Role of Hexokinase 2 (HK2) in Modulating Tumor Metabolism and Response to Therapy in Glioblastoma

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Abstract

Glioblastoma (GBM), similar to many other cancers, exhibits enhanced aerobic glycolysis with concomitant lactate production, a phenomenon known as the Warburg effect. We have demonstrated that preferential expression of Hexokinase 2 (HK2) is a critical mediator of metabolic reprogramming in GBMs and its inhibition is a potential therapeutic strategy for sensitization of GBM tumors to radiation (RAD) and/or temozolomide (TMZ). Our results indicate that conditional HK2 inhibition disrupts energy homeostasis and sensitizes GBMs to radiochemotherapy under hypoxia. In GBM xenografts, conditional HK2 loss sensitizes GBM tumors to concomitant RAD/TMZ and results in a significant survival benefit in the mice. Moreover, loss of HK2 resulted in GBM remodeling with HK2 knockdowns showing increased necrosis, hypoxia, inflammatory infiltration and reduced vascularization. We anticipate that targeting a key metabolic enzyme involved in the Warburg effect might improve the efficacy of current therapeutic regimen and provide a unique paradigm for the management of GBMs.
Acknowledgments

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Dedications

I dedicate this work to my lovely mother and others like her who have suffered and are suffering from brain tumor
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<tbody>
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<td>2DG</td>
<td>2-deoxyglucose</td>
</tr>
<tr>
<td>3-BrP</td>
<td>3-Bromopyruvate</td>
</tr>
<tr>
<td>AA</td>
<td>Anaplastic astrocytoma</td>
</tr>
<tr>
<td>ALD</td>
<td>Aldolase</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>ACL</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCNU</td>
<td>Carmustin</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminesence imaging</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrases</td>
</tr>
<tr>
<td>CBTRUS</td>
<td>The Central Brain Tumor Registry of the United States</td>
</tr>
<tr>
<td>C-Kit</td>
<td>tyrosine-protein kinase Kit</td>
</tr>
<tr>
<td>c-Met</td>
<td>Met protein oncogene</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalic virus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
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<tr>
<td>DCE</td>
<td>Dynamic contrast enhanced</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycyclinex</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organization for Research &amp; Treatment of Cancer</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFR</td>
<td>V-Erb-B2 erythroblastic leukemia viral oncogene homolog 2</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
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<td>The food and drug administration</td>
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<tr>
<td>F6P</td>
<td>Fructose-6-phosphate</td>
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<tr>
<td>FDG–PET</td>
<td>$^{18}$F-deoxyglucose positron emission tomography</td>
</tr>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GBM</td>
<td>Glioblastoma</td>
</tr>
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<td>GSC</td>
<td>Glioma stem cell</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>G6P</td>
<td>Glucose-6-phosphate</td>
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<td>Glucose-6-phosphate dehydrogenase</td>
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<td>Glucose transporters</td>
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<td>Glutathione</td>
</tr>
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<td>Histone deacetylase</td>
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<td>Human epidermal growth factor receptor</td>
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<tr>
<td>HGA</td>
<td>High grade astrocytomas</td>
</tr>
<tr>
<td>HIF1</td>
<td>Hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HK1</td>
<td>Hexokinase 1</td>
</tr>
<tr>
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<td>Hexokinase 2</td>
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<td>Isocitrate dehydrogenase 1</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>$K_{\text{trans}}$</td>
<td>Contrast transfer coefficient</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
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<td>LGA</td>
<td>Low grade astrocytoma</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MCT</td>
<td>Membrane monocarboxylate transporters</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporters</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>MVD</td>
<td>Microvascular density</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCIC</td>
<td>National Cancer Institute of Canada</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis 1</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Non-obese diabetic severe combined immunodeficient</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PFA</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphates</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositide 3-kinases</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PFK1</td>
<td>Phosphofructokinase 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase- B</td>
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<tr>
<td>PKM</td>
<td>Pyruvate kinase M</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK</td>
<td>Dehydrogenase kinase</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>RAD</td>
<td>Radiation</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>REMBRANDT</td>
<td>Repository of Molecular Brain Neoplasia Data</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>R5P</td>
<td>Ribose-5-phosphate</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentosephosphate pathway</td>
</tr>
<tr>
<td>PDGFA</td>
<td>Platelet derived growth factor A</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidenedifluoride</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis (SDS-PAGE)</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>T1w</td>
<td>T1-weighted</td>
</tr>
<tr>
<td>TIGAR</td>
<td>TP53-inducible glycolysis and apoptosis regulator</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TKT</td>
<td>Transketolase</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline response element</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
Chapter 1: Introduction and Literature Review

1.1 Glioblastoma

1.1.1 Epidemiology and prevalence

Human brain tumors are a heterogeneous group of neoplasms that display variable clinical behavior, histology, grading, proliferative aptitude and invasiveness. Despite years of research and numerous clinical trials, primary human brain tumors continue to be among the top ten causes of cancer-related deaths, accounting for 2.3% of cancer-related deaths in Europe and North America. In the United States the incident rate of primary non-malignant and malignant CNS tumors is 15.4/100,000 per year (7.07/100,000 for malignant tumors). The majority of the newly diagnosed primary brain tumors are classified as gliomas, which are one of the most aggressive of all brain tumors. Glial neoplasms represent ~ 30% of central nervous system (CNS) tumors and account for approximately 80% of the malignant tumors (Figure 1.1). The World Health Organization (WHO) classifies gliomas according to their presumed cell of origin, as determined by histological analysis. The main histological subtypes of gliomas are astrocytomas, oligodendrogliomas and ependymomas. Astrocytic tumors are the most common of gliomas accounting for about 76% of cases. Astrocytomas encompass a broad category of tumors with different growth rates, aggressiveness, clinical outcome and molecular/morphological features. Astrocytomas are graded on a WHO consensus-derived scale into four distinct clinicopathological entities with increasing malignancy; pilocytic astrocytomas (WHO grade I) and diffuse astrocytoma (WHO grade II) are described as low grade astrocytomas (LGAs) being slow growing and less aggressive; anaplastic astrocytoma (AA, WHO grade III) and glioblastoma (GBM, WHO grade IV) are described as high grade astrocytomas (HGAs). GBM is the most common and the most malignant astrocytic adult brain tumor accounting for 54% of all primary gliomas (Figure 1.2). As astrocytomas advance through the pathologic spectrum from grade III to GBM, the proliferative capacity, aggressiveness and the degree of infiltration increase substantially. GBMs are characterized by complex chromosome aberrations, intratumoral cytogenetic heterogeneity and histologically they display brisk mitotic activity, nuclear pleomorphism as well as dense cellularity. Specially,
relative to lower grade astrocytomas, GBMs exhibit more advanced phenotypical features of malignancies, including enhanced regions of necrosis and microvascular proliferation. GBMs have significant intra-tumor regional heterogeneity, which is comprised of foci of necrosis surrounded by hypercellular cells in the peripheral edge often called pseudopalisades. Furthermore, GBMs reside in a highly vascular stroma displaying microvascular hyperplasia, a form of neo-vascularization, that in extreme cases give rise to glomeruloid bodies by piling of endothelial cells (EC).

In Europe and North America, the incidence of GBMs is approximately 3 new cases per 100,000 persons annually. Similar to other type of cancers, increasing age correlates with the higher incidence of GBM with the median age of diagnosis being 64 years. GBM occurs in both men and women, however they are more frequent in males than females (with a ratio of 1.58:1) and more common in Caucasians than in African-Americans. The etiology of gliomas remains largely unknown. Specific causal elements have not been identified for gliomas, nonetheless some factors such as radiation, increased cell phone usage, pesticide exposure and head trauma have been suggested as potential risk factors, but most studies have been incapable of establishing strong and consistent associations between these exogenous factors and GBM development, with an exception of exposure to high-dose radiation.
Figure 1.1: Distribution of primary brain and central nervous system tumors (A) and gliomas (B). Source: Charts modified from The Central Brain Tumor Registry of the United States (CBTRUS).

A

Glioblastoma 17%

Ependymomas 2%
Nerve sheath 9%
Lymphoma 2%
All other 13%
Pituitary 12%
Meningioma 34%
Cranioopharyngioma 0.7%
Astrocytomas 7%
Oligodendrogliomas 2%
Embryonal, including medulloblastomas 1%

B

Glioblastoma 54%

Ependymoma
Protoplasmic and fibrillary astrocytoma 2%
All other astrocytoma 9%
Pilocytic astrocytoma 5%
Oligodendroglioma 6%
Anaplastic astrocytoma 7%
All other glioma 11%
1.1.2 Classification and molecular pathology

GBMs are subtyped into primary or secondary tumors based on their clinical and biological presentation. This distinction between primary and secondary GBMs was first noted by a German neuropathologist Hans-Joachim Scherer around 1940s[14,15]. Now it is well recognized that primary or de novo GBMs develop without any recognizable precursor lesions and have a short clinical history. Secondary tumors progressing from lower grades are rare (~5%) and tend to occur more in younger patients below the age of 45[16]. Primary GBMs account for the majority (90%) of GBM cases; present at an older age manifesting with faster rate of tumor progression, more aggressive clinical behavior and lower patient survival rate compared to secondary GBMs[16]. Remarkably, despite their variable clinical histories, primary and secondary GBMs are histologically and morphologically indistinguishable[16]. Although both subtypes have similar phenotypical features, it is now recognized that these entities evolve through different molecular mechanisms and may encompass two distinct sub-variants of GBMs that have different genetic and molecular aberrations[4].

Great progress has been made in deciphering the molecular and genetic pathology or evolution of GBMs in the last two decades. Malignant transformation of GBMs results partly from progressive accumulation of multiple molecular aberrations, occurring at the genetic and epigenetic levels, including chromosomal gains or losses, gene mutation, amplification, deletion, DNA and histone methylation or demethylation, and transcriptional regulation[7,17]. Primary GBMs are genetically characterized by epidermal growth factor receptor (EGFR) amplification and EGFR mutations (most commonly EGFRvIII variant), loss of heterozygosity (LOH) of chromosome 10q, and deletion of the phosphatase and tensin homolog (PTEN) and the cyclin-dependent kinase inhibitor 2A (CDKN2A) genes[5,18,19]. The genetic abnormalities in secondary GBMs include tumor protein p53 (TP53) and retinoblastoma (RB) mutations, overexpression of platelet derived growth factor A and platelet derived growth factor receptor alpha (PDGFA/PDGFRα) and LOH of 19q[4,7]. The identification of different pattern of genetic and molecular signatures that underlie GBM pathogenesis have opened a new and exciting therapeutic avenue with hope that this knowledge would identify patients that would most likely benefit from a distinct targeted therapies or treatment strategies.

The use of comprehensive genome-wide approaches to analyze genetic and epigenetic alterations
in GBMs, as is done by The Cancer Genome Atlas (TCGA), has strengthened our understanding of GBM’s genetic and molecular landscape and has revealed additional insights such as presence of unique prognostic or diagnostic biomarkers. Tools such as gene copy number, gene sequencing, epigenetic methylation, gene expression profile, and micro RNA profiling of GBM specimens have helped with sub-classification of GBM patients with differing prognosis and/or response to specific therapies. The three principal pathways consistently deregulated in most GBMs are receptor tyrosine kinase (RTK) signaling, TP53 and RB tumor suppressor pathways. In fact, 74% of GBMs harbored alterations in all the three pathways. The importance of mutations such as neurofibromatosis 1 (NF1), V-Erb-B2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) and isocitrate dehydrogenase 1 (IDH1) has also been recently illustrated by genomic analysis. Mutations in IDH1 are observed in about 70% of low grade and secondary GBMs suggesting a potential diagnostic marker of secondary GBMs. Interestingly, IDH1 mutations in primary GBMs are reported in very low frequencies of 3-7%, making IDH1 a positive prognostic factor.

In parallel, cDNA expression profiling has been profound in providing new data recognizing the distinct molecular subgroups of GBMs, each harboring unique molecular and genetic aberrations that drive tumorigenesis. Verhaak and coworkers proposed four distinct molecular patterns for primary GBMs: proneural, neural, classical and mesenchymal, each with distinct prognostic implications. Phillips, Aldape, and colleagues recognized proneural and mesenchymal as being the two principle categories in gliomas among other subclasses prior to TCGA subgrouping. These classifications provide new insights into patterns of disease progression and importantly confirms GBM’s heterogeneous landscape, with hope that current information would one day facilitate diagnosis and future personalized therapies for patients with distinct molecular signatures.

1.1.3 Treatment strategies in GBMs

The current treatment regimen for newly diagnosed GBM patients involves maximal safe surgical resection followed by radiotherapy with concurrent adjuvant chemotherapy, temozolomide (TMZ). While maximal surgical debulking is preferred, complete resection is not entirely possible owing to the highly infiltrative and invasive nature of GBMs together with surgical limitation in resection of lesions involving eloquent brain. These features make
complete surgical resection highly challenging and results in tumor recurrence/regrowth from the remnant invasive or resistant cells that evaded therapy.

Despite multimodal treatment approaches including surgery, concurrent or sequential chemoradiotherapy with TMZ, patients’ outcome is dismal for newly diagnosed GBMs with reported median survival being around 15 months post-diagnosis \(^{27,28}\). Only 10% of GBM patients survive five years post-diagnosis, regardless of the continual growth in wealth of knowledge in GBM pathogenesis and therapeutic advancements \(^{27-29}\). On the basis of several meta-analyses including the international randomized trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC), addition of TMZ to radiotherapy have shown significant survival benefit for newly diagnosed GBM patients \(^{30}\). TMZ is the only approved chemotherapy drug that has shown some efficacy in this aggressive disease, mostly owing to its radio-sensitization abilities \(^{30,31}\). TMZ is a DNA alkylating prodrug that is converted into an active diazonium salt under physiological pH. TMZ transfers an alkyl (methyl) group to three main locations on the DNA molecule: N\(^7\) guanine (70% of adducts), O\(^6\) guanine (5% of adducts, most toxic), and N\(^3\) adenine (9% of adducts), damaging the DNA and triggering tumor cell death \(^{32}\). A landmark clinical study carried out by Stupp et al, made concomitant radiation and TMZ, followed by adjuvant TMZ, the standard of care for newly diagnosed GBM patients – approximately a decade ago \(^{30}\). The Stupp regimen showed modest but clinically significant results by increasing the median overall survival from 12.1 months with radiation alone to 14.6 months in concurrent treatment group. The 2-year survival rate for patients with concurrent treatment was 26.5%, relative to only 10% for radiation alone \(^{30}\). Importantly, patients with tumors containing O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation displayed a better survival rate compared to those without a methylated promoter \(^{30}\). The 2-year survival rate of patients in combined TMZ and radiation treatment was 46% with MGMT promoter methylation compared to 13.8% with unmethylated MGMT promoter \(^{30}\). MGMT, among one of the strongest predictor of survival for newly diagnosed GBMs, is a DNA repair enzyme capable of counteracting cytotoxicity effects due to TMZ, thus its promoter methylation and epigenetic silencing can decrease DNA repair activity and increase the susceptibility of tumor cells to TMZ and cell death. Although MGMT methylation fails to reliably predict patient outcomes in response to therapy, it is nevertheless the only currently recognized or available prognostic biomarker of response to TMZ and radiation in GBMs \(^{33,34}\).
1.1.4 Novel targeted therapies and current clinical trials

The prognosis for GBM patients has remained relatively unchanged in the last 20 years, despite advancements in medical research and adjuvant therapies. This is partly owing to the hallmark feature of malignant glioma cells that display resistance to therapy, including radiation and chemotherapy and most recently targeted therapeutics such as anti-angiogenic therapy. In addition, the heterogeneous microenvironment of GBMs and variability in genetic profile confers additional layers of complexity to the pathology and response to therapy\(^1,2\). Successful treatment of GBMs requires thorough investigation of factors that underline GBM pathogenesis, with the ultimate goal of translating laboratory found data to the clinic to develop a more personalized and targeted therapy that encompasses the heterogeneity of GBMs. Many clinical studies are using new approaches to improve and overcome resistance to conventional therapies such as modifying conventional TMZ scheduling, combining TMZ and radiation with other targeted agents or testing novel molecular targeted agents with current standard of treatment\(^27\). Targeted therapies are aimed at directing the treatment towards specific and unique features of GBM tumor cells, including specific molecular aberrations, receptor targets, altered signaling or metabolic pathways, tumor angiogenesis or microenvironmental factors such as hypoxia\(^27\). The main objective of targeted therapies is to lay the structure for identification of specific predictive biomarkers such that patients who are most likely to benefit from a particular therapy can be selected for treatment. Currently there are numerous ongoing pre-clinical and clinical targeted therapies that aim at altering factors such as cell survival, proliferation, apoptosis, invasion, metabolism or angiogenesis in GBMs. **Table 1.1** provides an overview of selected targeted therapeutics in GBMs\(^35\).
Table 1.1: Selected molecular targets currently in clinical/pre-clinical trials in gliomas.

