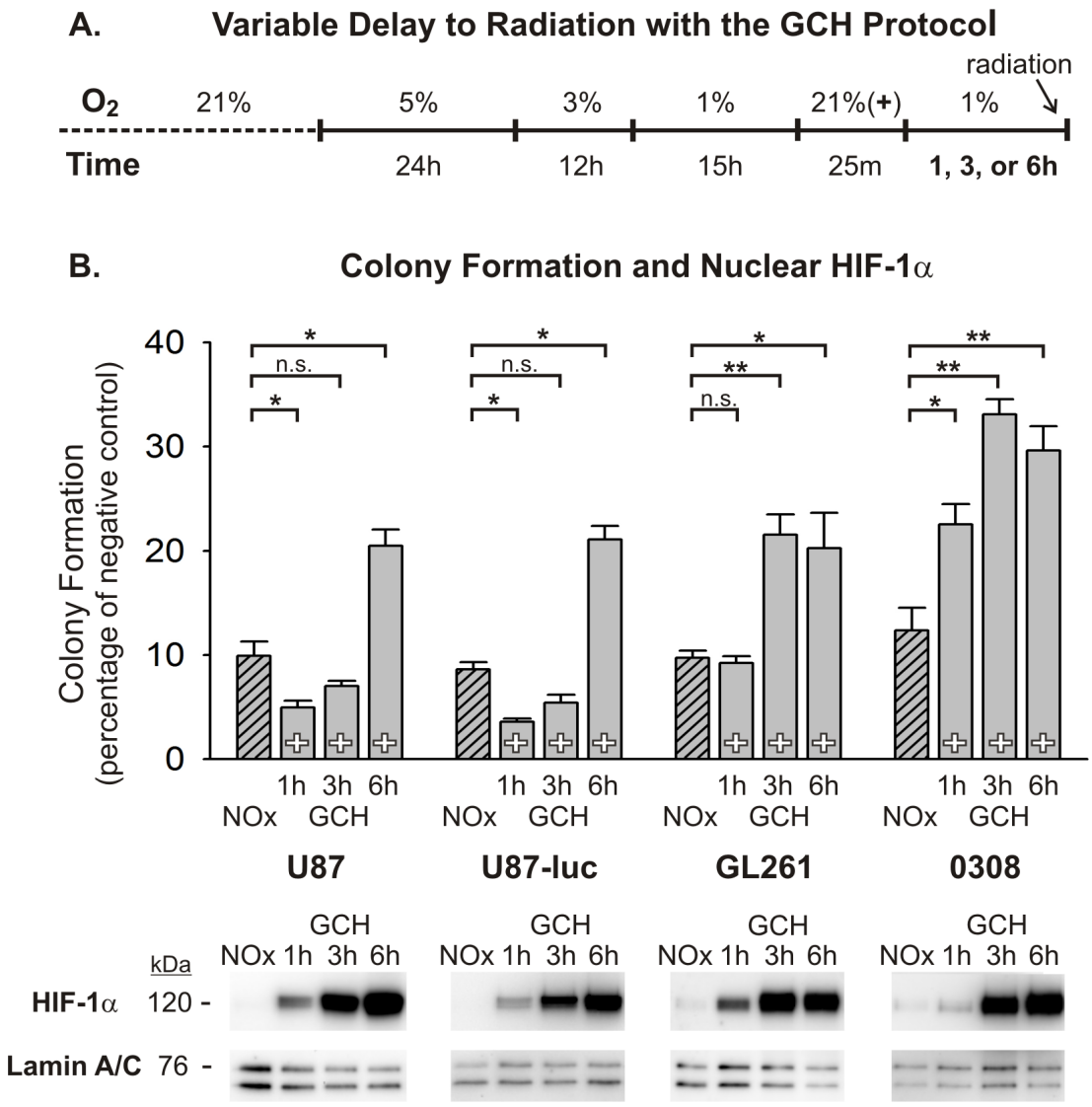
<u>Figure 2-3</u>: Normoxic pre-treatment sensitizes glioma cells to radiation following graded chronic hypoxic (GCH) exposure. (A) The graded chronic hypoxia (GCH) protocol is shown, depicting the timing and severity of hypoxic exposure to four cell lines. Cells either remain in a continuous hypoxic environment (-) or are transiently (25 min) exposed to normoxia 25 min prior to radiation (+).

Continuously normoxic cells (NOx) were irradiated as a positive control. (B) The results of anchorage-independent colony forming assays are shown for U87, U87-luc, GL261 glioma cells and 0308 GSCs following 5Gy radiation exposure under varying oxygen conditions. To allow for ease of comparisons among cell types, raw values are expressed as a percentage of the corresponding cell type's negative (non-irradiated) control and the means and SEMs are plotted. Each result represents at least three independent samples, plated in triplicate. Holm-Sidak comparisons for multiple groups were used for statistical comparisons of raw values (*$p<0.05$, **$p<0.01$). Also shown are Western blots of nuclear HIF-1α at the time of irradiation for each cell type. Corresponding Western blots of lamin A/C are shown as a loading control



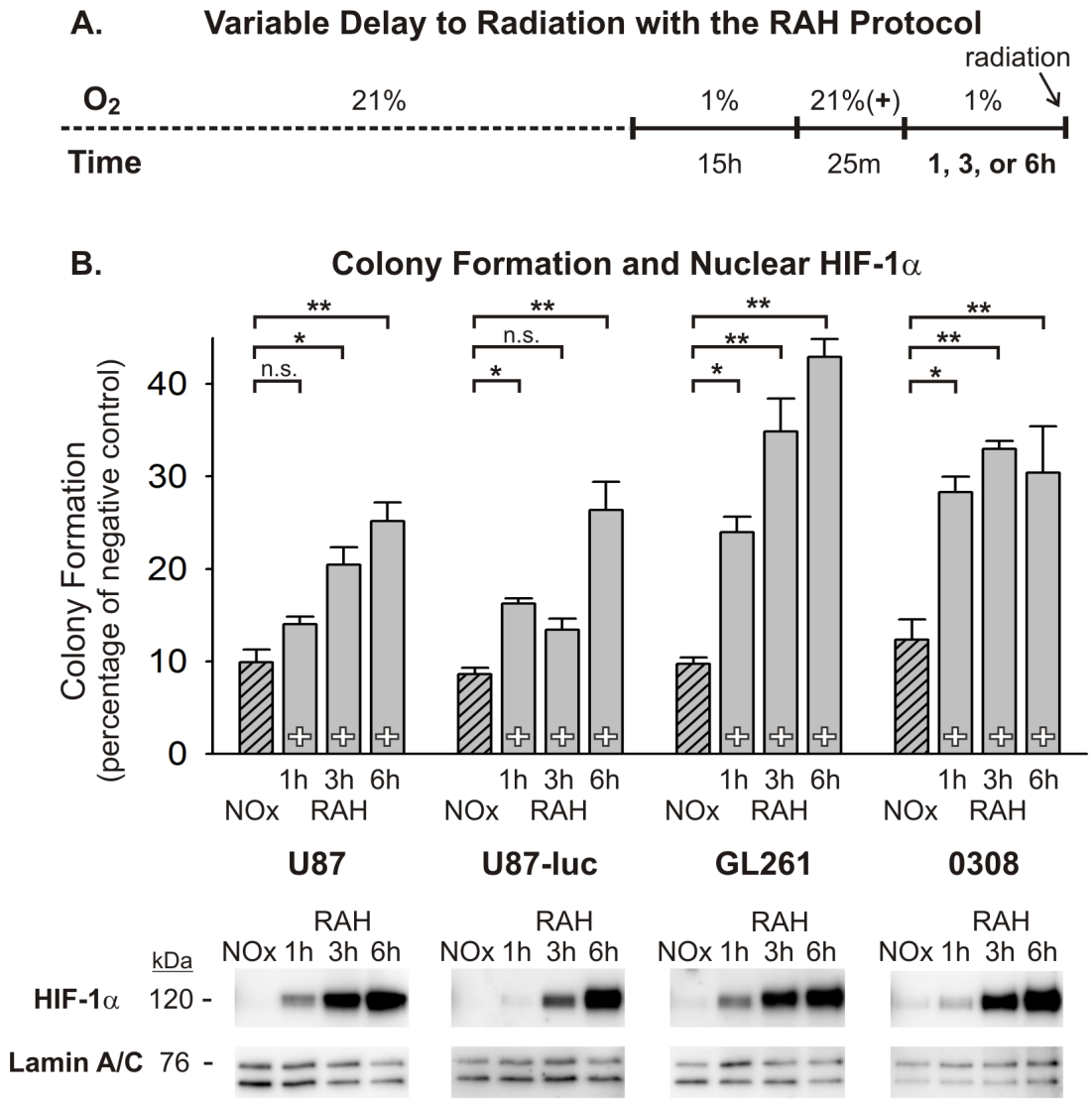
<u>Figure 2-4</u>: Normoxic pretreatment sensitizes glioma cells to radiation following rapid acute hypoxic (RAH) exposure. (A) The rapid acute hypoxia (RAH) protocol is shown depicting the timing and severity of hypoxic exposure. Cells either remain in a continuous hypoxic environment (-) or are transiently (25 min) exposed to normoxia 25 min prior to radiation (+). Continuously normoxic cells (NOx) were irradiated as a positive control. (B) The results of anchorage-