<table>
<thead>
<tr>
<th>PRIMARY TARGET</th>
<th>AGENT</th>
<th>OTHER TARGETS</th>
<th>MECHANISM OF ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Gefitinib (ZD1839)</td>
<td></td>
<td>TKI</td>
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<tr>
<td></td>
<td>Erlotinib (OSI-774)</td>
<td></td>
<td>TKI</td>
</tr>
<tr>
<td></td>
<td>Lapatinib (GW-572016)</td>
<td></td>
<td>TKI</td>
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<tr>
<td></td>
<td>PF-00299804</td>
<td>HER-2, HER-4</td>
<td>TKI</td>
</tr>
<tr>
<td></td>
<td>BIBW2992</td>
<td>HER-2, HER-4</td>
<td>TKI</td>
</tr>
<tr>
<td></td>
<td>Cetuximab</td>
<td></td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td></td>
<td>Nimotuzumab</td>
<td></td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>EGFR-vIII</td>
<td>CDX110</td>
<td></td>
<td>Vaccine</td>
</tr>
<tr>
<td>FGFR</td>
<td>Brivanib (BMS-582664)</td>
<td>VEGFR2</td>
<td>TKI</td>
</tr>
<tr>
<td>HDAC</td>
<td>Vorinostat (SAHA)</td>
<td></td>
<td>HDAC inhibitor</td>
</tr>
<tr>
<td>c-Met</td>
<td>XL184</td>
<td>VEGFR</td>
<td>TKI</td>
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<tr>
<td>mTOR</td>
<td>Sirolimus (rapamycin)</td>
<td></td>
<td>mTOR inhibitor</td>
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<tr>
<td></td>
<td>Everolimus (RAD001)</td>
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<td>mTOR inhibitor</td>
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<tr>
<td></td>
<td>Temsirolimus (CCI-779)</td>
<td></td>
<td>mTOR inhibitor</td>
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<tr>
<td></td>
<td>Ridaforolimus (AP23573)</td>
<td></td>
<td>mTOR inhibitor</td>
</tr>
<tr>
<td>PDGFR-a</td>
<td>MC3G3</td>
<td></td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>PDGFR-b</td>
<td>Imatinib</td>
<td>BCR/Abl, c-Kit</td>
<td>TKI</td>
</tr>
<tr>
<td></td>
<td>Dasatinib</td>
<td>Src, c-Kit,</td>
<td>TKI</td>
</tr>
<tr>
<td>Abbreviations: EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; FGFR: fibroblast growth factor receptor; VEGFR2: vascular endothelial growth factor receptor 2; HDAC: histone deacetylase; c-Met: met protein oncogene; HER: human epidermal growth factor receptor; mTOR: mechanistic target of rapamycin; HGF/SF: hepatocyte growth factor/scatter factor; PDGFR: platelet-derived growth factor receptor; BCR/Able: breakpoint cluster region/abelson murine leukemia viral oncogene; c-Kit: tyrosine-protein kinase Kit; Src: sarcoma; B-Raf: v-Raf murine sarcoma viral oncogene homolog B; 1Flt3: fms-like tyrosine kinase 3; RET: ret proto-oncogene; PI3K: phosphoinositide 3-kinase; STKI: serine-threonine kinase inhibitor; PKC: protein kinase C; VEGF: vascular endothelial growth factor; PlGF: placental growth factor; Tie-2: angiopoietin-1 receptor.</td>
<td>Tandutinib (MLN518)</td>
<td>Flt3, c-Kit</td>
<td>TKI</td>
</tr>
<tr>
<td></td>
<td>Afibercept (VEGF Trap)</td>
<td>VEGF-B, PlGF</td>
<td>Soluble decoy receptor</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab</td>
<td></td>
<td>Monoclonal antibody</td>
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</tbody>
</table>

| VEGF-A | Cediranib (AZD2171) | VEGFR-3, B-Raf, PDGFR, c-Kit, |
| | CT-322 | All VEGFRs, PDGFR, c-Kit |
| | Pazopanib | All VEGFRs, PDGFR, c-Kit |
| | Sorafenib | VEGFR-3, B-Raf, PDGFR, c-Kit, |
| | Sunitinib | PDGFR, Flt3, c-Kit |
| | Vandetanib (ZD6474) | EGFR |
| | XL-184 | c-Met, RET, c-Kit, Flt3, Tie-2 |

Current experimental strategies for GBMs and other gliomas include monotherapy by targeting and inhibiting kinases such as EGFR or PDGFR, combination of agents that inhibit complementary targets such as EGFR/PI3K and mTOR (e.g. erlotinib + temsirolimus) or combination therapy with new targeted agents combined with conventional radiochemotherapy (e.g. cetuximab + TMZ and radiation) \(^4,36\). In addition to these molecular targets, inhibiting blood vessel formation has been shown to be a promising strategy for GBM treatment. Anti-angiogenic therapies have been extensively tested in pre-clinical and clinical settings in North America and throughout Europe \(^37-40\). Bevacizumab (Avastin), a humanized monoclonal antibody that inhibits
VEGF, has been FDA (The food and drug administration) approved in the United States for treating recurrent GBMs. The use of anti-angiogenic therapies has been controversial due to concerns regarding infiltrative and a more aggressive pattern of recurrence following treatment. Currently a large international randomized double-blind phase III clinical trials (AVAglio, BO21990, NCT00943826) with anti-angiogenic agent, Bevacizumab, in combination with radiochemotherapy has been completed for newly diagnosed GBMs with results pending. This study will be instrumental in determining the safety and efficacy of combining Bevacizumab with standard therapies in GBMs.

Identifying and developing effective molecular targeted therapies faces several challenges mainly due to GBMs invasiveness and its intratumoral heterogeneity at the genomic and molecular level. Regardless, the acquired knowledge regarding GBMs aberrant signaling pathways, mutations and epigenetic changes have been instrumental in narrowing down the search to a limited set of players on which to focus further therapeutic investigations.

1.2 Metabolism

1.2.1 Metabolism and bioenergetics of normal Cells

In normal cell bioenergetics, carbohydrates, proteins and lipids are metabolized to synthesize essential cellular building blocks such as nucleic acids, nonessential amino acids, fatty acids and other precursor molecules. Furthermore, glucose metabolism is coupled to mitochondrial respiration to produce energy requirement in a form of adenosine triphosphates (ATP) for various cellular processes. Glutamine is another substrate catabolized in most mammalian cells; together with glucose they supply carbon, nitrogen, energy source and reducing equivalents required for cell growth and division. Besides ATP synthesis, glycolysis generates several metabolic intermediates that can be diverted for biosynthesis of essential macromolecules such as fatty acids from Acetyl coenzyme A (acetyl-CoA), nucleic acids from ribose, nonessential amino acids, and other biomolecules that make up cells and tissues.

1.2.2 Normal glycolytic metabolism

Glycolysis is amongst the most ancient and conserved molecular networks where a glucose molecule is metabolized and broken down across 10 enzymatic steps to produce pyruvate.
Figure 1.2 shows glycolysis and the cellular fate of glucose. Glucose enters the cell through facilitated glucose transporters (GLUTs) and is trapped intracellularly through phosphorylation by hexokinase (HK) enzymes to produce glucose-6-phosphate (G6P). Conversion of glucose to G6P is the first rate-limiting step allowing entrapment of glucose in the cytoplasm; otherwise glucose can be exported out from the cell. Phosphoglucone isomerase is the second enzyme catalyzing G6P to fructose-6-phosphate (F6P), which is converted to fructose-1,6-bisphosphate by the third enzyme phosphofructokinase 1 (PFK1). Aldolase in turn metabolizes fructose 1,6-bisphosphate to yield two three-carbon molecules of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Pyruvate kinase (PK) catalyzes the ultimate step of glycolysis by converting phosphoenolpyruvate (PEP) to pyruvate and a molecule of ATP. Glycolysis can occur independent of molecular oxygen. In anaerobic conditions of growth, the pyruvate molecule is ultimately reduced to lactate or ethanol by fermentation. Glycolysis forms 2 pyruvate molecules from degradation of the carbon skeleton of glucose, 2 molecules of ATP that are high-energy phosphate compounds, and 2 molecules of NADH (reducing agents) by transferring of a hydride ion to NAD$^+$–per molecule of glucose. Thus, during catabolism of glucose, some of the energy of the glucose molecule is conserved in the form of ATP and NADH, whilst much more energy remains in the end product, pyruvate. The overall product of glycolysis can be summarized in the following equation:

\[ \text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{Pi} \rightarrow 2 \text{pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O} \]

When oxygen is present in the environment, cytosolic pyruvate can enter the mitochondria and get converted to acetyl-CoA to initiate the Krebs cycle also known as the tricarboxylic acid (TCA) or citric acid cycle. Ultimately, for each glucose molecule (2 pyruvates), 8 NADH, 2 FADH$_2$ (flavin adenine dinucleotide) and 2 ATP molecules are produced in the Krebs cycle before oxidative phosphorylation starts.
Figure 1.2: Glycolysis and the enzymes involved. Glucose is broken down to two molecules of pyruvate through 10 enzymatic reactions and during the sequence of reactions some of the free energy released is conserved in the form of ATP and NADH. Pyruvate is further metabolized and reduced in anaerobic conditions to lactate or oxidized in the mitochondria.
1.2.3 Tricarboxylic acid (TCA) cycle and OXPHOS

Before the existence of atmospheric oxygen, anaerobic glycolysis or fermentation evolved to produce energy for anaerobic life forms \(^{45}\). After emergence of oxic environment, pyruvate was able to be oxidized by oxidative phosphorylation (OXPHOS) in the mitochondria to produce and maintain high-energy status in the cell. When oxygen is present, glucose-derived pyruvate is converted to acetyl-CoA and two molecules of NADH (Figure 1.3). Acetyl-CoA is further oxidized by mitochondrial enzymes via TCA cycle to produce reducing equivalents that fuel the respiratory chain (OXPHOS) to maximize ATP production. In the mitochondria, acetyl-CoA is broken down through 8 steps to produce intermediates of citrate, isocitrate, a-ketoglutarate, succinyl-CoA, succinate, fumarate, malate, and oxaloacetate. Most of the carbon for de novo fatty acid synthesis is driven from mitochondrial matrix intermediates (Figure 1.4). In this case, mitochondrial acetyl-CoA condenses with oxaloacetate to form citrate, which is transported outside of the mitochondria and is broken down into its constituents by cytoplasmic ATP citrate lyase (ACL). Fatty acid synthase (FASN) can synthesize long-chain fatty acids by using acetyl-CoA as a primer, malonyl-CoA as a two-carbon donor, and NADPH as a reducing equivalent. Oxaloacetate and a-ketoglutarate can also be used for the synthesis of non-essential amino acids. Similar to citrate, malate produced in the TCA cycle can be transported to the cytoplasm and converted to pyruvate and/or lactate plus NADPH. Citrate might also be converted to isocitrate and in turn to a-ketoglutarate, generating another molecule of NADPH by the action of the enzyme isocitrate dehydrogenase 1 (IDH1). Reducing equivalents generated by glycolysis and the TCA cycle, namely NADH and FADH\(^2\), are fueled into the respiratory chain in the mitochondria to be oxidized by the process of respiration. Electrons are passed on within the respiratory chain to generate an electrochemical gradient across the inner mitochondrial membrane. This generated proton gradient or mitochondrial membrane potential (MMP) is used to drive the synthesis of ATP from ADP and inorganic phosphate ion through OXPHOS. Thus, most non-proliferating and differentiated cells rely on the efficiency of OXPHOS to produce sufficient ATP to maintain their integrity.
Figure 1.3: Glycolysis, pentose phosphate pathway and TCA cycle. Source: Diagram was modified with permission from Pavlides et al. Cell Cycle 8(23): 2009, 3984-4001. PMID: 19923890.
1.2.4 Glutaminolysis

There are two major sources of carbon for the cell, glucose and glutamine. Glutamine is one of the most abundant naturally occurring amino acids in the body and it can supply the carbon backbone to the TCA cycle during catabolic conditions. Glutaminolysis can yield substrates such as α-ketoglutarate to feed and replenishing the TCA cycle (Figure 1.3), produce glutathione (GSH) for anti-oxidant defense of the cell and allocate building blocks for lactate, amino acid and nucleotide synthesis. Once in the cell, glutaminase enzymes generate glutamate by deamination of glutamine. Glutamate in turn can be converted to α-ketoglutarate and enter the TCA cycle. This process, referred to as anapleurosis, supplies carbon input to replenish the intermediates of the TCA cycle, permits the production of other amino acids, fatty acids, lactic acid and maintains the NADPH pool in the cell. In addition, glutamate can be converted into the reduced tripeptide GSH, which is an antioxidant molecule fundamental for controlling the redox state of cellular compartments. In the eukaryotic cells, several mechanisms such as GSH have been evolved to counteract and defend the cell against destructive effect of reactive oxygen species (ROS). In the biological system oxygen-derived free radicals are collectively known as ROS, comprise of hydrogen peroxide H₂O₂, hydroxyl radical HO• or superoxide O₂⁻. Mitochondria are the major producer of ROS. At normal physiological levels, ROS have been shown to be important in cellular signaling as second messengers to transmit signals such as pro-inflammatory or growth-stimulatory signals. However, at high concentrations, ROS can oxidize and damage macromolecules such as nucleic acids, proteins and fatty acids, and can influence the function of many biochemical reactions through redox-imbalance and create oxidative stress for the cell.