independent colony forming assays are shown for U87, U87-luc, GL261 glioma cells and 0308 GSCs following 5Gy radiation exposure under varying oxygen conditions. To allow for ease of comparisons among cell types, raw values are expressed as a percentage of the corresponding cell type's negative (non-irradiated) control and the means and SEMs are plotted. Each result represents at least three independent samples, plated in triplicate. Holm-Sidak comparisons for multiple groups were used for statistical comparisons of raw values (**$p<0.01$). Also shown are Western blots of nuclear HIF-1α at the time of irradiation for each cell type. Corresponding Western blots of lamin A/C are shown as a loading control. All bands shown that are non-adjacent to the negative control (NOx) are denoted with a separating black line.



<u>Figure 2-5</u>: The duration of enhanced radiosensitivity after normoxic pretreatment differs among cell lines undergoing Graded Chronic Hypoxia. (A) The GCH protocol is shown with variable delays to radiation after normoxic pretreatment. After GCH, all cells are transiently (25 min) exposed to normoxia for 25 min (+) and are then returned to severe hypoxia (1% O_2) for 1, 3, or 6 hours prior to radiation. Continuously normoxic cells (NOx) were irradiated as a positive control. (B) Results from anchorage-independent colony forming assays indicate that the decay of enhanced radiosensitivity differs among cell lines. To allow for

ease of comparisons among cell types, raw values are expressed as a percentage of the corresponding cell type's negative (non-irradiated) control and the means and SEMs are plotted. Each result represents at least three independent samples, plated in triplicate. Holm-Sidak comparisons for multiple groups were used for statistical comparisons of raw values (*$p<0.05$, **$p<0.01$). Also shown are Western blots of nuclear HIF-1α at the time of irradiation for each cell line. Corresponding Western blots of lamin A/C are shown as a loading control.



<u>Figure 2-6</u>: The duration of enhanced radiosensitivity after normoxic pretreatment of cells undergoing Rapid Acute Hypoxia. (A) The RAH protocol is shown with variable delays to radiation after normoxic pretreatment. In this protocol all cells are transiently (25 min) exposed to normoxia for 25 min (+) and then returned to severe hypoxia (1% O_2) for 1, 3, or 6 hours prior to radiation. Continuously normoxic cells (NOx) were irradiated as a positive control. (B) Results from anchorage-independent colony forming assays indicate that the decay of enhanced radiosensitivity for cells in the RAH protocol is generally more rapid than that observed for cells in the GCH protocol. To allow for ease of comparisons among

cell types, raw values are expressed as a percentage of the corresponding cell type's negative (non-irradiated) control and the means and SEMs are plotted. Each result represents at least three independent samples, plated in triplicate. Holm-Sidak comparisons for multiple groups were used for statistical comparisons of raw values (*$p<0.05$, **$p<0.01$). Also shown are Western blots of nuclear HIF-1α at the time of irradiation for each cell line. Corresponding Western blots of lamin A/C are shown as a loading control.

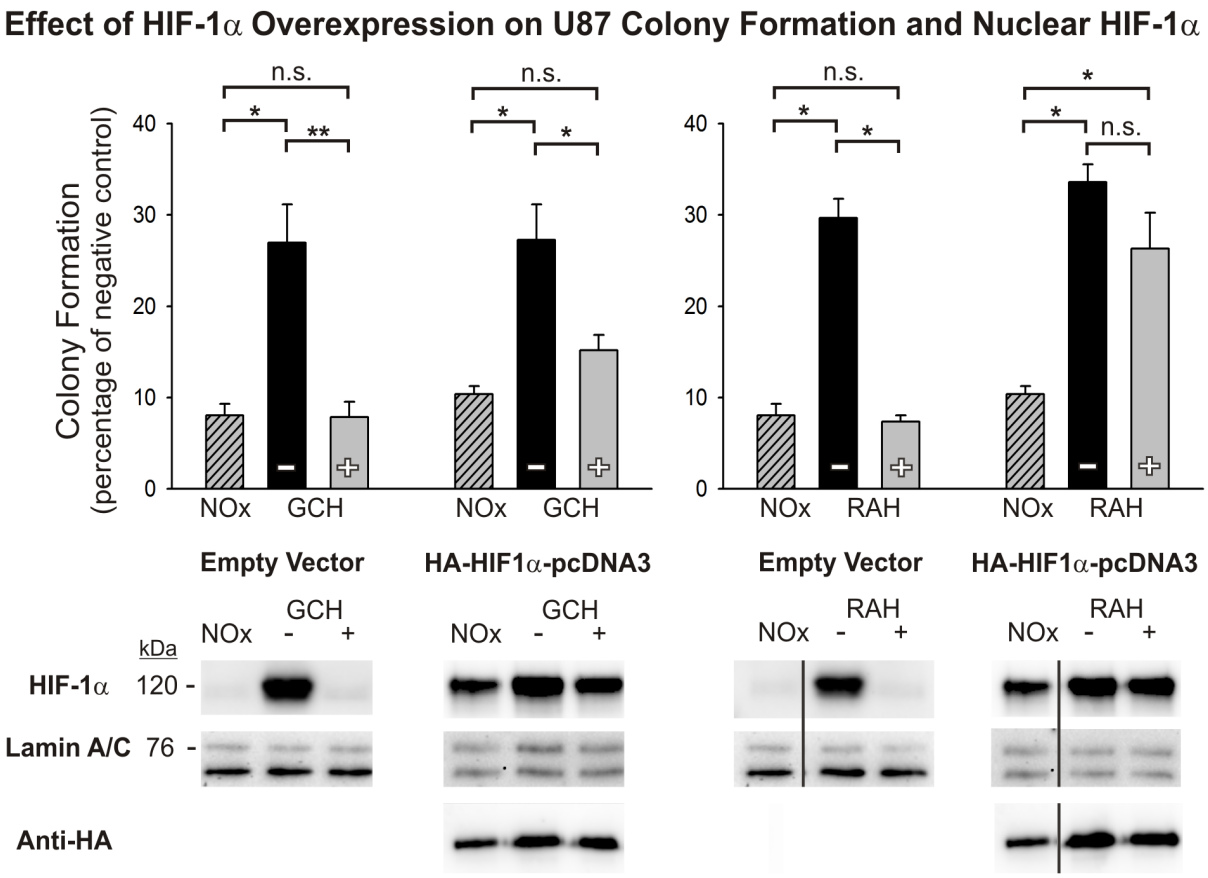


Figure 2-7: HIF-1α overexpression rescues oxygen-induced radioresistance in RAH-treated cells, but not GCH-treated cells. Results are shown for the anchorage-independent colony forming assays for U87 cells transfected with either an empty vector or HIF-1α expression vector and then exposed to GCH or RAH protocols without (-) or with (+) reoxygenation. Continuously normoxic cells (NOx) were irradiated as a positive control. To allow for ease of comparisons among conditions, raw values are presented as a percentage of that cell type's negative (non-irradiated) control and the means and SEMs are plotted. Each result represents at least three independent samples, plated in triplicate. Holm-Sidak comparisons for multiple groups were used for statistical comparisons of raw values (*$p<0.05$, **$p<0.01$). Western blotting analysis of nuclear HIF-1α at the time of irradiation is shown for each cell type below clonogenic results. Corresponding Western blots of lamin A/C are shown as a loading control and blots for

hemagglutinin (HA) are shown below HIF-1α overexpression vector results to demonstrate transfection efficacy. All bands shown that are non-adjacent to the negative control (NOx) are denoted with a separating black line.