1.2.5 Metabolic reprogramming in cancer cells: The Warburg effect

In 1926, Herbert G. Crabtree made an interesting observation that mitochondrial respiration can be suppressed by glycolysis in cells that possess approximately equal glycolytic and respiratory capabilities, a phenomenon commonly known as the Crabtree effect. Rapidly proliferative normal cells such as thymocytes or spermatozoa display the Crabtree effect, where a high rate of glycolysis in these cells significantly inhibits oxygen consumption. This phenomenon is also prevalent in malignant tumor cells where a high rate of glycolysis is accompanied with partial mitochondrial suppression/under-activation, impaired respiration, reduced oxygen consumption,
and lower ATP production. Unlike normal cells that undergo glycolysis or fermentation when oxygen is limited, cancer cells undergo glycolysis with increased lactate production even in the presence of adequate cellular oxygen \(^2\) (Figure 1.4). It has been shown that tumor cells exhibit various metabolic anomalies, however preferential metabolism of glucose to lactate regardless of oxygen availability, is perhaps best known, studied and the most relevant to tumor cell proliferation. This phenomenon is commonly known as aerobic glycolysis or the Warburg effect, since it was first discovered by the Nobel Prize winner Otto Warburg in the 1920s \(^{53,54}\). The Warburg phenomenon is often accompanied by increased glucose uptake, and this occurrence can be visualized in tumors using \(2-(^{18}\text{F})\)-deoxyglucose positron emission tomography (FDG–PET) imaging \(^5\). FDG–PET is a medical imaging test used as a staging tool for different types of cancers, where tracing of differentially metabolized glucose analogue –FDG– can distinguish cancer cells from their normal counterpart based on high cancer metabolism of glucose (FDG).

The Warburg effect is not specific to tumor cells and it can be seen in normal rapidly proliferating/dividing cells of the body such as proliferating T-cells or during reprogramming of fibroblasts into pluripotent stem cells \(^{55,56}\). Recently, the implications and better understanding of cancer metabolism has regained traction since the first observations by Otto Warburg that glucose metabolism is fundamentally different in cancer cells \(^{53}\). Warburg observed that normal cells produce most of their energy through mitochondrial respiration when oxygen is present. In contrast, cancer cells showed excessive “fermentation” of glucose to lactate acid even when ample oxygen was present for normal respiration such that over 50% energy in the cell was generated via glycolysis, and the rest through OXPHOS \(^5\). The reprogramming of energy metabolism is a robust metabolic hallmark of tumor cells and is seen in several types of cancers including GBMs \(^{57}\). Although Warburg mistakenly attributed the altered metabolism to irreversibly damaged mitochondria, nevertheless his contributions were pivotal to an early understanding of cancer metabolism. In contrast to Warburg’s original hypothesis, which stated that defective mitochondrion was at “the root” \(^{53,54}\) of the metabolic impairment, currently it is thought that in most cases mitochondrial metabolism is reprogrammed and is not necessarily defective \(^{58,59}\). An initial misconception was that proliferating cancer cells do not utilize their mitochondria. However, it has been shown that most proliferating cancer cells still derive a big proportion of their energy from oxidative respiration \(^{58,59}\). However, compared to quiescent cells, the OXPHOS-dependent production of ATP in these cells appears to be secondary to the use of mitochondrial enzymes in the synthesis of anabolic precursors to support biomass accumulation...
**Figure 1.4: Metabolism in resting versus proliferating cells.** Energetic demand of resting cells is mostly met through oxidative metabolism of glucose to produce large amount of ATP (left). In proliferating cells, energetic demand is mostly met through aerobic glycolysis (right) by providing bioenergetics, biosynthetic precursors and maintaining the redox balance.
In most cancer cells with a highly glycolytic phenotype, OXPHOS operates at a low capacity and in some cases shows remnants of the Crabtree effect: suppression of respiration by glycolysis \(^{58,59}\).

Several tumor-specific alterations in glycolytic and TCA cycle enzymes, aberrant oncogenic and hypoxia signaling have been shown to be linked to the distinct bioenergetic phenotype of cancer cells. However, the principal rational for aerobic glycolysis is still elusive and probably varies for different cancer types and tissues \(^{60}\). Some argue that altered tumor metabolism has arisen from the selective pressures of the hypoxic tumor microenvironment \(^{60}\). Under hypoxic conditions, the reliance on aerobic glycolysis can become fundamental for proliferating cells, where hypoxic stress can activate almost all the enzymes of glycolysis and kick start glycolysis \(^{57}\). However, the basis for the metabolic switch cannot be explained solely by cellular adaptation to hypoxia and some have argued that proliferative cancer cells may already contain a reprogrammed metabolism before encountering a hypoxic environment \(^{57,60}\). Strong evidence now exists to suggest that other factors such as proto-oncogenes, tumor suppressors and other microenvironmental factors may evolve to regulate metabolism \(^{58}\).

Regardless of its origin, cancer cells exhibit multiple alterations in substrate (glucose and glutamine) metabolism to support their uncontrolled growth and proliferation. Although the mechanistic transition from normal respiration to aerobic glycolysis is not well understood, different players and factors have been discovered and implicated in regulating the Warburg effect in a number of malignancies. In order to better explain the metabolic remodeling, we can discuss putative advantages that exist with altered glycolysis in rapidly proliferating tumor cells.

### 1.2.6 The putative advantages of the Warburg effect

The question arises as to what are the advantages or functional rational for alteration of cancer cell metabolism. The reprogramming of cellular metabolism is accompanied by several key events namely enhanced glucose uptake and glycolysis, increased synthesis of intermediates for biosynthetic pathways, increased lactate levels both intra- and extracellular and decreased rate of ATP production (Figure 1.4 and 1.5). The maintenance of the glycolytic switch is accompanied by uncoupling of glycolysis from OXPHOS, with partial suppression of respiration by OXPHOS \(^{58,59}\). At first glance increased glycolysis seems inefficient for energy production, since anabolic
metabolism is not focused on maximizing ATP, and substantially less ATP per glucose (2 ATPs compared to ~36 ATPs) is produced through aerobic glycolysis. However, most cancer cells do adopt this inefficient glycolytic mode of ATP synthesis, leading to the postulate that this altered metabolism renders cancer cells a survival advantage. The evidence is developing for the proposal that proliferating cells have requirements that extend beyond ATP or energy maximization\textsuperscript{52}. Besides providing a steady supply of ATP, glycolysis assures constant level of metabolic intermediates as a fuel for rapidly growing cells\textsuperscript{52}. Since proliferating cells have to divide faster and make new membrane, they are in much greater need for reduced carbon and nitrogen source for macromolecule biosynthesis such as lipids, proteins and nucleic acid than ATP. Thus an abnormally high rate of glucose import and glycolysis is implemented to meet the these demand\textsuperscript{52}. In essence, aerobic glycolysis allows for rechanneling of intermediate metabolites towards anabolic pathways to promote cell growth and proliferation. It is therefore postulated that metabolic rewiring in cancer is possibly an adaptive response to meet the challenges of rapidly proliferating tumor cells\textsuperscript{52}. As suggested by Vander Heiden, aerobic glycolysis satisfies the metabolic requirement of cancer cells by providing biosynthetic precursors for rapidly dividing cells, and that the high flux of substrates down the glucose pathway provides carbon and nitrogen source needed for generation of macromolecules such as lipids, proteins and nucleotides (Figure 1.4 and 1.5)\textsuperscript{52}. For instance, in order to sustain the rapid cellular proliferation, membrane formation and DNA assembly, tumor cells have to increase de novo synthesis of fatty acids and nucleotide precursors\textsuperscript{52}. One mechanism that has evolved to help this process is the diversion of glycolytic intermediates –G6P or fructose 6-phosphate– into the pentose phosphate pathway (PPP) to synthesize ribose 5-phosphate (R5P), which in turn can be used to synthesize nucleotides for DNA and RNA\textsuperscript{52}. The entry of G6P into this pathway is governed by glucose-6-phosphate dehydrogenase (G6PD) enzyme, converting G6P to R5P producing two molecules of NADPH. NADPH is vital for cellular redox control as a reducing agent, where a high ratio of NADPH to NADP can prevent or reduce intracellular oxidative damage. NADPH is also vital as a cofactor for biosynthetic reactions to synthesize fatty acids, nucleotides, and amino acids.
Figure 1.5: Metabolic demand in resting versus proliferating cells. Normal cells implement catabolic metabolism to satisfy energetic demand of the cells (top). In tumor cells there is an increase in cellular uptake of glucose and glutamine to meet the anabolic demand of macromolecular biosynthesis, as well as changes in redox balance (bottom).
The molecular mechanism of aerobic glycolysis is complex and research has focused on elucidating deregulated roles of glycolytic enzymes and their effect on the metabolic network. In this regard, overexpression of key enzymes of oxidative and non-oxidative branches of the PPP, G6PDH and transketolase (TKT), have been shown to play an important role in cancer by facilitating nucleotide synthesis. Also, changes in de novo fatty acid biosynthesis have been documented in many cancers to facilitate membrane assembly in rapidly dividing tumor cells. Normal cells tend to use circulating lipids (exogenous lipids from dietary intake) for the synthesis of new structural lipids. However, tumor cells have been shown to utilize de novo synthesis of fatty acids to produce more than 93% of triacylglycerol fatty acids, which is necessary to maintain a constant supply of lipids to power membrane production in a highly proliferating cell. In glycolytically-converted tumor cells, citrate generated from the mitochondria can be transported to the cytoplasm where it is converted back to acetyl-CoA and used as a building block for fatty acid synthesis. In fact, ATP citrate lyase (ACL) activity has been shown to be increased in some cancer cell lines, while inhibition of ACL may lead to cancer growth termination. Also, hyperactivity and overexpression of fatty acid synthase (FASN), the key enzyme responsible for the terminal catalytic step in fatty acid synthesis, is one of the most frequent alterations in cancer cells.

Excess lactate production is one of the most prominent features of altered metabolism towards fermentation. Excess pyruvate generated by glycolysis that is not fed into the TCA cycle is either converted to lactate and alanine in the cytoplasm or re-wired for lipid synthesis. Surplus lactate accumulated in the glycolytic cell has to be excreted from the cell though a family of monocarboxylate transporters (MCT) along with a proton to maintain intracellular alkalization. This acidic extracellular environment created by lactate can potentially favor tumor cell invasion through activation of cathepsins and metalloproteinases to help degrade extracellular matrix and basement membrane. Also, extracellular lactate has an immune suppressive effect, enhancing cancer cell survival and apoptosis resistance to harsh environmental conditions such as hypoxia or radiation and chemotherapy. In addition, it has been recently proposed that lactate in tumor microenvironment can be used as a potential fuel, where it can re-enter cells within the tumor and act as a fuel for oxidative phosphorylation in oxygenated areas. This metabolic symbiosis or the reverse Warburg effect carried out by lactate or other metabolites has to be re-evaluated further in various cancers.
In addition, metabolic reprogramming confers a survival advantage for the tumor cells and leads to resistance to apoptosis and other stressful environmental conditions.\textsuperscript{52} Cancer cells that undergo aerobic glycolysis might have the ability to suppress the intrinsic mitochondrial apoptotic program through mechanisms regulated by pro-apoptotic proteins of the Bcl-2 family. It has been shown that metabolic enzymes such as hexokinases (mostly HK2) can bind to the mitochondrial outer membrane and suppress the binding and activation of pro-apoptotic proteins such as Bax and Bak. This may help protect the cell against mitochondrial-associate apoptosis and promote cancer cell survival\textsuperscript{69}. In addition, changes in mitochondrial membrane potential have been documented in cells that undergo aerobic glycolysis. It has been shown that the glycolytic shift in tumor cells makes the outer mitochondria membrane less susceptible to permeabilization and promotes hyper-polarization of the mitochondrial membrane potential and subsequent resistance to mitochondrially-mediated cell death\textsuperscript{69-71}. Glycolytic enzymes such as HK1 and 2 are able to bind to the mitochondrial voltage dependent anion channel (VDAC) and can trigger a chain of events that would regulate the intrinsic apoptotic pathway abrogating apoptosis\textsuperscript{70,72}. Furthermore, strong evidence now exists to support that enhanced diversion of glycolytic intermediates such as G6P into the pentose phosphate pathway (PPP) can lead to increased production of NADPH, which may help to promote cellular antioxidant defense via reduction of glutathione to scavenge ROS, thus conferring another survival benefit for the tumor cells\textsuperscript{52,73}. Repression of respiration or underutilization of mitochondria through the Warburg effect can be accompanied by the reduction of ROS production. As mentioned before, during mitochondrial respiration, ROS can leak out of respiratory chain to the cytoplasm and in pathological states where ROS is in excess, it can lead to cellular damage and induction of apoptosis. Thus, suppression of OXPHOS or mitochondrial respiration by the Warburg effect may modulate cellular redox balance and protect cells from ROS-induced oxidative stress and associated cellular damage. It is interesting to note that cells without mitochondrial DNA are resistant to ROS generated apoptosis\textsuperscript{60,74}. In addition to the above mentioned survival advantages, the metabolic shift in cancer may promote adaptation of tumor cells to environmental stressors such as conditions of fluctuating oxygen (hypoxia) levels or nutrient levels that can be brought about by vascular irregularities in tumors such as GBMs\textsuperscript{75}. 
1.2.7 Beyond the Warburg effect

The metabolic adaptation in the tumor microenvironment can be influenced by various environmental factors such as hypoxia, the status of oncogenes and tumor suppressors and most likely factors that are not yet realized. Tumor hypoxia has been recognized as one of the most prominent features of the neoplastic microenvironment. Solid tumors such as GBMs frequently harbor populations of cancer cells with dynamic oxygen gradients thought to be brought about by a disorganized and chaotic tumor vascular network. Low oxygen tension or hypoxia (chronic and acute hypoxia or anoxia) have been shown to have different effects on cellular processes; One of the major players in hypoxia adaptation is activation and stabilization of HIF1α and 2α resulting in activation of a cascade of downstream regulators with a broad functional consequence in various cancers. The most fundamental result of HIF1α activation is a shift in energy status by inducing genes involved in aerobic glycolysis and angiogenesis, ultimately resulting in regulation of oxygen delivery and consumption. Hypoxia orchestrates adaptation of tumors to metabolic microenvironmental stressors by induction of almost all the genes that encode glycolytic enzymes, glucose transporters (GLUT1 and GLUT3), as well as lactate exporters and pH regulators (MCTs, CAs). HIF1α can promote the expression of multiple metabolic proteins such as hexokinase (HK), phosphofructokinase 1 (PFK-1), aldolase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDHA) or plasma membrane MCTs (MCT1 or 4) or carbonic anhydrases (CAXI and CAXII) to stimulate the glycolytic flux and facilitate lactate shuttling into the extracellular space. Simultaneously, HIF1α can limit OXPHOS and suppress pyruvate entry to TCA cycle by stimulating pyruvate dehydrogenase kinase 1 (PDK1), which inhibits the mitochondrial pyruvate dehydrogenase (PDH) activity, thus attenuating pyruvate oxidation in the Krebs cycle and increases the generation of lactate from pyruvate. It has been also shown that HIF1α can cooperate with oncogenic MYC (v-myc myelocytomatosis viral oncogene homolog) to promote OXPHOS-uncoupled glycolysis and aid in the anapleurotic process in the mitochondria. HIF1α also augments angiogenesis and endothelial cell proliferation by increasing the expression of angiogenic factors such as vascular endothelial growth factors (VEGF)-A and angiopoietins. Other molecular mechanisms involving non-metabolic genes such as known oncogenes and tumor suppressors have been implicated to converge and regulate different steps of the metabolic pathways. In fact, most metabolically linked molecular changes lie directly
downstream of various oncogenes or tumor suppressor pathways\textsuperscript{58}, and it has been proposed that these metabolic and non-metabolic traits of malignancies may have been co-selected or co-evolved during transformation to maximize tumor growth\textsuperscript{58,59}.