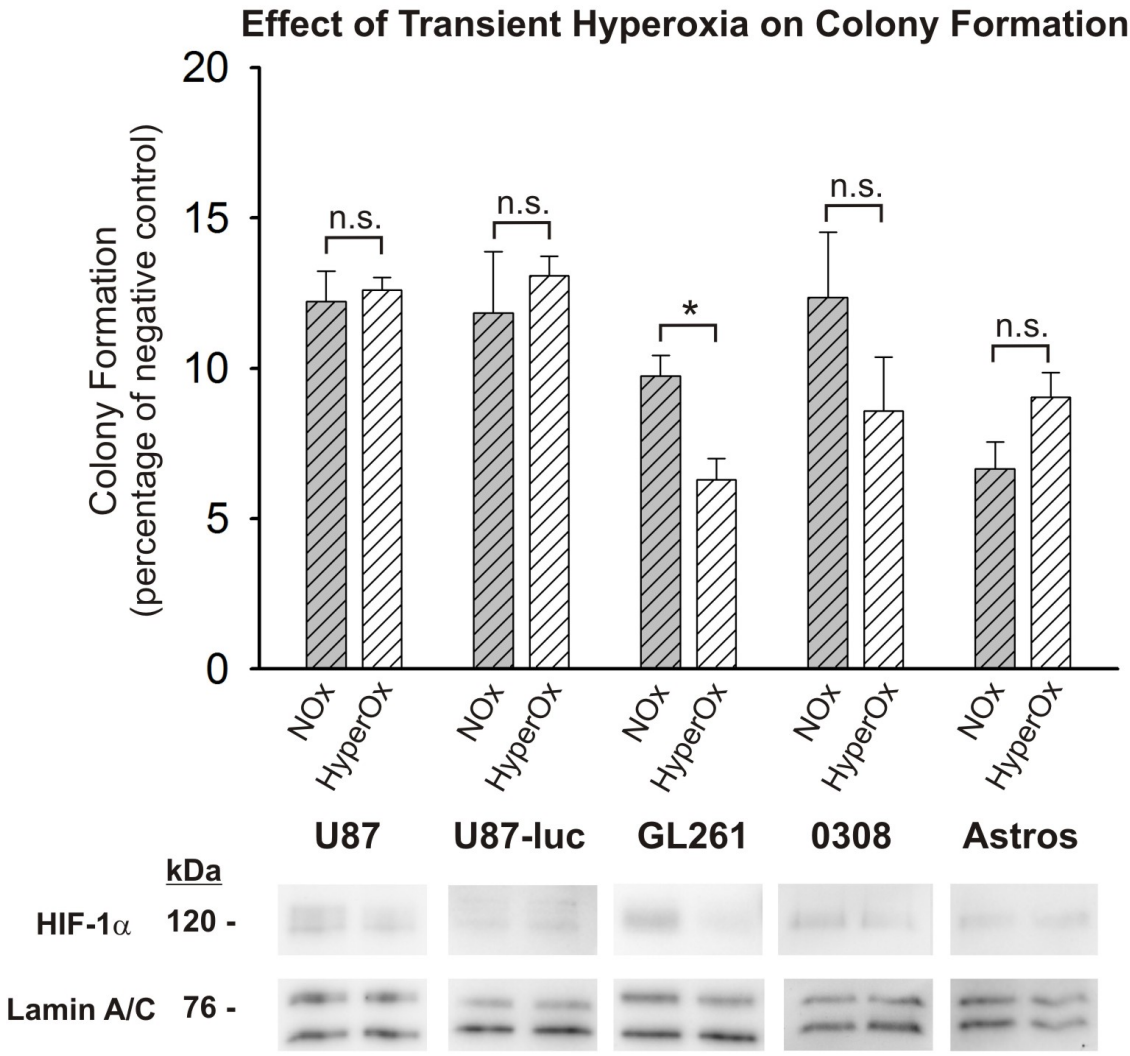
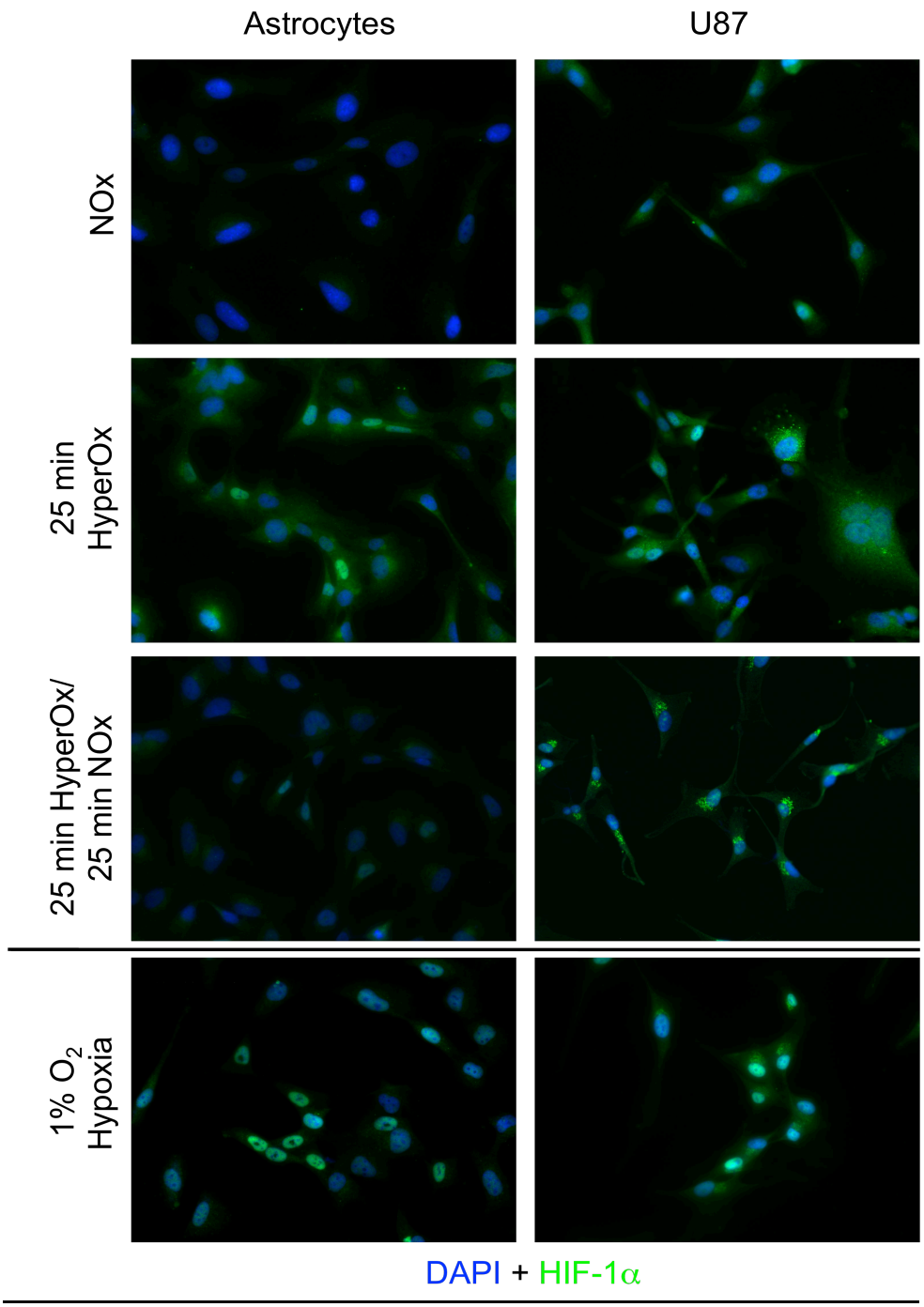