The close interplay between metabolic enzymes and transcriptional regulators along with tumor metabolites may establish a heterogeneous metabolic microenvironment within tumors. For instance, \textit{PI3K/AKT}, \textit{TP53} and \textit{Myc} are three key genes that are often altered in cancers, which can drive and promote changes in metabolism\textsuperscript{58}. Data has indicated that these networks of regulation orchestrate a state of high glucose uptake/flux and activate genes involved in the glycolysis (\textit{PI3K/AKT/TP53} pathway)\textsuperscript{58,85,86}, upregulate glutaminolysis (\textit{TP53/MYC}), lipid synthesis (\textit{PI3K/AKT})\textsuperscript{87,88} and alleviate glycolysis repression (\textit{TP53})\textsuperscript{89,90}. Thus, it is suggested that oncogenic and tumor suppressive mutations may foster tumor growth and survival by metabolic reprogramming to meet the bioenergetic demand of the cell.

\subsection*{1.2.8 Alterations in glycolytic enzymes}

Aberrant expressions of metabolic enzymes or altered isoform patterns are potential mechanisms involved in the metabolic reprogramming with ensuing metabolic modification. A plethora of metabolic-related molecular changes have been demonstrated in various cancers including GBMs such as altered expression (mostly overexpression) of GLUT receptors, HK, PK, LDH, PDH, and PDK. It is well documented that GLUT mRNA and protein levels are higher in tumor cells compared to normal tissues. The predominant overexpressed isoforms are GLUT1 and 3, however, other isoforms, which are not usually found in the tissue of origin, may also be overexpressed\textsuperscript{91}. An example of altered isoform expression is the switch in splice isoforms from the adult pyruvate kinase M1 (PKM1) to the embryonic PKM2 such that the expression of PKM2 isoform is prevalent in primary tumors and cell lines compared to normal cells\textsuperscript{92-94}. It is proposed that PKM2 provides a metabolic and growth advantage by slowing glycolysis to allow shunting of carbon metabolites to key subsidiary biosynthetic pathways\textsuperscript{92-94}. In addition to enhancing glycolysis, the Warburg effect generates copious amount of lactate as a byproduct, which is facilitated by lactate dehydrogenase A (LDHA) isoform over-expression in many tumor types. LDHA governs the irreversible conversion of pyruvate to lactate in the last step of glycolysis. Increased LDHA expression can increase the extracellular acidification, facilitating the maintenance of glycolysis, as well as contributing to invasion and dampening of immune
surveillance\textsuperscript{95}. PDK1 is another perpetrator of aerobic glycolysis, which is upregulated in most cancer cells including GBMs. PDK can inhibit pyruvate entry to TCA cycle by phosphorylating and inactivating PDH enzyme\textsuperscript{96}. Inhibitors of PDK1 such as dichloroacetate (DCA) have been shown to have anti-cancer effect by shifting the metabolism from glycolysis to glucose oxidation and inducing apoptosis in many cancers including GBMs\textsuperscript{97,98}. Likewise, in highly proliferating tumor cells, HK2 has been shown to be the predominant isoform rather than HK1 or HK4 (liver). In GBMs, upregulation and over-expression of HK2 plays a key role in regulating the metabolic switch and the Warburg phenotype\textsuperscript{99}. Extensive studies of Pedersen and colleague in 1970s have been instrumental in discoveries regarding HK2’s relevance in cancer and specifically the Warburg phenotype\textsuperscript{100-103}. Recently, we have shown that HK2 is highly expressed in GBMs compared to low-grade tumors and normal brain tissue\textsuperscript{99}, the latter expressing mainly the HK1 isoform (Figure 1.6A). In highly glycolytic GBM cell lines, mitochondrial-bound HK2 was shown to play a critical role in the Warburg effect and its inhibition by constitutive HK2 knockdown was shown to inhibit aerobic glycolysis; leading to an increase in normal oxidative respiration, decrease lactate production and induction of apoptosis specially under hypoxic conditions\textsuperscript{99}. In addition, HK2 expression analysis using the TCGA GBM database shows a statistically significant enrichment of HK2 in the mesenchyme subtype of GBM, which is the most aggressive of all subtypes (Figure 1.6B).
**Figure 1.6: HK2 expression in gliomas (A) and different GBM subgroups (B).** HK2 expression analysis using the TCGA GBM database showing a statistically significant enrichment of HK2 in GBMs relative to lower grade gliomas or normal brain (A) and in the mesenchymal subgroup of GBMs relative to other subgroups (B). The TCGA portal data browser is accessible online at cancergenome.nih.gov. Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001).

**Abbreviations:** TCGA: The Cancer Genome Atlas; NHA: normal human brain; OG: oligodendroglioma; AA: anaplastic astrocytoma; GBM: glioblastoma.
1.3 Hexokinase and its isozymes

The first step of glycolysis is catalyzed by an evolutionarily conserved enzyme called hexokinase (HK)(ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1). HKs catalyze the phosphorylation of glucose by transfer of a γ-phosphoryl group from adenosine triphosphate-magnesium complex ([ATP-Mg]2⁺) to glucose, generating G6P. By phosphorylating glucose, negatively charged G6P is entrapped in the cytoplasm for commitment to two alternative metabolic routes: used for ATP generation by glycolysis or channeled into PPP for biosynthetic processes. Glucose is the preferred substrate for HKs, but it can also phosphorylate other hexoses to varying degrees, as recognized by the name hexo-kinase. There are four mammalian isozymes of HK namely types HK1, HK2, HK3 and HK4, with the latter commonly known as glucokinase, which were initially separated by ion exchange chromatography and electrophoresis in the mid 1960s from different mammalian tissues. HK isoforms display different and distinct subcellular localization, tissue specificity and catalytic or regulatory properties (Table 1.2). In general, HK isozymes have different catalytic and regulatory properties, which makes them suitable for specific metabolic roles in the cells (anabolic or catabolic). Physiologically, isozymes are analogous enzymes with different but customized characteristics to suit them for specific tissue requirements or metabolic conditions. Isozymes may exhibit different transcriptional regulation, which can facilitate their selective expression in different organs/tissue under particular metabolic status. Thus, owing to different isoforms of HK, glucose metabolism may be compartmentalized to different subcellular locations, allowing G6P to channel through particular metabolic pathways. Hence, it is reasonable to assume that different HK isozymes have evolved to undertake particular roles in the mammalian metabolism and to facilitate metabolic fine-tuning in different conditions.

i) Subcellular localization: HK1 and HK2 are present in the cytosol and also are localized to the outer mitochondrial membrane (OMM) through a N-terminal 15-21 amino acid sequence that forms a hydrophobic alpha helix necessary for insertion into the OMM hydrophobic core. These amino-acid determinants are necessary and sufficient for stabilization of binding to the mitochondrion and if missing will result in HK displacement from the OMM. Mitochondrial binding of HK1 and 2 is functionally and enzymatically driven. Functionally, mitochondrial binding has been shown to allow for
preferential access of HKs to mitochondrial ATP to accelerate the catalytic function. This strategic localization may vary across different tissues or metabolic conditions. HK3 is localized in the perinuclear region in many cell types, yet it lacks definitive nuclear targeting sequence. HK4 is predominately cytosolic, however it can reside in a mitochondrial complex with pro-apoptotic machinery in liver and pancreatic β-cells.

ii) Tissue specificity: HK1, also know as brain HK, is abundant in the body and is predominantly present in high metabolic tissue such as the brain and kidneys. HK2 expression is much more restricted in the body and is predominantly found lower levels in insulin-sensitive tissues such as skeletal muscle, heart and adipose tissue. HK3 is relatively abundant in the lungs, liver and spleen, while HK4 is mostly found in the liver and pancreatic tissue.

iii) Catalytic and regulatory properties:

HK4 is the only HK isoform with a mass of approximately 50 kDa. HK1-3 isoforms have molecular mass of approximately 100 kDa and are postulated to have evolved by duplication and fusion of a gene encoding a 50 kDa ancestral HK. The N-and C-terminal halves of HK1-3 exhibit significant sequence homology, on the other hand, HK4 contains sequences similar to the C- and N-halves of HK1-3. Catalytic function resides exclusively in the C-terminal half of the HK1 and 3 isozymes; whereas HK2 displays catalytic activity in both C and N terminal halves. Thus, it is assumed that HK2 likely resulted from duplication and fusion of an ancestral gene encoding a 50 kDa HK (similar to HK4). While HK1 and 3 likely evolved from further duplication of the gene encoding an ancestral 100 kDa HK, with specific mutations that lead to functionally different halves: catalytic and regulatory.

HK isozymes show different degrees of production, (G6P) inhibition, substrate binding and ATP affinity. HK1-3 show high product inhibition by G6P. G6P can act as an allosteric inhibitor and compete with ATP for enzyme binding and also inhibit HK localization to mitochondria. Both halves of HK2 are susceptible to product inhibition, however only the N-terminal half of HK1 and 3 are regulated by G6P. In terms of substrate affinity, HK1-3 have a 250 fold higher affinity for glucose (low $K_m$) compared to HK4 suggesting enzyme saturation with the substrate at normal glucose concentrations. In addition, inorganic phosphates (Pi) are thought to be other
important regulators of HK binding to the OMM. Pi can bind to N-terminal half of HK1 and antagonize the ability of G6P to release HK from mitochondria, however HK2 is not affected by Pi. It is proposed that the response of HK1 to Pi, its low G6P inhibition and presence of only one catalytic domain makes it more suited for the catabolic function in the cell\textsuperscript{107}. In contrast, since HK2 is insensitive to Pi, has double the catalytic capacity and is inhibited by G6P, it is proposed to be more suited for anabolic roles in the cell such as providing G6P to PPP for biosynthetic reactions or for glycogen synthesis\textsuperscript{107}.

Table 1.2: Tissue specificity, subcellular localization and catalytic or regulatory parameters of mammalian hexokinase (HK) isozymes\textsuperscript{107}.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HK1</th>
<th>HK2</th>
<th>HK3</th>
<th>HK4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue specificity</td>
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<td>Muscle, adipose</td>
<td>Lung, spleen</td>
<td>Pancreas, liver</td>
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<td>Molecular weight (kDa)</td>
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<td>~100</td>
<td>~100</td>
<td>~50</td>
</tr>
<tr>
<td>Catalytic subunit</td>
<td>C-terminus</td>
<td>C &amp; N-terminus</td>
<td>C-terminus</td>
<td>C-terminus</td>
</tr>
<tr>
<td>Substrate</td>
<td>Hexoses</td>
<td>Hexoses</td>
<td>Hexoses</td>
<td>Glucose</td>
</tr>
<tr>
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<td>0.3 (high)</td>
<td>0.003 (high)</td>
<td>6 (low)</td>
</tr>
<tr>
<td>Product inhibition by G6P</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Effect of Pi on G6P inhibition</td>
<td>Decrease</td>
<td>Increase</td>
<td>Increase</td>
<td>?</td>
</tr>
<tr>
<td>Mitochondrial binding</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
1.3.1 Hexokinase 2

The human HK2 gene (HXKII or muscle form HK) is mapped to chromosome 2p13 and contains 18 exons and encodes a protein of 917 amino acids. Compared to other HK isozymes, HK2 protein is limited in its expression throughout the body, however mammalian muscle, heart and adipocytes contain low but significant levels. HK2, similar to HK1, has mitochondrial binding motifs that allow for HK2 binding to OMM VDAC\textsuperscript{101,109}. However, the extent of HK2-mitochondrial binding can change based on metabolic conditions, growth factor signaling or different tissue localization. As mentioned, the key HK isozyme involved in cancer promotion is HK2. HK2 is overexpressed in many cancer cells, which could be an adaptation to the requirement for enhanced glycolysis or a necessary genetic modification occurring during carcinogenesis. Understanding the localization of HK2 under different metabolic conditions (high/low glucose, hypoxia or under dynamic growth factor stimulation) in conjunction with differences in regulatory mechanism of different HK isoforms could provide vital clues as to HK2’s altered functioning during tumorigenesis.

1.3.2 Hexokinase 2 versus hexokinase 1

Based on the differences in glucose binding affinity, the selection of HK1-3 over HK4 is deemed favorable from a metabolic standpoint, as isoforms of the former can harness glucose with over 100-fold higher affinity than the latter enzyme, making HK1-3 more suitable for glycolysis\textsuperscript{107,111}. Next, the selection of HK1 and 2 in contrast to HK3 isoforms is likely due to the existence of a 15-21-residue N-terminal\textsuperscript{112} hydrophobic domain for attachment to OMM\textsuperscript{108,113,114}, in the former. This attachment is achievable through VDAC, also know as mitochondrial porin, which is the most abundant protein of OMM and is a major channel for exchange of metabolites and ions between the inner mitochondria and cytoplasmic regions of the cell\textsuperscript{112,114}. HK2 association with OMM provides several kinetic benefits: 1) provides HK2 with preferential access to mitochondrially generated ATP to facilitating the catalytic reaction, 2) increases HK2's binding affinity to ATP (≈ 5-fold), 3) reduces HK2’s sensitivity to product inhibition by G6P, thus facilitating HK2’s catalytic function and production of G6P, and 4) protects HK2 against proteolytic degradation\textsuperscript{101,102,115}. This mechanism in normal cells ensures coordination of glucose phosphorylation with the TCA cycle, avoiding the buildup of potentially toxic lactate. Considering HK1 and HK2, it is only HK2 that has retained the catalytic activity in both the C
and N termini, thus having superior catalytic power in highly glycolytic cells. Notably, HKs
response to Pi (inorganic phosphate such as ATP) may further provide a clue for HK2’s selection
over HK1 in cancer cells. As mentioned, Pi antagonizes G6P inhibition in HK1 only, whereas in
HK2 Pi adds to G6P inhibition. An increase in cellular Pi levels from hydrolysis of ATP to
ADP and a decrease in cellular G6P due to increased flux through downstream glycolytic
reaction generally occur during periods of increased energy demand and glycolytic metabolism
as in brain, a tissue known to rely on glycolytic metabolism to sustain energy. The resulting
increase in the [Pi]/[G6P] ratio increases HK1 activity, leading to the suggestion that HK1
functions primarily in a catabolic role in glycolytic metabolism for ATP production. In
contrast, HK2 may be more suited for an anabolic role in shunting G6P through the PPP and
facilitating biosynthetic reactions. Differences in catalytic power in conjunction with Pi
inhibition reinforce the differences in physiological roles of HK1 and HK2, suggesting a more
frontline position for HK2 in cancer metabolism.

1.3.3 Regulation of hexokinase 2

HK2 is known to be one of the most regulated HK isoforms, especially at the transcriptional
level, containing binding sites for numerous transcription and regulatory factors such as glucose,
glucagon, insulin, G6P, cyclic AMP, wild type and mutant p53, Akt or catecholamines. The
HK2 proximal promoter contains several transcription initiating cis-regulatory elements
(TATA and CAAT elements) allowing strong transcriptional regulated by several systemic and
cellular stimuli. The presence of distinct response elements on the HK2 proximal promoter in
contrast to other HKs may play an important role in promoting HK2 overexpression in cancer
cells. Of note, HK2 contains hypoxia response elements (HRE) for HIF1α binding and both its
proximal and distal promoter regions are critical for HIF1α binding and transcription activation
under hypoxia. This hypoxic-regulation is crucial for HK2’s role in GBMs since they are known
to be highly hypoxic in nature. It has also been shown that c-MYC overexpression leads to
MYC-HIF cooperation, inducing HK2 expression via c-Myc E-box-mediated transcriptional
activity on the HK2 promoter. Functional response element for p53 are also shown to be
located on the distal region of the HK2 promoter and recently p53-inducible gene TIGAR
(TP53-induced glycolysis and apoptosis regulator) has been shown to form a complex with HK2
on the mitochondria under hypoxia, sensing glucose and oxygen availability and contributing to
HK2’s ability to modulate energy metabolism and cell survival\textsuperscript{89,90}. It is reasonable to assume that the HK2’s promoter is tailored to provide an enhanced response to microenvironmental changes such as hypoxia or metabolite levels, resulting in greater HK2 synthesis\textsuperscript{115}. Thus, HK2 overexpression in metabolically reprogrammed tumor cells is partly driven by the presence of glycolysis-supportive regulatory elements that are suited to provide a greater response to external microenvironmental stimuli, resulting in greater HK2 synthesis to facilitate and maintain a high glycolic flux in the cell.

1.3.4 Hexokinase 2 overexpression in malignant tumors

Evidence suggests that HK2 has a critical role in cancer promotion and its overexpression and association with poor prognosis is well documented in several types of cancer malignancies such as hepatocellular carcinoma\textsuperscript{123}, pancreatic cancer\textsuperscript{124}, gastric carcinoma\textsuperscript{125}, lung cancer\textsuperscript{126} or malignant GBMs\textsuperscript{127}, among others. The link between HK2 overexpression and highly glycolytic malignant tumors was first demonstrated by mRNA analysis where HK2 message was increased by ~100-fold relative to background mRNA signal\textsuperscript{118,128,129}. While HK1 overexpression is also seen in some highly glycolytic tumors\textsuperscript{102}, to date its precise regulatory role in cancer metabolism has not been studied. Studies on the relevance of HK2 on GBM or brain tumor progression and tumorigenesis are limited. Our group was the first to focus on the role of HK2 in GBMs in detail. Results from our group have shown HK2’s overexpression as a negative prognostic factor in GBM, even after age standardization\textsuperscript{99}. The potential mechanisms of HK2 overexpression may vary in different cancer types encompassing gene amplification, increased gene expression, and mRNA translation, post-translational regulation by protein-protein interactions or via microRNAs\textsuperscript{115}. There is also evidence suggesting the involvement of other processes that can work separately or in concert with regulatory mechanisms to influence HK2’s overexpression such as epigenetic changes or enhanced mRNA stability\textsuperscript{130,131}. However, the extent of their contribution to HK2 overexpression in different tumor type is still an open issue. Regardless of the mechanism, a considerable body of evidence has now shown that elevated HK2 expression promotes cell proliferation and survival in cancer cell lines and malignant tumors and inhibition of HKs through broad spectrum inhibitors have shown anti-cancer effects alone or in combination with other therapeutics.
1.3.5 The survival role of hexokinase 2

In addition for HK2’s vital role in metabolic reprogramming, HK2-mitochondrial interactions have been proposed to be crucial for promoting cancer survival via modulation of signaling events related to intrinsic apoptosis of the cells\textsuperscript{72,132}. There is abundant evidence to support the fact that the interaction of HK2 with VDAC regulates cellular apoptotic cascades and provides anti-apoptotic signaling in the cell that ultimately decides the fate of a tumor\textsuperscript{72,111,133}. Increased anti-apoptotic capacity of the cells by HK2-mitochondria interaction is mediated via two mechanisms. HK2’s association with mitochondria prevents the opening of the mitochondrial permeability transition pore (PTP). PTP is a multi-protein complex spanning the outer and inner mitochondrial membrane composed of VDAC, ANT, cyclophilin D, HK2 and other proteins. Once open it can lead to loss of mitochondrial membrane potential, organelle swelling and release of apoptotic inducers such as cytochrome c and ultimately cell death. It has been well documented that the association of HK2 with the mitochondria can influence VDAC’s response to Bcl family proteins and inhibit pro-apoptotic proteins from binding to the OMM. HK2-VDAC binding acts as a block and displaces pro-apoptotic factors such as Bax and Bak from VDAC and antagonizes their action by facilitating the interaction of VDAC with anti-apoptotic proteins\textsuperscript{70,134}. In addition, release of pro-apoptotic proteins protects the mitochondria against PTP-opening and mitochondria depolarization. Alternatively, some recent evidence suggests that HK2 binding leads to displacement of VDAC-bound anti-apoptotic protein Bcl-XL and increases its availability to neutralize pro-apoptotic proteins. Regardless of the mechanism, the “guardian of the mitochondria”, HK2, plays an important role in inhibiting VDAC conductance and ultimately regulates apoptosis and fosters cellular survival. When apoptosis is required in the cells, multiple regulatory mechanisms are in place to facilitate displacement of HK2 from the mitochondria. As pointed out, several mechanisms can regulate this binding. It is shown that HK2 binding to VDAC is inhibited by high concentration of G6P, which leads to conformation changes in HK2 and displacement from OMM. Also divalent cations, such as Mg\textsuperscript{2+} that are essential for HKs catalytic function, have been shown to regulate HK2’s binding to the mitochondria\textsuperscript{117}. There is evidence that HK2 binding to mitochondria can be regulated by protein kinases such as Akt\textsuperscript{72,134}. In fact, Akt hyper-activation has been associated with increased glycolysis in several tumor types by phosphorylating and activating glycolytic enzymes such as HK2 and GLUTs\textsuperscript{135}. When activated, Akt can positively regulate binding of HK2 to the mitochondria, however this only
works when glucose is available. This implies that the enzymatic role of HK2 is necessary for this interaction. This model is supported by the finding that 2-deoxyglucose (2DG), an analogous form of glucose, is able to protect the cells under irradiation and serum starvation, however, this non-phosphorylatable and non-metabolizable form of glucose, could not confer this protection. Other evidence has suggested that Akt directly stimulates HK2 binding with VDAC via phosphorylation of HK2 and/or VDAC. In cardiomyocytes, it was shown that activated Akt is localized to the mitochondria following PI3K activation and it results in an enhanced binding of HK2-VDAC through phosphorylation of HK2. Others groups have shown that VDAC can be phosphorylated by glycogen synthase kinase 3 (GSK3) activation (downstream of Akt) leading to HK2 detachment, mitochondrial dysfunction and injury. Thus GSK3 inhibition of Akt has a protective effect on mitochondria and promotes HK2 binding and stabilization. Interestingly, mutant constructs of HK1 and HK2 including N-terminal truncated mutant kinase constructs failed to confer protective effects of HK1 and 2 under cytotoxic H$_2$O$_2$ treatment, suggesting that the catalytic function of HKs are essential for their survival role. Thus, in addition for HK2’s vital role in metabolic reprogramming, HK2-mitochondrial interaction provides the cancer cell with resistance to apoptosis and a survival advantage that can be exploited therapeutically with HK2 inhibitors.

**1.3.6 The antioxidant role of hexokinase 2**

As mentioned previously, reactive oxygen species (ROS) are by-products of cellular respiration and are usually found in low concentrations in normal physiological conditions. When production of ROS is deregulated such that there is increased generation and accumulation of ROS, it can become toxic and lead to cellular toxicity, damage and ultimate induction of senescence or apoptosis. In cancer cells, mechanisms regulating ROS production have shown to be altered to promote cellular survival. HK2 is one of many molecules that have been shown to harbor an anti-oxidant role for the tumor cell. This protective role of HK2 is predominately achieved through mitochondrial binding of HK2 (as discussed above) to reduce mitochondrial membrane permeability and ROS leakage from the mitochondria. HK2-mitochondria binding leads to decreased mitochondrial permeability and may reduce ROS production and potentially contribute to HK2’s protective effect. In addition, HK2 augments PPP and in turn increases generation of NADPH. NADPH, besides its reducing role, is an important cellular mechanism
for defense against oxidative stress via ROS scavenging, protecting the cell against oxidative damage. Proper kinase activity as well as VDAC interaction of HK2 was found to be essential for attenuating ROS generation and maintaining a reduced state of mitochondrial membrane depolarization. Evidence has shown that mitochondrial bound HKs specifically decrease the production of ROS such as H$_2$O$_2$ from the mitochondria. When HK-depleted mitochondria were incubated with HKs that were unable to bind to VDAC, it resulted in a much less efficient reduction in rate of H$_2$O$_2$ generation.

### 1.4 The Warburg effect in GBMs

GBMs have a highly glycolytic nature with a propensity to metabolize glucose to lactate at an accelerated rate even when ample oxygen is present, a feature common to most malignant/proliferating tumors. These metabolic anomalies are in contrast with non-transformed normal brain tissue where glycolysis primarily occurs under anaerobic conditions. Glycolysis has been shown to be upregulated in GBMs by more than 3 fold. Also, high glycolytic GBM tumors have been shown to redirect pyruvate away from mitochondrial oxidation and largely reduce it to lactate in the cytoplasm. On the same line, $^{13}$C-nuclear magnetic resonance spectroscopy measurements have shown that GBM cultures convert about 90% of glucose and 60% of glutamine into lactate or alanine, instead of shuttling them to the mitochondria for respiration. Using stereotactic microdialysis Tabatabaei et al investigated the changes in glucose, glutamate and glycerol metabolites in GBMs during 5 days of radiotherapy and found that the baseline metabolite levels were lower in tumors compared to adjacent brain tissue, with the lactate/pyruvate ratio markedly higher in tumor compared to adjacent brain.

Previous studies by us and others have demonstrated differential expression of tumor specific enzyme isoforms or receptors such as HK2, LDHA, PKM2, PDK1, GLUT1/4 and MCTs relative to normal brain tissue. Although aerobic glycolysis is the most characterized phenomenon in regards to metabolic modification, many other aspects of GBM metabolism are likely to be deregulated and need to be further elucidated to allow for development of cancer therapeutics that take into account the aberrant metabolism of GBMs.
1.4.1 Heterogeneity in GBM metabolism

The major limitations in treatment of GBMs are the extreme heterogeneity of the tumor microenvironment and the multifactorial nature of this disease\(^2\). While there might not be a consensus regarding the association of innumerable molecular, genetic or epigenetic alterations with prognostic factors, one thing that most would agree on is the extent of the heterogeneous landscape in GBM\(^2\). In brain tumors and specifically GBMs, heterogeneity in oxygen concentration can modulate the metabolic signature of the cells and act as a catalyst for expression of factors regulating aerobic glycolysis, lactate production or signaling pathways that regulate aerobic glycolysis\(^{146}\). GBM’s regional heterogeneity in hypoxia ie necrotic and non-necrotic tissue in combination with molecular, mutational and epigenetic variation is considered to have a profound effect on tumor metabolism, increased resistance to treatment or tumor progression of GBMs \(^{18,21,24,77,147-151}\). GBMs are known to reside in a heterogeneous microenvironment with respect to tumor cells (glioma stem cells), parenchymal cells (stroma cells), hypoxia level or acidity\(^{152}\). GBMs harbor populations of cancer cells with dynamic oxygen gradients. Hypoxia is mostly present in high-grade gliomas, with GBMs dominated by moderate to severe hypoxic cells and with a subset of oxic cells \(^{153-155}\). Evans et al. have nicely demonstrated GBMs’ dynamic oxygenation with oxygen concentrations ranging between 2.5% and 0.5% for mild hypoxia and 0.5%–0.1% for moderate/severe hypoxia \(^{156}\). Several reports demonstrated variability in GBMs with respect to glucose dependency, enhanced glucose uptake and the extent of mitochondrial respiration\(^{157,158}\). Similarly, in GBMs that were reliant on glycolysis, several tumor specific enzyme isoforms were found to be overexpressed compared to normal brain, contributing to metabolic autonomy of tumor in comparison to normal cells \(^{158}\).

Interestingly, in a recent report HK2 expression was found to be elevated in brain metastasis from breast cancer, when it was compared to the primary breast tumor expression of HK2 \(^{159}\). This supports the importance of unique microenvironmental influences present in the brain that can modulate expression and activation of HK2 in tumor growth. Although now recognized as a major player in cancer pathogenesis, the precise role and the significance of metabolic heterogeneity in GBM treatment and prognosis still requires further evaluation. Understanding and decoding the interconnected microenvironmental network of genes involved in metabolic, hypoxic and angiogenic reprogramming of the cells can potentially provide diagnostic and prognostic values.
1.5 Therapeutic targeting of metabolism

The identification of commonly altered metabolic enzymes and mechanisms has led to the development of number of compounds that can selectively or globally target cancer metabolism, some of which are currently being tested in pre-clinical and clinical settings. Since results from monotherapies have not been very promising, these drugs are now being tested in combination with radiotherapy and/or chemotherapy for increased efficacy. Several anti-metabolic compounds have been developed that target and inhibit tumor-specific enzyme-isoforms, transporters or suppress enzyme activities such as GLUTs, HK, GAPDH, PDK, LDHA, PKM2, MCTs, CAs, FASN or ACLY. 2DG, 3-BrP and lonidamine are global glycolysis blockers. 2DG, a glucose analogue, inhibits glucose metabolism. 2DG can compete with glucose for phosphorylation by the enzyme HK to produce 2DG-6-phosphate, which is a competitive inhibitor of enzymes that metabolize G6P. 2DG has been used in pre-clinical and clinical settings as a potential anti-cancer agent in several types of cancers including GBMs. Evidence suggests that 2DG can potentiate the effects of standard cytotoxic radiochemotherapy by HK inhibition, ATP depletion, oxidative-stress mediated cell death and histone regulation. 3-BrP, initially proposed as a HK inhibitor, is a pyruvate analogue that is known for its inhibitory effect on glycolysis and its alkylating properties. 3-BrP is shown to be effective as a potential anti-cancer drug by inhibiting mitochondrial OXPHOS, ATP generation and ultimately cell death in several cancer types such as gliomas, hepatocellular carcinoma or human leukemia cell lines. Although in vivo pre-clinical studies have been promising, its off-target effects have raised concerns for clinical use. Lonidamine, another metabolic blocker, inhibits mitochondrial respiration by inhibiting the mitochondria-bound HKs and enhancing the activity of several alkylating agents. Lonidamine has been tested in clinical trials for solid tumors including advanced breast, ovarian or lung cancer, with some encouraging results. Phase I/II studies with recurrent malignant glioma patients did not show any therapeutic efficacy. Interestingly, phase II clinical studies with this compound in combination with the anxiolytic drug diazepam demonstrated promising results of tumor stabilization in 50% of cases in GBMs but no effect on tumor regression.
Figure 1.7: Selected targeting of tumor metabolism. Gluts are upregulated to facilitate the glucose transport across the plasma membrane. HK2 overexpression enhances the glycolytic flow. PKM2 is the predominate isoform expressed to enable shuttling of glycolytic intermediate into anabolic pathways rather than to pyruvate. PDK blocks PDH activity and decreases pyruvate entering to the TCA cycle. Glutamine uptake is increased to fuel aerobic glycolysis.