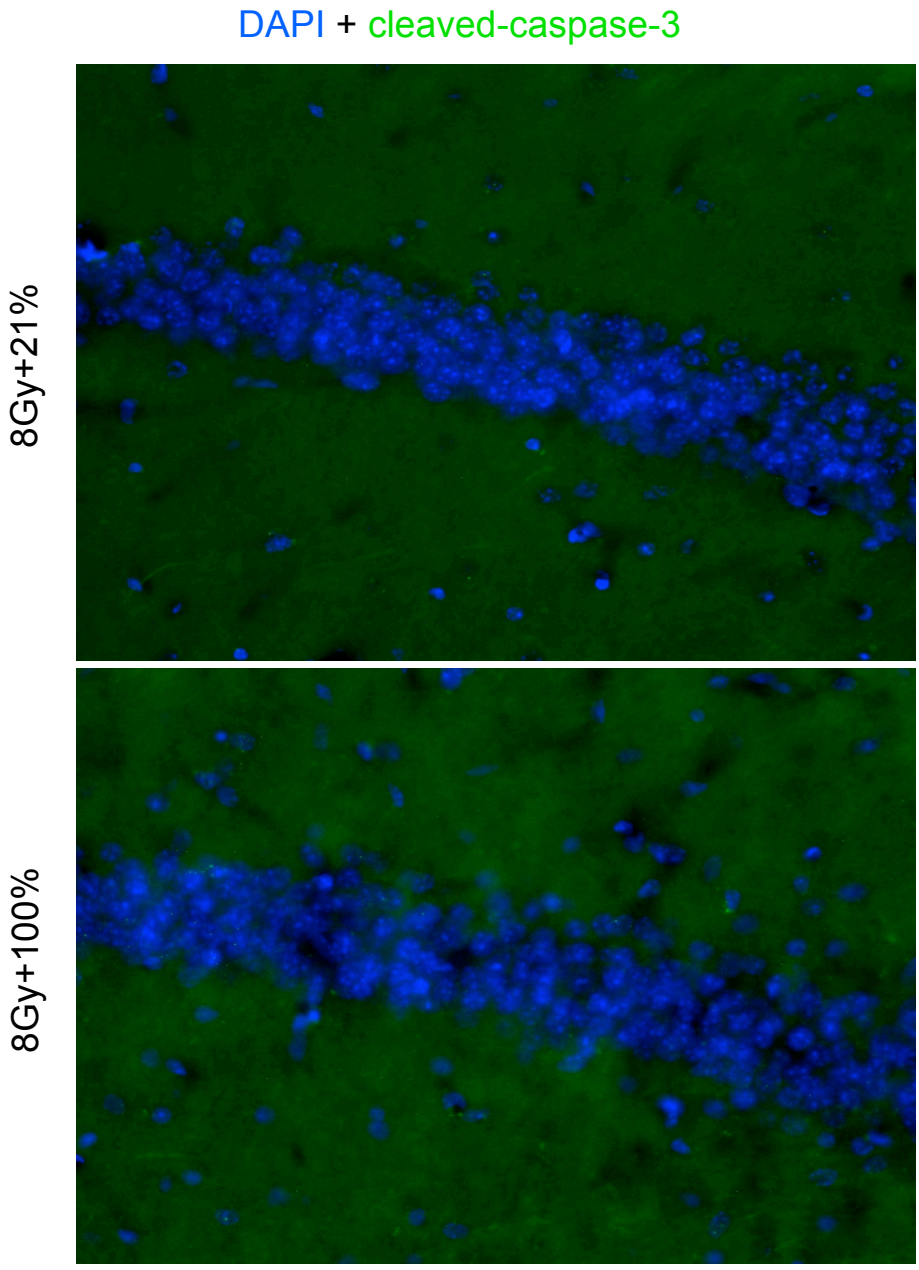
Figure 2-8: Transient hyperoxia does not sensitize normal human astrocytes to radiation. Results are shown for the colony forming assays for all previously assayed cell lines and a normal human astrocyte cell line (Astro). Cells were continuously maintained under normoxic conditions (NOx) or exposed to 25 min of hyperoxia (50% O_2) and then returned to normoxic conditions for 25 min (HyperOx) before being treated with a 5Gy dose of radiation. To allow for ease of comparisons among cell types, raw values are expressed as a percentage of the corresponding cells type's negative (non-irradiated) control and the means and SEMs are plotted. Each result represents three independent samples, plated in

triplicate (*p<0.05, Student's t-test). Also shown are Western blots of nuclear HIF-1α at the time of irradiation for each cell line. Corresponding Western blots of lamin A/C are shown as a loading control.



<u>Figure 2-9</u>: Differential expression of HIF-1α occurs after transient hyperoxia in normal human astrocytes and U87 glioma cells. Fluorescent images of cultured normal human astrocytes and U87 glioma cells exposed to various oxygen conditions are shown. The presence and localization of HIF-1α (green) was assessed, and DAPI (blue) was used as a nuclear counterstain. Under normoxic

conditions (NOx), little or no HIF-1α was observed in the nuclei of either cell type. Diffuse HIF-1α staining was observed in the cytoplasm of U87 cells, but was barely detectable in the cytoplasm of astrocytes. Under hyperoxic conditions (25 min HyperOx), diffuse HIF-1α cytoplasmic staining and sporadic nuclear staining was seen in both cell types. After returning to normoxic conditions from hyperoxia (25 min HyperOx/25 min NOx), HIF-1α staining was very faint in the cytoplasm of astrocytes. In contrast, prominent, punctate perinuclear staining was observed in the U87 cells. Very limited HIF-1α staining was observed in the nuclei of either cell type after returning to normoxic conditions. As a positive control, both cell types were first maintained under normoxic conditions and then subjected to hypoxia (1% O_2 Hypoxia) for 1 hour. Under hypoxic conditions, nuclear localization of HIF-1α was increased in both cell types. Low to moderate cytoplasmic HIF-1α staining was observed in U87 cells and, to a much lesser extent, in astrocytes.



<u>Figure 2-10</u>: No differences in cleaved-caspase-3 activation in healthy hippocampal tissue 24-hours after radiation. Fluorescence images from coronal sections of CA1 hippocampal regions from tumor-bearing mice are shown. Mice were treated with a whole-head dose of 8Gy radiation 24-hours prior to perfusion and slices were stained with cleaved-caspase-3 antibody to assess for apoptotic activity. No cleaved-caspase-3 activation was observed in either treatment group at this time point. DAPI was used as a nuclear counterstain.