Abbreviations: Glut: glucose transporter; HK2: hexokinase 2; 3-BRP: 3-bromopyruvate; 2-DG: 2-deoxyglucose; PKM2: pyruvate kinase M2; PDH: pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; DCA: dichloroacetate; G6P: glucose 6-phosphate; PEP: phosphoenolpyruvate. TCA: tricarboxylic acid cycle. ATP: adenosine triphosphates; NADPH: nicotinamide adenine dinucleotide phosphate.
To date, no specific inhibitor of HK2 has been developed. Unacceptable toxicity seen with global and broad spectrum HK inhibition warrants development of selective HK2 inhibitors (without effects on the normal brain isoform HK1) that may lay the substructure for successful clinical trials and more effective treatment strategies in combination with classical radiochemotherapy.

The small molecule DCA is another compound involved in the down-regulation of glycolysis, commonly used to treat patients with lactic acidosis. DCA targets and inhibits of PDK, expression of which is enhanced in several cancers including GBMs. A clinical trial in small group of 5 GBM patients showed promising results by altering mitochondria membrane potential, reactivating PDH and increasing mitochondrial respiration. Of note, reduction in mitochondrial localization of HK2 with DCA compared to pre-treatments rendering cells more sensitive to apoptotic cell death.

Inhibiting lactate production or lactate transport is another strategy to target the Warburg effect in highly glycolytic tumors. The LDHA isoform (LDH-5), which converts pyruvate to lactate, has been shown to be expressed at a high level in tumors and correlates with poor prognosis in human colorectal, gastric and lung cancers. Although selective LDHA inhibition has not been tested on GBMs, in breast cancer cells LDHA knockdown by RNA interference methods stopped tumor proliferation and reduced tumor initiation in mice. LDHA inhibition by inhibitor FX11 in human lymphoma and pancreatic cancer xenografts resulted in attenuated ATP generation and tumor growth. Thus LDHA may be a potential GBM target to stop tumor initiation and growth.

Regulating of intracellular pH is essential for tumor cells and tumors have cleverly upregulated pH-regulating proteins including MCTs or carbonic anhydrases (CA) and these proteins can be readily targeted using antibodies or drugs. A recent study used a carbonic anhydrase inhibitor in combination with temozolomide and showed enhanced apoptotic response in glioma cell lines in the combination therapy. Combining a MCT1 inhibitor with radiotherapy has been shown to result in tumor growth delay in gliomas and tumor xenografts. Although still in pre-clinical stages, determining the effect of disrupting lactate transport in clinical settings in gliomas is an attractive therapeutic avenue.

Overall, investigation of the anticancer potential of metabolic enzymes along with selection of rational target combinations will be crucial for delivery of effective therapies in many tumors.
types including GBMs.

1.6 Rational for radio-chemo sensitization of hexokinase 2

Cancer cells are notorious for their regulatory mechanisms that allows them to survive in stressful environments with varying oxygen and nutrients levels\textsuperscript{173,174}. Although cancer cells exhibit increased glycolysis and are more dependent on glucose as a fuel, inhibition of glycolysis alone may not be sufficient to kill growing tumor cells. It has been suggested that as a cellular adaptation to glucose deprivation, cancer cells can use other carbon sources such as glutamine for fuel or can switch to a less favorable oxidative respiration if they contain normal functioning mitochondria\textsuperscript{175-177}. One way to enhance sensitivity of tumor cells to glycolysis inhibition and improve therapeutic activity would be to combine multiple agents with different or coinciding mechanisms of action. Indeed, earlier studies have demonstrated that combination of glycolytic inhibitors and DNA-damaging agents seems to be an effective therapeutic strategy to kill cancer cells\textsuperscript{178}. It was shown that the cells dependency on aerobic glycolysis could determine their sensitivity to DNA-alkylating drugs and poly (ADP-ribose) polymerase (PARP)-induced cell death\textsuperscript{178}. In cells undergoing the Warburg effect, PARP activation due to DNA damage resulted in a rapid decline in cellular ATP and NAD\textsuperscript{+}, two factors necessary for glycolytic reactions. Thus, their shortage indirectly inhibits glycolysis and sensitizes the cells to other stressors. In addition, it has been suggested that glycolytic inhibitors may change the outcome of radiation therapy due to their radiosensitization effect. By favoring mitochondrial respiration, blocking glycolysis may shift the total cellular redox state in favor of increasing intracellular ROS leading to oxidative stress-mediated cell death in tumor cells and this may render cancer cells more susceptible to ROS/free radical-based treatment strategies such as radiotherapy\textsuperscript{179-181}.

Multiple groups have investigated the possibility of developing and testing inhibitors of glycolysis as a novel therapeutic approach either singularly or in combination with other anti-cancer agents such as radiotherapy or chemotherapy in different cancer types including gliomas. As mentioned, there are several lines of evidence showing increased efficiency of pharmacological inhibitors of glycolysis such as 3-BRP and 2DG by combining them with cytotoxic therapies including radiation and chemotherapy through promotion of apoptotic cell death\textsuperscript{71,182}. Combination of 2DG (HK2-inhibitor) with adriamycin or paclitaxel resulted in an enhanced therapeutic activity in animal tumor models of osteosarcoma or non-small-cell lung
Huang and colleague have tested novel strategies to selectively kill tumor cells based on their metabolic alterations and recently have shown that combination of a derivative of 3-BRP, 3-bromo-2-oxopropionate-1-propyl ester (3-BrOP) and carmustine (BCNU) exhibited a synergistic killing effect in GBMs, especially in hypoxic conditions. The cytotoxic effect was mostly attributed to ATP depletion and reduced ability of the cells to repair BCNU-induce DNA damage\(^{184}\). Different tumor cells have shown variations in 2DG-induced radio-chemo sensitization independent of ATP levels, suggesting that other factors such as p53, Bcl2/Bax ratio or imbalances in the oxidative stress may influence cellular responses to damage caused by radiation and chemotherapeutic drugs. Aerobic glycolysis involves reduced respiration and ROS production coupled with increased formation of NADPH. Thus, inhibition of glycolysis and induction of respiration is expected to sensitize the cells to agents that induce oxidative stress through ROS such as ionizing radiation or chemotherapy. Along the same line, some studies have shown that 2DG may be a good radio-chemo sensitizer due to its ability to alter the redox homeostasis of the cells and sensitize them to DNA damage induced by chemotherapy agents such as cisplatin and doxorubicin\(^{160,185,186}\). Also combination of 2DG with radiation showed a profound decrease in the glutathione content and enhanced DNA damage leading to apoptotic and necrotic cell death, while reducing the damage to normal cells and normal brain tissue in glioma patients\(^{180,187,188}\). We have also shown that GBM cells are more sensitized to radiation alone or TMZ alone when HK2 is depletion \textit{in vitro} (HK2 siRNA knockdown) and this effect was associated with depolarization of the mitochondrial membrane, increased release of cytochrome c and activation of apoptosis\(^{99}\). To date, there has been no report of a paired trial of glycolysis inhibitors and radio-temozolomide in GBMs. Due to the important roles of HK2 in both cancer metabolism and apoptosis, HK2 is an attractive target to combine with current GBM therapeutics. This thesis aims to profile the effect of metabolic modulations on the sensitivity of primary brain tumors to standard therapeutic interventions including radiochemotherapy.
1.7 Hypothesis and experimental aims

Failure to respond to available therapies namely radiochemotherapy, underscores the need to develop more effective treatment strategies for GBM patients. Our aim is to use metabolic modulations as an approach to enhance the cytotoxicity of radiochemotherapy. We hypothesize that altering tumor metabolism through conditional HK2 inhibition is a potential therapeutic strategy for sensitization of GBM tumors to radiation (RAD) and/or temozolomide (TMZ) \textit{in vivo}. We postulate that conditional HK2 loss in GBM orthotopic tumors can enhance the overall efficacy of radiochemotherapy and improve the survival of glioma-bearing mice.

To test our hypotheses, our experimental aims are as followed:

**Aim 1: Determine the impact of conditional HK2 inhibition on \textit{in vitro} growth and sensitization to therapeutics**

\textbf{Q1: Can conditional HK2 inhibition reverse the Warburg phenotype in GBM cell lines?}
\textbf{Q2: Can conditional HK2 inhibition sensitize GBM cell lines to cytotoxic therapies of TMZ and/or RAD or hypoxia?}

**Aim 2: Determine the impact of conditional HK2 inhibition on \textit{in vivo} growth and response to therapy**

\textbf{Q1: What is the impact of conditional HK2 inhibition on \textit{in vivo} tumor growth or regression?}
\textbf{Q2: Can conditional HK2 inhibition sensitize GBM tumors to RAD and/or TMZ?}
Chapter 2: Materials and Methods

2.1 Cell lines and cell culture conditions

GBM cell lines used in this study included human U87 MG cells lines obtained from the American Type Culture Collection (ATCC, Manassas, VA) and human GS2 glioma stem-like cell lines that were kindly provided by Dr. David James (University of California, San Francisco). The U87 cell lines were maintained in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). GS2 cell lines were maintained as adherent cultures in McCoy's 5A Modified Media supplemented with 5% non-essential amino acids and 10% FBS. For normoxic conditions, cells were incubated as adherent cultures in 10cm or 6cm plates in a humidified chamber at 37°C with 5% CO2. For hypoxic conditions, cells were incubated in a hypoxia chamber (Whitley H35 HypoxyStation) equipped with a glove box controlled by a hypoxic gas mixer at 37°C with 0.2% or 1% oxygen levels. Cells were plated in 10cm or 6cm plates and exposed to hypoxia for 48 hours at a confluency of 70-60%. Initial hypoxia experiments were performed in both 0.2% and 1% O2 levels. Since results were comparable, future experiments were performed and only reported in 1% O2 levels.

2.2 Generation of inducible cell lines

2.2.1 Inducible lentivirus system

The pTRIPZ lentiviral inducible shRNAmir vector system was purchased from Open Biosystems containing lentivirus elements, tet-on elements and a puromycin selection gene (Figure 2.1). HK2 expression would be conditionally inhibited by three independent small hairpin RNAs (shRNAs) against HK2 denoted as V1, V2 and V3 and a controlled scrambled shRNA. The addition of doxycycline (Dox, tetracycline derivative) induces expression of shRNAmir and turbo red fluorescent protein (turboRFP) in the pTRIPZ vector. shRNA and RFP reporter are expressed from the same transcript under the control of a tetracycline response element (TRE) promoter, which allows for quick assessment of viral titer, transduction efficiency and level of knockdown in vitro and in vivo.
Figure 2.1: pTRIPZ lentiviral vector features.

<table>
<thead>
<tr>
<th>Vector Element</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRE (minCMV) promoter</td>
<td>Tetracycline responsive RNA Polymerase II promoter</td>
</tr>
<tr>
<td>UBC promoter</td>
<td>Drives expression of rTA3 and IRES-puro</td>
</tr>
<tr>
<td>rTA3</td>
<td>Reverse tetracycline transactivator</td>
</tr>
<tr>
<td>shRNA</td>
<td>microRNA adapted shRNA engineered based on miR-30 design</td>
</tr>
<tr>
<td>cPPT</td>
<td>Central Polypurine tract for translocation into the nucleus of non-dividing cells</td>
</tr>
<tr>
<td>WRE</td>
<td>Enhances the stability and translation of transcripts</td>
</tr>
<tr>
<td>TurboRFP</td>
<td>Marker to track shRNAmir expression</td>
</tr>
<tr>
<td>Puro</td>
<td>Mammalian selectable marker</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin (carbenicillin) bacterial selectable marker.</td>
</tr>
<tr>
<td>5'LTR</td>
<td>5' long terminal repeat</td>
</tr>
<tr>
<td>pUC</td>
<td>High copy replication and maintenance of plasmid in E.coli</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SIN-LTR</td>
<td>Self inactivating long terminal repeat</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev response element</td>
</tr>
<tr>
<td>Zeo</td>
<td>Bacterial selectable marker</td>
</tr>
<tr>
<td>Ψ</td>
<td>Psi packaging sequence allowing viral genome packaging during transduction</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site allows expression of rtTA3 puro on a single transcript</td>
</tr>
</tbody>
</table>

### 2.2.2 Lentivirus production

Lentiviral particles were generated by co-transfection of HEK293T (human embryonal kidney) cells with a third generation packaging plasmids encoding the vesicular stomatitis virus G envelope, gag-pol genes and respective shRNA lentivirus vector. The supernatant containing the lentiviral particles were harvested 48 and 72 hours post-transfection and were centrifuged at 2000 rpm for 10 minutes and filtered through a 0.22µm sterile filter. The virus was aliquoted and stored at -80°C for later use.

### 2.2.3 Lentivirus transduction, selection and shRNA induction

Cells were plated at a density of 100,000 cells/well in a 24-well tissue culture plate in antibiotic-free medium one day prior to transduction. At the time of transduction, lentivirus was added to the cells as per instructions for pTRIPZ and incubated at 37°C for 12-24 hours. Later, the medium was removed and replaced with new media for 48 hours. To select for stably transduced cells, 1 ug/mL puromycin medium was added 72 hours post-transduction. To assay for transduction efficiency following infection or to induce shRNA expression, 1µg/mL Dox was added to the media. 48 hours post Dox the proportion of cells expressing red fluorescent protein (RFP) were determined using a fluorescence microscope to approximate the transduction efficiency. Western blot analysis was performed to quantify the level of knockdown in three
independent shRNA constructs. For knockdown conditions, cells were maintained on puromycin (1µg/mL) and Dox (1µg/mL) throughout the experiment.

2.3 Generation of stable cell lines

2.3.1 Luciferase lentiviral system

U87 cell lines were transduced with a lentiviral vector containing a stable firefly luciferase (Fluc) expression under the control of the cytomegalic virus (CMV) promoter. The lentiviral luciferase vector also contains a green fluorescent protein (GFP) reporter gene. GS2 cell lines were already modified with a lentiviral vector containing firefly luciferase\textsuperscript{189}. Viral particles were harvested from supernatant 48 and 72 hours post-transfection and were used to infect U87 cells. GFP-positive cells were purified and sorted by fluorescent activated cell sorting (FACS) (transfection efficiency > 90%). GFP-positive cells were lysed, treated with luciferin (D-luciferin potassium salt, 150 mg/kg, Gold Biotechnology, St Louis, MO) and a luciferase assay was performed. Luciferase levels were read with a luminometer (Berthold LB 96V MicroLumat). Luciferase levels (expressed as Relative Light Units) were normalized to total protein content of each sample.

2.4 Cell growth assay

Cell viability was measured by direct cell count using the Vi-CELL Coulter Analyzer (Beckman). 50,000 to 80,000 cells were seeded into 6 well plates and incubated for up to 6 days. Cells were trypsinized and counted in triplicates at days 2, 4 and 6 post-incubation.

2.5 Colony formation assay

1000-2000 cells were seeded in 6 cm plates and incubated for 14 days in the normoxic incubator. At day 14 cells were removed, fixed by acetone-methanol (1:1) and stained with crystal violet. The number of colonies was counted manually in triplicates and the graphs were the mean results relative to the control in each condition. For radiation arm, adherent cells in 10cm or 6cm plates were irradiated in medium at room temperature at dose rate 1 Gy/min for a total of 4 Gy. After irradiation cells were immediately trypsinized, counted and plated for 14 days. For TMZ arm, cells in10cm or 6cm plates were exposed to 100µm TMZ for 6 h.
2.6 Comet assay

DNA damage was visualized with comet assay using Trevigen method (Trevigen, INC, Helgerman CT. Cat#4250-050-01). Cells were trypsinized and counted. Next cells were pelleted, washed once in ice-cold 1XPBS, resuspend at 1 x 10^5 cells/ml in ice cold PBS and placed on ice. In treatment conditions, cells were kept on ice before lysis. Cells were combined with molten low melt agarose (LMAgarose) at 37°C at a ratio of 1: 10 (v/v) and immediately pipetted 100µL onto a CometSlide™. Slides were placed flat at 4°C in the dark for 30 minutes and immersed in pre-chilled lysis solution at 4°C for 45 minutes. Slides were treated with alkaline solution, pH>13 for 60 minutes at room temperature in the dark to unwind and denature the DNA. Next, slides were transferred to a horizontal electrophoresis apparatus containing alkaline electrophoresis solution and the power was set to 1 volt. After 20 minutes slides were removed, dipped in 70% ethanol, air-dried and stored with desiccant at room temperature. For scoring, slides were stained with 1X ethidium bromide for 25 minutes, coverslipped and scored immediately by epifluorescence microscopy. For each sample more than 50 comets were scored and analyzed. The comet parameter considered by the software in this study was the Olive tail moment, which is the product of percent tail DNA and the distance between the head and tail along the x-axis of the comet.

2.7 Lactate assay

Equal numbers of cells were plated in 96 well plates and the culture media were collected after 24 or 48 hours and lactate concentration was measured in triplicates with a colorimetric kit according to the manufacturer’s instructions (Eton Bioscience Inc. San Diego, CA, USA). The absorbance was determined at 490nm using a plate reader.

2.8 Oxygen consumption assay

Oxygen consumption rate was directly measured using the Instech Fiber optic oxygen monitor (model 110, Instech Laboratories, Plymouth Meeting, PA) as per the manufacturer’s instructions. Cells were trypsinized, counted and 10 million cells were re-suspended in 500µL of media and warmed at 37°C water bath. Oxygen tension was measured in triplicates and the rate of oxygen consumption per unit time was calculated and expressed as nmol O2/million cells/min.
2.9 Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded 5µm thick sections were de-paraffinized in xylene and rehydrated in graded ethanol and rinsed in dH2O. Heat-Induced Antigen Retrieval was used by pressure-cooking the sections for 20 minutes in citrate buffer (pH=6). Next endogenous peroxide activity was blocked in 3% hydrogen peroxide in methanol for 20 minutes. Slides were incubated with primary antibodies with appropriate conditions. Detection was performed using Vectastain ABC reagent and DAB chromogen (Vector Labs, Burlingame, CA). Slides were counterstained in Meyers Haematoxylin for 1 minute; dehydrated through ethanol (70%, 95%, 100% and 100%) and coverslipped using Permount (Fisher). Flash-frozen 5µm sections were air-dried and rehydrated in graded ethanol. Sections were permeabilized in PBS 0.1% Triton X-100 and endogenous peroxidase activity was inhibited by 3% hydrogen peroxide. Slides were blocked with 10% fetal bovine serum in PBS and incubated with primary antibodies with appropriate conditions. Detection was performed using Vectastain ABC reagent and DAB chromogen (Vector Labs, Burlingame, CA). Slides were counterstained in Meyers Haematoxylin for 1 minute, dehydrated through ethanol (70%, 95%, 100% and 100%) and coverslipped. Primary antibodies used were: MIB-1/Ki67 (Dako), HIF1α (BD Transduction), HK2 (Cell Signaling), CD31 (Millipore), cleaved caspase 3 (Cell signaling). CAIX antibody was kindly provided by Dr. Wouters’ laboratory. TUNEL staining was performed with the DeadEnd™ colorimetric TUNEL System (Promega) according to manufacturer’s instructions. All the slides were scanned using Zeiss Mirax scanner and analyzed by Mirax Viewer software.

2.10 Western blotting and antibodies

To analyze protein expression cells were grown on 10 cm plates, were scraped off or trypsinized, spun and resuspend in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Samples were incubated at 4°C for 30 minutes to be lysed sufficiently and then centrifuged at 4°C, 14000 g for 10 min, and the supernatant was collected. Protein concentrations were determined by BCA (bicinchoninic acid) assay as per the manufacturer’s instruction (Pierce Chemical Co., Rockford, IL). A total of 20-30 µg of protein was separated on 8% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidenedifluoride (PVDF) membranes (NEN Research Products/Du Pont, Boston, MA) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). After blocking the membrane with PBS (or TBS) containing
5% nonfat dry milk and 0.1% Tween 20 (Sigma-Aldrich) for 1 hour, the membranes were incubation with primary antibodies overnight at 4 degrees or 1 hour at room temperature. The following antibodies were used: 1:20,000 βactin (Sigma-Aldrich), 1:1000 HK2 (Cell Signaling) and 1:1000 HK1 (Cell Signaling). Membranes were washed and later incubated with horseradish peroxidase-conjugated secondary antibodies against the species the primary antibody was raised against (BioRad, Hercules, CA). Protein bands were detected with Chemiluminescence Reagent Plus (PerkinElmer Inc., Massachusetts, USA). Densitometric analysis was performed using ImageJ software (http://rsbweb.nih.gov/ij/).

2.11 Mouse xenograft studies

All animal procedures were performed in accordance with UHN institutional animal care guidelines. 6-8 weeks old male NOD-SCID mice (PMH/OICR) were used for intracranial xenograft studies. All intracranial injections were performed under general anesthesia (intraperitoneal injections of Avertin 1.25% solution, 0.2 mL/10 g body weight approximately 0.45 mL for 6 week old mice). Mice were monitored for any change in their status and were sacrificed according to animal protocol upon reaching a pre-moribund state, which is typically characterized by hunched or abnormal posture, decreased movement, lethargy, paralysis or weight loss. For intracranial injections, approximately 150,000 cells were suspended in 5µL PBS and were injected into the right frontal lobe of mice. The head was cleaned with 70 % ethanol, the scalp was opened by a 1 cm incision and the landmark was identified for injections (coordinates calculated from bregma). A small hole was drilled over the target area and the cells were injected using a Hamilton syringe. The cells injected include 1) parental U87; 2) parental GS2; 3) U87 scrambled shRNA and 4) U87 HK2shRNA.

For intracranial window (ICW) generation, mice were anaesthetized, a mid-line scalp incision was made to expose the skull and a circular bone flap was generated on the right frontal skull using a high-speed drill with a 2.7 mm trephine (Fine Science Tools) and approximately 150,000 cells suspended in 5µL of PBS were injected intracranially. A 5 mm glass coverslip (Warner Instruments) was placed over the exposed skull to create the window and self-curing dental acrylic (Bosworth) was used to seal the coverslip onto the skull surface\textsuperscript{190}. We imaged and examined the brains at the site of the ICWs by 2Photon Laser Microscope (2PLM) high-resolution \textit{in vivo} imaging. This imaging strategy allows for real-time, \textit{in vivo} visualization of
established tumors at different time points in the brain\textsuperscript{190}.

\subsection*{2.11.1 Treatment arms}

Mice received intracranial injections of U87 cells with conditional HK2shRNA construct. 7 days post implantation, mice were imaged by bioluminescence imaging (BLI, see below for detailed methods) to assess baseline tumor presence/size and mice without tumors or smaller BLI signals than the average were excluded from the study. Mice with comparable BLI measurements were randomized into Dox (HK2 knockdown) and no Dox groups (Control). 14 days post intracranial injections mice with established tumors were randomized into 3 treatment groups:

1) TMZ alone (TMZ administrated by oral gavage 66 mg/kg daily × 3 days)
2) RAD alone (2 Gy daily × 3 days – every other day)
3) Concomitant TMZ and RAD (TMZ given two hours before RAD).

Mice were followed for survival analysis and were sacrificed when symptomatic (weight loss, decreased oral intake, seizures, loss of fur). Post-sacrifice, brains were harvested to acquire pathological data. For brain pathology, a group of mice were perfused with ice-cold saline followed by 4\% paraformaldehyde (PFA) and the brains were harvested, incubated in PFA overnight followed by snap-freeze in liquid nitrogen store at -80\(^\circ\)C. Another group of mice were euthanized, brains were removed immediately and fixed in formalin followed by paraffin embedding. Only for Dox timing optimization experiments, Dox was administered from: 1) day 7-end; 2) day 7-day21 and 3) day21-end. For \textit{in vivo} HK2 knockdown experiments, mice were fed sterile doxycycline-containing food pellets.

\subsection*{2.12 Bioluminescence imaging (BLI)}

Bioluminescence imaging (BLI) was conducted using the Xenogen IVIS system of the Xenogen 100 series coupled with Living Image software for data acquisition (Xenogen Corp.). Mice were administered luciferin (D-luciferin potassium salt, 150 mg/kg, Xenogen Corp.) via IP injection and were anesthetized with isoflurane after 4 minutes and serial images were obtained following injections. The time of peak distribution within each group of mice was determined by serial image acquisition. For BLI signal analysis, Living Image software was used to define fixed regions of interest surrounding the intracranial area of signal and the total number of photons per
second per steradian per square centimeter was recorded.

2.13 Radiation

*In vitro* irradiation of cells was done using a Gamma Cell 40 irradiator (Nordion International) with a dose rate of 1 Gy/min for a total of 4 Gy. For *in vivo* radiation treatments, mice were anesthetized using isoflurane and placed in an in-house custom-built immobilization device. Stereotactic irradiation was delivered using a cone-beam CT image-guided small animal irradiator (XRad 225Cx micro-IGRT unit, Precision X-Ray, Inc). Radiation was prescribed with anterior-posterior parallel-opposed beams using a 1 cm collimator and irradiation was guided to the right cortex of the brain. Fractionated radiation schedule was used, delivering 6 Gy in 3 fractions of 2 Gy (6.2 Gy/min) every other day.

2.14 Magnetic resonance imaging (MRI)

A 7-Tesla BioSpec 70/30 MRI (Bruker Corporation) was used for imaging, equipped with the B-GA12 gradient coil and 7.2 cm inner diameter linearly polarized volume resonator coil for radiofrequency (RF) transmission as detailed previously. Two mice with HK2 knockdown U87 tumors and three with U87 control tumors were subjected to contrast-enhanced MRI at 3 weeks post-implantation to visualize tumor and evaluate vascular permeability through contrast transfer coefficient ($K_{trans}$). Mice were anesthetized with isoflurane and placed on the MR bed. Imaging sessions were aimed to include I) T2-weighted-RARE (T2w) anatomical images to confirm the presence of gross tumor; II) Dynamic contrast-enhanced (DCE) imaging; contrast delivery after 6 baseline images (0.38 mmol/kg Gadolinium/diethylene triamine pentaacetic acid via tail vein catheter injection over 6 sec) and III) Contrast enhanced T1-weighted RARE (T1w); start imaging at 5 min post-contrast to measure vascular permeability parameter ($K_{trans}$). MIPAV software (National Institutes of Health) was used to analyze the MRI images.

2.14 Statistical analysis

*In vitro* experiments were performed in triplicates. Means and standard deviations (SD) or standard error of the means were computed. Student’s t-test was used for pairwise comparison analysis of variance (ANOVA) in combination with Tukey's multiple-comparison post hoc test was performed for multivariate analysis. Significance was defined at p < 0.05. On the graphs,
asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001). In vivo survival analysis was performed using Kaplan-Meier curve generated in Prism 6 (GraphPad), and statistical significance was measured using log-rank (Mantel-Cox) test.
Chapter 3: Results

3.1 Cell lines with stable expression of luciferase

We generated GBM cell lines with stable expression of luciferase. Tagging GBM cell lines with luciferase allows for non-invasive, longitudinal and real-time detection and monitoring of tumor growth and response to therapy\textsuperscript{189}. GS2 cells already contained stable luciferase expression\textsuperscript{189}. U87 cell lines were transduced with a lentiviral vector containing firefly luciferase as described in section 2.3. The lentiviral luciferase vector contains a green fluorescent protein (GFP) marker that allows for assessment of viral titer and transduction efficiency. 7 days post-transduction, GFP-positive cells were purified and sorted by FACS and found to have transfection efficiency > 90\%. To measure \textit{in vitro} luciferase signal U87Fluc GFP-positive cells were lysed, treated with luciferin (substrate) and luciferase assay was performed using a luminometer. Luciferase signal was significantly higher in U87Fluc GFP cells compared to U87 parental control (Figure 3.1A). The U87Fluc cells had 71-fold higher luciferase level compared to non-luciferase U87 control cells (Figure 3.1A, \textit{p} < 0.0001). GS2 cell lines already carry stable luciferase expression and the luciferase assay showed 108-fold increase in the luciferase level when luciferin was added to the cell lysate (Figure 3.1B, \textit{p} < 0.0001). Luciferase activity was measured in triplicates for each group. Data were analyzed for significance by standard Student’s t-test. A value of \textit{p} < 0.05 was considered significant.
Figure 3.1: Luciferase activities in U87 (A) and GS2 (B) cells. U87 (A) and GS2 (B) Cell lysates were assayed for luciferase activity in the presence of luciferin substrate. Bars represent mean values ± SD, and asterisks denote a significant difference (* indicates $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$).
3.2 Cell lines with inducible expression of HK2shRNA

GBM cell lines with stable expression of luciferase, U87Fluc and GS2, were then used to make lines with inducible knockdown of HK2. pTRIPZ lentivirus system was used to generate cell lines that would only express the HK2shRNA insert in the presence of Doxycycline (Dox). The pTRIPZ lentiviral inducible HK2shRNA vector system was obtained from Open Biosystems containing the tet-on elements and a puromycin selection gene (Figure 3.1). Lentivirus particles carrying 3 HK2shRNA pTRIPZ plasmids (V1, V2 and V3) and the scrambled vector were produced as described in section 2.2.3. The 3 hairpin sequences were screened for HK2 protein expression using Western blots. Two hairpin sequences V1 and V2 were found to be the most efficient in HK2 Knockdown. V1 and V2 significantly reduced HK2 protein expression when Dox was added to the media for 72 hours, compare to parental cells and ones infected with scrambled shRNA as confirmed on Western blots and densitometric analysis (Figure 3.2). In U87 cells, V1 and V2 knockdowns showed 95% (p = 0.0211) and 93% (p = 0.0291) decrease in HK2 protein expression compared to control based on densitometric analysis (Figure 3.2A and C). In GS2 cells, V1 and V2 knockdowns showed 63% (p = 0.0211) and 79% (p = 0.0340) decrease in HK2 protein expression compared to control, respectively (Figure 3.2B and D). HK1 protein expression remained unchanged after the induction of HK2shRNA demonstrating specificity of shRNA sequences (Figure 3.2A and B). ANOVA followed by Tukey's post hoc test was used to compare groups to determine which clone had the lowest HK2 expression. A value of p < 0.05 was considered significant.
**Figure 3.2: Conditional HK2 knockdown.** Three independent HK2shRNA doxycycline (Dox) inducible pTRIPZ hairpins V1, V2 and V3 were used to knockdown HK2. Knockdown by V1 and V2 results in > 60% reduction in HK2 protein levels in both U87 (A,C) and GS2 (B,D) cell lines. After transduction, cells with pTRIPZ HK2shRNA or with scrambled-shRNA were treated with Dox (1 and 1.5 µg/µl) for 72 hours, lysed and total protein were extracted and analyzed by Western blotting. HK1 protein expression is unchanged upon HK2 knockdown. The HK2 protein levels were quantified relative to parental U87 (C) and parental GS2 (D) lines using imageJ. Bars represent mean values from three independent blots ± SD.
C