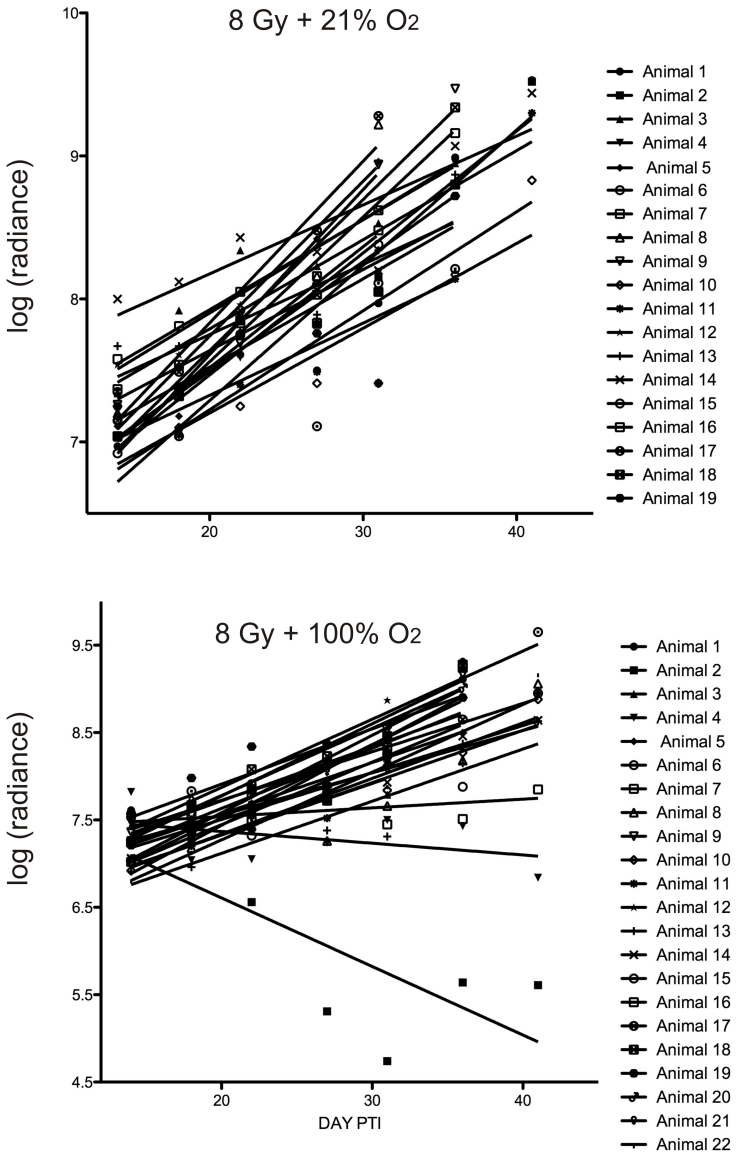


Figure S1. Tumor growth plots: linear regressions for individual animals.

Linear regression plots of tumor growth over time are shown for animals in the 8Gy+21% O2 and 8Gy+100% O2 treatment groups. Individual data points represent the log of tumor radiance, as assessed by IVIS at each tumor measurement time point. Slope values for tumor growth slope were generated from these linear regression plots and are shown in Table S2.

IVIS Data: Linear Regression Slope Values for Individual Animals

8Gy+21% O_2	8Gy+100% O_2
0.09461	0.06282
0.08526	-0.07847
0.06487	0.0594
0.07869	-0.0135
0.1154	0.0554
0.08464	0.04843
0.06683	0.00997
0.1083	0.05991
0.1087	0.06976
0.05931	0.06364
0.06921	0.05891
0.05011	0.09178
0.06232	0.05967
0.04817	0.05379
0.05192	0.07805
0.05773	0.09406
0.1131	0.07376
0.1041	0.08482
0.0631	0.06534
	0.08625
	0.0785
	0.07746

Table S1. Tumor growth slopes for individual animals. Slope values for tumor growth were generated from the linear regression plots of IVIS measurements presented in Figure S2. Slope values are shown for each animal in the 8Gy+21% O_2 and 8Gy+100% O_2 treatment groups.

Discussion

Recent advances in our understanding of the cellular and molecular characteristics of gliomas have provided hope for developing promising alternatives to the traditional mainstays for treatment of this lethal disease (Aboody et al, 2013; Hamza and Gilbert, 2014; Jackson et al, 2014). Nonetheless, the vast majority of patients still receive surgical resection, radiotherapy, and chemotherapy as a standard of care. Even with this aggressive approach to treatment, tumor recurrence is universal, after which continued radiation and chemotherapy are the primary treatment modalities (Preusser et al, 2011; Omuro and DeAngelis, 2013). The presence of hypoxic niches within the solid tumor microenvironment represents a serious challenge to the success of standard therapies. It is therefore of critical importance to identify new and effective ways to optimize the targeting of hypoxic glioma cells. The present study reveals a novel means by which a well-studied non-invasive, non-pharmocological adjuvant sensitizes glioma cells to radiotherapy. Transient elevation of tissue oxygen via normobaric hyperoxia provides a window of enhanced glioma cell vulnerability to ionizing radiation that persists well after tumor tissue has returned to hypoxic conditions.

The ability of ionizing radiation to damage cells is related to the concentration of molecular oxygen present in the tissue at the time of radiation (Gray et al, 1953). The low level of molecular oxygen in hypoxic tumor environments renders radiotherapy less effective than would be achieved under normoxic conditions. Consequently, considerable effort has been directed toward increasing the levels of molecular oxygen in the tumor region during radiotherapy

(Kohshi et al, 1999; Kaanders et al, 2002; Overgaard, 2011). However, elevating tissue oxygen introduces the risk of increasing the vulnerability of radiation injury to healthy tissue. As shown here and elsewhere (Duong et al, 2001; Hou et al, 2011; Hou et al, 2012), systemic oxygen treatment creates hyperoxic conditions in normal brain tissue (Figure 2-1), which can place non-target cells at a higher risk for injury when radiation is administered while oxygen is elevated. In fact, we show that the magnitude of oxygen elevation in healthy tissue in response to 100% FiO_2 is considerably greater than that observed in tumor tissue, and the rate of this increase in normal tissue is more rapid. This underscores the potential risk to healthy tissue that is present when radiation is delivered during hyperoxia. Nonetheless, the goal of elevating tumor tissue oxygenation is also achieved by increasing FiO_2. This elevation is considerable, nearly reaching normal physiological levels within 15 minutes of the onset of hyperoxia. There has been a general consensus that the presence of elevated oxygen in tissue is necessary at the time of radiation for oxygen to sensitize radioresistant cells. However, our findings indicate that oxygen-induced radiosensitization is not completely dependent on the presence of elevated oxygen during radiotherapy. We show that there is in fact an extended window of cellular vulnerability that persists well beyond the point at which these cells return to their baseline hypoxic conditions. It is therefore reasonable to assume that other cellular or molecular modifications underlie this shift in tolerance, and that these mechanisms operate in addition to the direct radiosensitizing effects of molecular oxygen.

A key assumption regarding this shift in vulnerability is that oxygen induces persistent radiosensitivity via mechanisms that are intrinsic to the glioma cells. We tested this concept using simplified *in vitro* models of tumor hypoxia, representing two different durations and severities of hypoxic conditions. We were able to confirm, in multiple glioma cells lines, the radiosensitizing effects of transiently increasing oxygen levels. The findings show that radiosensitization is produced, at least in part, by a direct effect on glioma cells, indicating that a change in intrinsic glioma cell resistance mechanisms contributes to oxygen-induced enhanced tumor vulnerability.

Based on the critical role that the transcription factor HIF-1 is known to play in GBM progression and treatment resistance, we also assessed HIF-1α expression levels in the glioma cell lines at multiple time points following hypoxic conditions and reoxygenation exposures. HIF-1 regulates numerous genes responsible for tumor cell survival, growth, and invasiveness at the transcriptional level (Zagzag et al, 2000). The HIF-1α subunit is over-expressed in many human cancers, due in large part to extensive areas of tumor hypoxia and loss-of-function and gain-of-function mutations in genes regulating HIF-1α (Semenza, 2012). This contributes to the aggressiveness and treatment-resistance of tumors through the promotion of anti-apoptotic genes (e.g. BCL-xL), and inhibition of pro-apoptotic genes (e.g. IAP-2) (Dong et al, 2001; Chen et al, 2009). The availability of HIF-1α to act on target responsive elements when heterodimerized with HIF-1β is dependent upon its nuclear translocation. Western blot analyses of nuclear fractions from each of the cell lines used in this study demonstrated elevated nuclear HIF-1α accumulation

under hypoxic conditions. Notably, transient reoxygenation of these cells following both the GCH and the RAH protocols reduces nuclear HIF-1α protein expression to near pre-hypoxic levels. And, these decreased levels of HIF-1α persist for at least one hour after the cells have returned to severe hypoxic conditions, a phenomenon that could potentially be explained by the persistence of elevated HIF-prolyl-hydroxylase levels in cells exposed to prolonged hypoxia. Marxsen et al (2004) demonstrate that accumulation of HIF-1α in cells during prolonged hypoxia leads to a concomitant accumulation of the HIF-a-prolyl-hydroxylases, PHD2 and PHD3. Upon reoxygenation, elevated levels of PHD2 and PHD3 facilitate the rapid degradation of HIF-1α, and while PHD protein levels begin to decline soon after reoxygenation, there persists a relative elevation in PHDs for up to 24 hours post-reoxygenation. Based on this evidence, it is reasonable to suggest that the refractory accumulation of HIF-1α in the nucleus of glioma cells following reoxygenation and subsequent return to hypoxia in our models is at least partly due to an upregulation of PHD2 and PHD3 activity.

The recovery of high HIF-1α nuclear expression after oxygen treatment corresponds with the recovery of radiation resistance in cells subjected to RAH, but to a lesser extent in cells subjected to GCH. Interestingly, when we overexpressed HIF-1α in U87 cells exposed to GCH or RAH, the reduction of nuclear HIF-1α resulting from transient reoxygenation was abated. Artificially maintaining HIF-1α at high levels during and immediately following reoxygenation rescued radioresistance in RAH-treated, but not GCH-treated, cells. This suggests that the specific hypoxic history of each cell within a given tumor dictates the characteristics

of the cellular response to transient reoxygenation and the subsequent period of radiosensitization. This concept speaks to the heterogeneity of the tumor microenvironment and the complexity of developing therapeutics aimed at targeting these treatment-resistant tumor cells.

From these results, it is reasonable to speculate that a temporary reduction in nuclear HIF-1 activity contributes to a subsequent repression of the pro-survival mechanisms normally being maintained in certain hypoxic tumor cells. This could help explain the increased vulnerability of transiently reoxygenated cells to ionizing radiation under hypoxic conditions compared with cells that have not been exposed to reoxygenation. The reported roles played by the HIF-1α subunit and HIF-1 transcription factor within the context of the hypoxic response in both healthy cells and cancer cells are very complex (Majmundar et al, 2010; Huang, 2013). The hypoxia models used in our studies demonstrate a difference in the influence of HIF-1α on cell vulnerability following reoxygenation and radiation after chronic versus acute hypoxic exposure. Further studies are needed in order to elucidate how such a temporary shut down of HIF-1 activity might impact the expression of its myriad downstream targets, and how alterations in the availability of these gene products might contribute to the extended vulnerability observed in oxygen pretreated glioma cells. Based on previous investigations into the effects of silencing HIF-1α on radiation survival outcomes in U87 and U251 glioma cells (Lanvin et al, 2013; Luo et al, 2014), we know that enhanced radiotherapeutic efficacy following downregulation of HIF-1 can result from delayed cell cycle progression and increased incidence of mitotic catastrophe and apoptosis. It will be important to

investigate whether the temporary absence of nuclear HIF-1α and attendant increase in hypoxic cell vulnerability to radiation following transient oxygen exposure in our models results in a similar range of cellular and molecular changes that lead to multiple cell fates.

The potential clinical relevance of oxygen-induced tumor cell vulnerability is that it holds the possibility to be effective by selectively increasing the sensitivity of treatment-resistant tumor cells to radiation, while not sensitizing non-target, healthy brain tissue. Many preclinical and clinical investigations have attempted to use elevated tumor oxygenation during radiotherapy in efforts to sensitize radio-resistant glioma tissue (Kohshi et al, 1999; Kaanders et al, 2002; Kohshi et al, 2007; Overgaard, 2011). A major drawback to these approaches, however, is that while increasing oxygen levels in the blood can elevate oxygen levels in hypoxic tumor tissue, it also produces hyperoxic levels of oxygen in the surrounding healthy tissue. The work conducted by Kohshi et al (Kohshi et al, 2007) on HBO therapy as a radiotherapeutic adjunct clearly demonstrates this point. Even with the use of more focused radiation techniques such as stereotactic radiotherapy for the treatment of brain tumors, it remains difficult to fully compensate for the irregular tumor margins present in high-grade gliomas, making collateral brain tissue damage an unfortunate reality. In the Kohshi et al study, the delay between HBO therapy and radiation was intentionally kept very short (7 minutes) in order to administer the radiotherapy during the period of elevated tissue oxygenation (Beppu et al, 2002). The resulting symptomatic radionecrosis in a significant portion of enrolled patients

(28%) emphasizes the need for alternative approaches when combining these two treatment modalities.

In contrast to this previous work, the approaches presented herein transiently elevate tissue oxygenation, but allow for tissue oxygen to return to baseline levels in both healthy and tumor tissue prior to treatment. Thus, the risk posed to eloquent tissue by elevated molecular oxygen at the time of radiation exposure is minimized. In fact, it is conceivable that hyperoxic preconditioning could actually reduce the vulnerability of healthy normoxic tissue (Liu et al, 2012). In order to investigate this possibility, we exposed normal human astrocytes maintained under normoxic conditions to transient hyperoxia and then returned them to normoxia before exposing them to radiation. Importantly, transient hyperoxia did not sensitize normal astrocytes to radiation. Rather, there was a trend toward desensitizing the astrocytes, as suggested by a slight increase in the clonogenic capacity of these cells. In contrast, GL261 cells were sensitized to radiation following transient hyperoxia, while the radiosensitivity of the other glioma cell lines was not affected by hyperoxic exposure. Western blot analyses of these cell lines 25 minutes after hyperoxic exposure showed no discernable differences in nuclear HIF-1α levels. Interestingly, however, when we looked at the cellular intensity and localization of HIF-1α expression in normal human astrocytes and U87 glioma cells using immunohistochemical analysis, there a striking difference between the two cell lines. While normal human astrocytes showed moderate cytoplasmic and some high nuclear HIF-1α protein expression during hyperoxic exposure, immunoreactivity in both cellular components was at near

baseline levels after returning cells to normoxia for 25 minutes. U87 glioma cells showed a similar elevation of HIF-1α protein levels during hyperoxia, but, in contrast to normal astrocytes, had highly localized HIF-1α positive cytoplasmic staining in what appeared to be the endoplasmic reticulum. Although it remains unclear what effects, if any, the observed difference in HIF-1α expression between these two cell lines has on the subsequent cellular response to radiation, this presents an attractive area for future investigation. If hyperoxic exposure prior to radiotherapy does indeed provide some form of cellular protection to normal human astrocytes and not to glioma cells, then the differential regulation of HIF-1α following cessation of hyperoxic exposure in these cell types would be a potential mechanism to explore.

We were able to clearly demonstrate that the *in vivo* oxygen treatment used in these studies was sufficient to create hyperoxic levels of oxygen in healthy brain tissue, and that these oxygen levels returned to baseline at the time of radiation (Figure 2-1). When we analyzed healthy brain tissue from animals in both radiation treatment groups for cleaved-caspase-3 positivity 24 hours after radiation treatment, few, if any, positively stained cells were observed in healthy hippocampal tissue in either group. The absence of cleaved-caspase-3 signal in these treated brains is most likely due to the low radiation dose used in our studies, as post-mitotic cells tend to be quite resistant to radiation damage. Nevertheless, these results suggest that transient elevation of molecular oxygen in healthy brain tissue prior to radiation exposure does not sensitize non-target cells. This outcome is encouraging because it is a central goal of this research to establish a radiation

treatment paradigm that is able to sensitize glioma tissue without jeopardizing healthy tissue. It will be important for future work to look into the effects of higher radiation doses on healthy brain tissue toxicity in both treatment groups using similar cell damage assessments. It will also be critical to assess multiple post-radiation time points, as the cellular radiation damage response can take many days to play out (Kesari et al, 2011). Taken together, the results from our hyperoxia experiments suggest that transient oxygen pretreatment prior to radiation therapy may allow for a divergence in the vulnerabilities of hypoxic tumor vs. healthy brain tissue that optimizes the injury produced in the target tumor tissue while limiting injury to normal tissue. The possibility of divergent tissue vulnerabilities underscores the potential benefit of using this type of adjuvant oxygen therapy during the course of radiation treatment in patients with GBM.

It is worth noting that the radiation treatment protocol used in our study employed a moderate, single dose of whole-head radiation. It will be valuable for future studies to evaluate the impact of oxygen pretreatment in conjunction with multiple radiation treatments using a fractionated dose protocol. In addition, other variants of hypoxic stress, such as cycling and intermittent hypoxia, are known to occur in solid tumors (Martinive et al, 2006; Dewhirst, 2009; Hsieh et al, 2010; Chou et al, 2012), and certain courses of hypoxia and reoxygenation have been shown to aid in tumor treatment resistance and progression via ROS signaling and regulation of stress granules (Moeller et al, 2004). It will therefore be important to assess the cellular and molecular impacts of transient oxygenation treatment for glioma cells under various forms of hypoxic challenge, as well as to evaluate the response of

other important cell populations, such as endothelial cells, both in the tumor microenvironment and in the healthy brain. Additional investigations of this type will aid in our understanding of the dynamics of oxygen-induced glioma cell radiosensitization, which may lead to the implementation of a novel, safe, and non-invasive approach for selective tumor cell treatment.

Chapter 3

Conclusions, Implications, and Future Directions

Overview

While some advances have been made in recent years towards the development of more effective treatments for GBM, the prognosis for patients with this disease remains dismal. The shortage of effective alternatives to traditional treatment modalities for GBM highlights the need to develop novel therapeutic approaches. A recent effort in the field to define the molecular characteristics of GBM has resulted in a new sub-classification system for these tumors that is based on tumor cell genetic mutation profiles. However, even these more rigorous guidelines for tumor classification fail to capture the complexity of individual tumor heterogeneity, and, except for a few promising emerging gene and immune approaches (Aalbers et al, 2011; Mao and Wu, 2010; Jackson et al, 2014), have fallen short in terms of leading to successful new drug discoveries.

One of the issues facing researchers in the push to develop targeted therapies for GBM is the inherent plasticity of glioma cells. This can allow tumor cells to escape from the therapeutic inhibition of certain oncogenes and pathways that promote tumor cell survival and aggression, often through redundant signaling pathways. Much like what has been observed in drug development for other cancers and neurological diseases (Ginsberg, 2009; Quick et al, 2010), there has been no "silver bullet" for the treatment of GBM. For this reason, it is likely that the focus of future therapeutic development for GBM will need to shift away from single molecules or pathways, and instead be directed towards therapies that target multiple genes and/or signaling pathways.

As the studies presented here demonstrate, oxygen pretreatment as an adjunct to radiotherapy is capable of enhancing the sensitivity of glioma cells, even under hypoxic conditions. This work also begins to define a role for the transcription factor subunit HIF-1α in regulating the radiotherapeutic response of glioma cells after oxygen pretreatment. As indicated by Western blot analyses of nuclear HIF-1α protein, and by HIF-1α overexpression experiments, this molecule is clearly implicated in the enhancement of radiosensitivity after oxygen pretreatment. HIF-1 is generally considered to be the master regulator of the cellular response to hypoxia and has a well-defined role in cancer cell agression, progression, and treatment resistance (Semenza, 2003; Powis and Kirkpatrick, 2004; Semenza, 2010). This makes HIF-1 an excellent target for therapeutic development because it holds the potential to influence many aspects of glioma cell resistance to traditional therapeutics. At this point, however, the consequences of manipulating HIF-1α nuclear expression via oxygen pretreatment remains unclear, in terms of which downstream effector gene targets and cell survival pathways contribute to enhanced radiosensitivity.

As mentioned previously, HIF-1 is known to influence hundreds of downstream gene targets that participate in the various cellular responses to low oxygen levels. Gene expression changes following exposure to hypoxic conditions have already been defined in some established glioma cell lines using gene microarrays (Ragel et al, 2007; Mendez et al, 2010). These types of studies are helpful because they reveal candidate genes that are known targets of HIF-1. They are thus prime candidates to help explain enhanced hypoxic cell vulnerability after

oxygen pretreatment. It will be important to determine which of these known targets are also affected after oxygen pretreatment. Because of the abundance of gene targets of HIF-1, gene expression analyses and large-scale proteomic screens would be valuable approaches for identifying leading candidates contributing to enhanced vulnerability after oxygen pretreatment. However, because each glioma cell line is unique in its genetic makeup, it will be important to evaluate potential transcriptional, translational, and post-translational alterations across multiple cell lines in an effort to narrow down the relevant candidates. Now that we have identified HIF-1α as a regulator of the glioma cell response to oxygen pretreatment, these approaches would cast a broader net for the next stage of research.

Enhanced cellular vulnerability to radiation after oxygen pretreatment could also result from residual changes in the regulation of reactive oxygen species (ROS). Increased production of ROS within cells has been linked not only to the mutagenic processes that lead to the transformation of normal cells to cancer cells, but also to the aggression and proliferation of cancer cells once a tumor has formed (Martin and Barrett, 2002). This holds true for glioma cells, which are generally highly glycolytic and, as a result, rely on the increased production of antioxidant species, such as glutathione, to mitigate elevated levels of oxidative stress. These antioxidants are an integral part of the glioma cell adaptation to ischemic environments and, ultimately, contribute to ROS-generating therapeutics such as radiation (Ogunrinu and Sontheimer, 2010; Jamal et al, 2010; Pena-Rico et al, 2011).

Based on the results of my studies, it is unlikely that oxygen pretreatment itself causes cellular damage. Rather, it may actually be the case that signaling

cascades triggered by the reoxygenation of hypoxic glioma cells aid in dampening regulatory mechanisms responsible for the neutralization of ROS that are already elevated in hypoxic glioma cells (Meijer et al, 2012). An oxygen-induced reduction in the endogenous levels of antioxidants within these cells would likely result in increased vulnerability to radiotherapeutic challenges. In addition, it is possible that oxygen pretreatment could lead to an overall increase in ROS production in glioma cells. A sudden influx of oxygen to a previously hypoxic cell could lead to a metabolic shift that overwhelms the mitochondria, resulting in a subsequent increase in ROS levels. The elevated levels of ROS during radiotherapy might then, in turn, contribute to the DNA damage caused by radiation exposure.

In order to fully understand the role of ROS in hypoxic cell vulnerability following oxygen pretreatment, future investigations will need to carefully consider the timing of both the oxygen and radiation treatments. Given the transient nature of ROS and their influence on cell signaling and DNA damage, assessing ROS levels during treatments, and at multiple time points following treatments, will be critical. In fact, there is evidence to show that delayed spikes in ROS production in glioma cells can influence vulnerability to radiation days after treatment (Gao et al, 2008). It will be useful then to look at both the immediate and more persistent impacts of oxygen pretreatment on ROS activity in order to determine the role they may be playing in enhancing cell sensitivity.

As research into the origin and progression of malignant tumors moves forward, a greater understanding is emerging regarding the complexity of intratumor structure and composition. Solid tumors are a heterogeneous mixture of

cells with a diverse set of genetic make-ups, differentiation states, and functions. Non-tumor cells, including endothelial, stromal, and immune cells, play a major role in determining how a tumor will grow and function within the body by establishing what is known as the tumor microenvironment. The interaction of tumors cells with their microenvironment is critical for tumor growth and survival, and, as such, has become an important area of research in the field of cancer biology and therapeutics (Hanahan and Coussens, 2012). In recent years, one subpopulation of tumor cell in particular has attracted attention in the field of cancer research because of its demonstrated ability to resist standard cancer therapeutics and drive tumor growth: the cancer stem cell. Hierarchical models of the developmental make-up of tumors state that only a small number of cells within a given tumor give rise to the rest of the tumor mass. These so-called cancer stem cells, referred to as glioma stem cells (GSCs) in the case of gliomas, exhibit similar characteristics to normal stem cells, including self-renewal and ability to differentiate; however, they lack the important self-regulatory abilities that ordinarily would keep proliferation and tumorigenicity in check. They are defined by their ability to maintain long-term clonal growth of a tumor, and typically possess properties that provide them greater resistance to cancer treatments when compared to their more differentiated tumor cell counterparts (Kreso and Dick, 2014).

Similar to normal stem cell populations that are found within defined areas in the body, GSCs appear to congregate in distinct niches within the tumor mass. And, similar to normal neural stem cells found in the brain, oxygen appears to play a critical role in the regulation and maintenance of GSC populations within the tumor

mass in GBM and other gliomas (Pistollato et al, 2009). There is evidence that endothelial cell regulation of oxygen supply to neural stem cell (NSC) niches controls the state of differentiation of these cells. Along with additional cell signaling components, lower physiologial oxygen tensions will activate NSCs while, conversely, increased oxygen availability will suppress stem cell proliferation (Shen et al, 2004; Pardal et al, 2007). In this way, endothelial cells, via the vascular supply, can keep NSCs in, or release them from, a quiescent state as they are needed during development and in normal adult brain functioning. Similarly, GSCs within brain tumors have been found to reside in perivascular niches and are regulated by endothelial cell signaling (Filatova et al, 2013; Pietras et al, 2014). Importantly, however, GSCs are also found in hypoxic and perinecrotic regions of tumors where oxygen tensions are very low. These severe hypoxic conditions generally lead to genomic instability and maintenance of a dedifferentiated state in cancer cells (Jogi et al, 2002; To et al, 2005). More specifically, a critical role for HIF-1α has been defined in the regulation of GSC differentiation states. The high levels of HIF-1α protein present in cells within these hypoxic regions allow the HIF-1α subunit to interact with Notch, activating this signaling pathway and promoting survival, proliferation, and dedifferentiation (Qiang et al, 2012). Considering, then, the established roles of hypoxia and HIF-1α in GSC regulation, the effect of oxygen pretreatment on GSC populations becomes an attractive area of future research. Relevant to this potential area of research is the fact that GSCs maintain very high levels of antioxidants within the cell in order to counter an elevated production of ROS resulting from excessive metabolic activity (Vlashi et al., 2011). This balance of

ROS and antioxidants is critical for maintenance of the stem cell phenotype. In conjunction with the ROS experiments outlined previously in this chapter, it will be of interest to look at how any potential changes ROS levels following oxygen pretreatment could be influencing GSC populations, as well.

As indicated in Chapter 2, the 0308 GSC line was used in our studies, in conjunction with multiple well-established differentiated glioma cell lines, as a means to determine the efficacy of oxygen pretreatment on a known treatment-resistant tumor cell population. Our results demonstrate that the use of oxygen pretreatment does indeed sensitize this GSC line to radiation, although the recovery of treatment resistance is substantially faster when compared to the differentiated glioma cell lines (see Figures 2-5 and 2-6). Analysis of nuclear HIF-1α levels in these GSCs indicate that this subunit may be playing an important role in the response to radiation. As is the case with all of the cell lines used in our studies, the mechanisms downstream of HIF-1α remain unclear. Nonetheless, given the known links among the maintenance of cancer stem cell populations, areas of tumor hypoxia, and HIF-1α, it is reasonable to posit that changes in stem cell composition within the glioma cell lines used in our studies may contribute to the observed alterations in radiosensitivity. The type of transient reoxygenation used in our treatment protocols could alter the oxygen tensions within GSC niches sufficiently to interrupt signaling cascades that are maintaining the dedifferentiation states of certain stem cell populations; this could ultimately change the composition and radiosensitivity of the tumor tissue. This type of phenotypic change in glioma tissue could help explain, in part, the vulnerability to radiation treatment seen following oxygen

pretreatment. It is certainly possible, however, that the brief nature of the type of oxygen preconditioning used here is not sufficient to bring about any type of phenotypic shift in these stem cell populations. If this is the case, then the finding would further support a central role for the changes in the cellular protective mechanisms regulated by the HIF-1 transcription factor in the increased vulnerability of tumor tissue following oxygen pretreatment, rather than any influences of the HIF-1α subunit acting on its own.

There have been a number of recent studies that have aimed to define the prevalence and role in treatment resistance of GSC in gliomas, both in rodent models and human tumors (Bao et al, 2006; Chen et al, 2012). One of the most widely used stem cell markers for the identification of GSCs in glioma tissue is Prominin-1 (CD133). CD133 is a transmembrane glycoprotein known to be a distinct marker for stem cell populations (Singh et al, 2003; 2004). Characterization of the distribution and response of $CD133^{+}$–positive cells would be a worthwhile next step for investigating whether or not oxygen pretreatment influences the differentiation states of GSCs within the cell lines used in these studies. One caveat to this approach is that, while CD133 markers provide a reasonable indication of the presence of cancer stem cells, their ability to properly define stem cell populations within tumors is still an area of much debate (Chen et al, 2010; He et al, 2011). Thus, in addition to using CD133 positivity for the evaluation of the effects of oxygen pretreatment on stem cell makeup, it will be valuable to use additional markers of stem cells (e.g. Olig2, ALDH, CD90) in order to properly define any potential phenotypic shifts.

Over the course of the last few decades, there has been tremendous advancement in our understanding of the complex components that make up fast-growing solid tumors such as GBM. As the cell types and interactions within the tumor microenvironment continue to be defined, it is becoming clear that future therapies for tumors like GBM will need to take into account both inter- and intratumor heterogeneity. While a number of important oncogenes and tumor-specific molecular targets have recently been identified, the development of future novel therapeutics for this disease will likely favor approaches that influence a broad range of oncogenic pathways in order to more effectively manage tumor cell plasticity and treatment resistance. The work presented in this thesis provides a case for the use of oxygen pretreatment as an adjunct for radiation therapy based on the evidence that this approach is able to be effective in the treatment of multiple glioma cell lines, including a GSC line. Additionally, my work demonstrates that these effects are mediated by the HIF-1 transcription factor subunit HIF-1α, a known regulator of a number of important downstream oncogenic elements. Continued research into elucidating the effects of oxygen pretreatment on HIF-1 downstream targets, ROS regulation, and GSC differentiation in hypoxic glioma cells will begin to identify the precise mechanisms behind this form of increased vulnerability. In doing so, we may be able to create optimal treatment conditions for radioresistant cells in a given tumor, based on patient-specific tumor hypoxia profiles and tumor cell composition. This type of tailored treatment approach to GBM could ultimately provide benefit in terms of increased survival and improved quality of life for patients who are in desperate need of therapeutic alternatives.

Concluding Remarks

The results presented in these studies demonstrate that oxygen pretreatment of hypoxic glioma cells represents a novel method by which treatment resistant cancer cells may be selectively sensitized to radiation therapy. This thesis defines working animal models of GBM and *in vitro* models of tumor hypoxia that may be used to advance our understanding of how transient elevation of oxygen leads to hypoxic cell vulnerability. My work has begun to identify a role for the transcription factor subunit HIF-1α in this phenomenon, and I have outlined a number of future areas of study that will help to further define the cellular and molecular mechanisms behind this oxygen-induced vulnerability. Clearly, more research is needed in order to understand the optimal treatment conditions for this type of adjunct therapy. Nonetheless, there now exists the potential for translational benefit for patients suffering from what is currently an incurable and deadly disease. The critical need for novel clinical approaches to the treatment of GBM, the excellent safety profile of medical oxygen, and the non-invasive nature of this therapeutic treatment approach renders this an especially attractive strategy for continued investigation.

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