U87

Relative HK2 density

DOX (µg/µl)

0 0 1 1.5 0 1 1.5 0 1 1.5 1.5

Control V1 V1 V1 V2 V2 V3 V3 V3 Scr

D

GS2

Relative HK2 density

DOX (µg/µl)

0 0 1 1.5 0 1 1.5 0 1 1.5 1.5

Control V1 V1 V1 V2 V2 V3 V3 V3 Scr
3.3 The metabolic profile of HK2 knockdown cells

The changes in the metabolic parameters of GBM cell lines were examined by measuring the rate of oxygen consumption and the level of extracellular lactate formation in the cells after HK2 knockdown. Measuring oxygen consumption rates and extracellular lactate levels in HK2 knockdowns would determine whether conditional loss of HK2 could reverse or restore the key readouts or endpoints of the Warburg effect including decreased oxidative phosphorylation (OXPHOS, oxygen consumption) and increased lactate production. U87 and GS2 cells with HK2 knockdown (With Dox) had a significantly greater oxygen consumption rate (Figure 3.4A and B), as well as a significant lower rate of lactate production (Figure 3.4C and D) compared to control no knockdown (No Dox). Oxygen consumption was increased by 55% (p < 0.016) and 44% (p < 0.033) in U87 V1 and V2 HK2 knockdown cells compared to control (no Dox), respectively. In GS2 cells, oxygen consumption was increased by 54% (p < 0.013) and 55% (p < 0.014) in V1 and V2 HK2 knockdown cells compared to control, respectively (Figure 3.4A and B). Lactate levels were decreased by 60% (p < 0.0181) and 65% (p < 0.0096) in U87 V1 and V2 HK2 knockdown cells and by 59% (p < 0.0016) and 47% (p < 0.0131) in GS2 V1 and V2 HK2 knockdown cells, respectively (Figure 3.4C and D). Experiments were performed in triplicates. Data were analyzed for significance by standard Student’s t-test. A value of p < 0.05 was considered significant.
Figure 3.3: Conditional HK2 knockdown increases oxygen consumption rates and represses lactate production levels. Increased in oxygen consumption rate in U87 (A) and GS2 (B) cells upon HK2 knockdown (With Dox) is accompanied with a decrease in lactate production levels (extracellular) in U87 (C) and GS2 (D) cells. No Dox represents control cells without HK2 knockdown. With Dox represents cells with HK2 knockdown. Scra represents cells with scrambled shRNA treated with Dox. Bars represent mean values ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; and ***, p < 0.001).
3.4 Cell viability in HK2 knockdown cells in normoxia and hypoxia in vitro

We used HK2shRNA V2 clones for the remaining experiments since they showed the lowest HK2 protein expression upon Dox administration (Figure 3.2). We examined viability of U87 and GS2 cells with conditional HK2 knockdown in normoxic conditions. Cell viability was measured by direct cell count for days 2, 4, and 6 post incubation in 21% O₂ incubator. Cell viability was unchanged in HK2 knockdown (With Dox) U87 (Figure 3.4A) and GS2 (Figure 3.4B) lines, compared to scramble or control (No Dox). Since HK2 knockdown did not produce a sufficient or a significant change in cell viability, we evaluated the cell viability under hypoxic conditions of 1% oxygen. For hypoxic conditions, cells were sensitized by incubation in hypoxic chamber for 48 hours and later were harvested and plated for 6 days in the normoxic incubator. Marked reduction in cell viability was observed upon HK2 knockdown (With Dox) under hypoxia (Figure 3.5A and B). The viability of the U87 HK2 knockdown cells were significantly decreased by 50% (p = 0.021) and 32% (p = 0.032) compared to control group (No Dox) at 4 and 6 days post-incubation, respectively (Figure 3.5A). In GS2 HK2 knockdown cells viability was decreased by 51% (p = 0.0051) and 33% (p = 0.021) compared to the control (No Dox) at 4 and 6 days post-incubation, respectively (Figure 3.5B). Experiments were performed in triplicate. Differences between multiple groups were examined using ANOVA followed by Tukey's post hoc test. A value of p < 0.05 was considered significant.

3.5 Cell viability in HK2 knockdown cells upon treatments with radiation (RAD) and/or temozolomide (TMZ) in normoxia and hypoxia in vitro

Hypoxia sensitization significantly reduced the cell viability of U87 and GS2 cells with HK2 knockdown when combined with treatments including TMZ, RAD or RAD/TMZ, compared to controls with related treatments (Figure 3.5A and B). This sensitization was not seen in normoxic conditions. Differences between groups were examined using ANOVA followed by Tukey's post hoc test. A value of p < 0.05 was considered significant.
Figure 3.4: No changes in cell viability in normoxia in relation to conditional HK2 knockdown. Cell viability on day 2, 4 and 6 post-incubation in normoxic conditions in U87 (A) and GS2 (B) cell lines upon treatments including HK2 knockdown (With Dox), TMZ alone (100um), RAD alone (4 Gy) and combined RAD/TMZ. No changes in cell viability upon HK2 knockdown compared to No Dox in each treatment group. Bars represent mean values ± SD.
B

- GS2 No Dox
- GS2 With Dox
- GS2 No Dox + TMZ
- GS2 With Dox + TMZ
- GS2 No Dox + RAD (4Gy)
- GS2 With Dox + RAD (4Gy)
- GS2 No Dox + TMZ + RAD (4Gy)
- GS2 With Dox + TMZ + RAD (4Gy)

Average number of cells

Day2 | Day4 | Day6
Figure 3.5: Conditional HK2 knockdown reduces the cell viability in hypoxia. Cells were sensitized by incubation in hypoxia (1% O₂) for 48 hours and were harvested and plated for 6 days in the normoxic incubator. Bars show average number of cells in U87 (A) and GS2 (B) cell lines upon treatments including HK2 knockdown (With Dox), TMZ alone, RAD alone (4 Gy) and combined RAD/TMZ. HK2 knockdown further decreased the cell viability in cells treated with RAD and/or TMZ at days 4 and 6 compared to no knockdown (No Dox) with treatment. Bars represent mean values ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; and ***, p < 0.001).
3.6 DNA damage in HK2 knockdown cells in normoxia and hypoxia in vitro

We next hypothesized that combining HK2 knockdown with standard therapeutics of RAD and/or TMZ may lead to enhanced DNA damage in GBM cell lines. Alkaline comet assay was utilized to detect DNA damage at the level of individual cells post-treatment with HK2 knockdown (With Dox), RAD alone, TMZ alone, and combined RAD/TMZ under normoxia. Cells were processed immediately post-irradiation and 48 hours post-TMZ treatment. U87 and GS2 cells showed no increases in DNA damage in respect to HK2 knockdown compared to no knockdown (No Dox) (Figure 3.6). HK2 knockdown cells treated with TMZ did not show an increase in DNA damage when compared to control cells (No Dox) treated with TMZ (Figure 3.6A). DNA damage was significantly increased in U87 and GS2 HK2 knockdown cells treated with RAD alone compared to control cells treated with RAD without HK2 knockdown (Figure 3.6B, p < 0.0001). Furthermore, DNA damage was significantly increased in GS2 cells using combined RAD/TMZ treatment on HK2 knockdown cells compared to controls (Figure 3.6C, p < 0.0001). We detected no changes in combination of RAD/TMZ treated U87 cells with respect to HK2 knockdown (Figure 3.6C). Differences between multiple groups were examined using ANOVA followed by Tukey's post hoc test. A value of p < 0.05 was considered significant. Above results correspond to normoxic conditions.

In hypoxic conditions, no sign of DNA damage was observed in U87 and GS2 cells upon HK2 knockdown alone (With Dox) relative to control without knockdown (No Dox) (Figure 3.7). However, both U87 and GS2 HK2 depleted cells showed enhanced degrees of DNA damage upon treatment with TMZ in hypoxia (Figure 3.7A), RAD (Figure 3.7B) and combined RAD/TMZ (Figure 3.7C), compared no knockdown treatments (p < 0.0001). Differences between multiple groups were examined using ANOVA followed by Tukey's post hoc test. A value of p < 0.05 was considered significant.
Figure 3.6: DNA damage in U87 and GS2 cells upon conditional HK2 knockdown in normoxia. Cells are treated with TMZ alone (A), RAD alone (B), combined RAD/TMZ (C) and (D) Dox for HK2 knockdown (With Dox). Box plots show median Olive tail moment as a measurement of DNA damage. Enhanced DNA damage is observed in U87 RAD + HK2 knockdown (B), GS2 RAD + HK2 knockdown (B) and GS2 RAD/TMZ + HK2 knockdown (C) compared to no knockdowns (No Dox) in each treatment group. Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001, ****; and p < 0.0001).
Figure 3.7: DNA damage in U87 and GS2 cells upon conditional HK2 knockdown in hypoxia. Cells are treated with TMZ alone (A), RAD alone (B), combined RAD/TMZ (C) or (D) Dox for HK2 knockdown. Box plots show median Olive tail moment as a measurement of DNA damage. HK2 knockdown (With Dox) enhances the DNA damage in all treatment groups in hypoxia. Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001).
3.7 Clonogenic potential of HK2 knockdown cells in normoxia and hypoxia

We assessed the ability of different treatments including HK2 knockdown (With Dox), TMZ alone, RAD alone or combined RAD/TMZ to inhibit clonogenic survival of U87 and GS2 cells. Under normoxic conditions, clonogenic potential of the cells was unchanged in U87 and GS2 HK2 knockdowns with treatment compared to cell without HK2 knockdown (No Dox) with related treatments (Figure 3.8A and B, graphs on the left). Only in U87 cells did combination of HK2 knockdown with RAD/TMZ show a significant decrease in clonogenic potential relative to RAD/TMZ treated controls (No Dox) in normoxia (Figure 3.8A, \( p = 0.032 \)). In contrast, when cells were sensitized under hypoxia, the clonogenic potential of U87s (Figure 3.8A, graph on the right) and GS2 (Figure 3.8B, graphs on the right) cells with HK2 knockdown were significantly inhibited relative to control without HK2 knockdown. Furthermore, the combination of HK2 knockdown with RAD, TMZ, or combined RAD/TMZ significantly decreased the surviving fraction of the cells relative to treatment controls without HK2 knockdown (Figure 3.8A and B, graphs on the right). Differences between groups were examined using ANOVA followed by Tukey's post hoc test. A value of \( p < 0.05 \) was considered significant.
**Figure 3.8:** Conditional HK2 knockdown reduces the clonogenic potential of the cells in hypoxia. HK2 knockdown (With Dox) decreased the clonogenic potential of U87 (A) and GS2 (B) cells under hypoxic conditions but not normoxic conditions. Bars represent mean relative values compared to U87 NT control ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; and $$$, p < 0.001).
3.8 Growth kinetics of U87 and GS2 intracranial xenografts

We initially used bioluminescence imaging (BLI) to determine the growth kinetics of parental U87 and GS2 tumors, and to establish our BLI technique to detect intracranial tumor growth. These measurements were used as baseline values for future HK2 knockdown experiments. Orthotopic GBM xenograft models were established with U87 and GS2 stable cells expressing luciferase, with 80% tumor take intracranially. We quantified and monitored growth kinetics of parental U87 (n=10) and GS2 (n=10) intracranial xenograft tumors longitudinally using weekly BLI starting at baseline from week 1 to week 5. BLI confirmed a consistent pattern of increase in the luminescence signal from week 1 to 4 or 5 for U87 and GS2 cell lines, respectively (Figure 3.9A). Mean luminescence signals revealed readily detectable and quantifiable signals from tumors starting from 7 days post-implantation. Results suggest that GS2 tumors have a slower growth rate compared to U87 tumors, however they do grow to reach the same luminescence measure by week 5. Figure 3.9B shows the heat map image corresponding to each cell line from week 1 to week 4.
Figure 3.9: Growth kinetics of U87 and GS2 xenograft tumors monitored by bioluminescence imaging (BLI). *In vivo* BLI of xenografted parental U87 (n=10, black line) and GS2 (n=10, purple line) cells (A) with representative heat map image for each tumor line from week 1 to week 5 (B). Each dot represents mean luminescence signal ± SD.
3.9 Tumor response to conditional HK2 knockdown

To study the impact of HK2 knockdown in intracranial GBM models we generated intracranial xenografts using U87 cell lines with HK2shRNA construct and U87 scrambled shRNA lines. We utilized the U87 cell line for the remaining knockdown and treatment experiments. Mice injected with U87 cells with HK2shRNA construct were randomized into Dox (HK2 knockdown) and no Dox (control) groups at day 7 post-implantation. As a control, mice were injected with U87 cells with scrambled shRNA construct and similarly treated with Dox. We also utilized our intracranial window models (ICW) to assess the time it takes for RFP-HK2-shRNA to be expressed post-doxycline (Appendix 1).

BLI was used to monitor the tumor growth in all groups from week 1 to week 5. Whereas U87 controls and U87 scramble treated with Dox rapidly generated tumors, the growth of U87 HK2 knockdown tumors was significantly decreased (Figure 3.10A). In comparison to the control group, luminescence signal in U87 HK2 knockdown mice was substantially reduced from week 3 ($p = 0.015$), and remained low after 5 weeks following implantation (Figure 3.10A, $p = 0.0022$). Figure 3.10B shows the representative heat map image corresponding to U87 control and U87 HK2 knockdowns from week 1 to week 5. Survival analysis revealed that the median survival of mice with HK2 knockdown was significantly increased compared to mice without HK2 knockdown (47.5 days vs. 36 days, $p < 0.0001$, Figure 3.10C). Kaplan-Meier survival curve were generated in Prism 6 (GraphPad), and statistical significance was measured using log-rank (Mantel-Cox) test.
Figure 3.10: Tumor response to conditional HK2 knockdown. Bioluminescence imaging (BLI) of xenografted U87 HK2 knockdown (With Dox) (n=10), U87 scrambled with Dox (n=10) and U87 control (No Dox) (n=10). Quantification of BLI signal over tumor region from week 1 to 5 (A) with representative heat map image (B) of mice with U87 tumors +/− HK2 knockdown. Doxycycline (Dox) was administered from day 7 to the end of the experiment. Kaplan–Meier survival curve showing mice with HK2 knockdown survived significantly longer compared to control mice (C). Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001).
3.10 Temporal dependence of HK2

In order to determine whether there is a temporal profile in expression and the role of HK2, we examined changes in tumor growth and survival based on different stages of HK2 knockdown. We examined 4 different patterns of HK2 knockdown by varying Dox administration timing in mice:

1) Early Dox (Day 7-21), loss of HK2 at early stages and HK2 rescued at day 21
2) Late Dox (Day 21-end), loss of HK2 at late stages and allowing HK2 to be present at early stages of tumor take
3) Throughout (Day 7-end), loss of HK2 throughout all stages of tumor growth
4) Control (No Dox), no loss of HK2

Analysis of survival data between the 4 groups revealed that the mice that were administered Dox throughout the experiment (group 3) had the most significant increase in the survival time relative to control (45 days vs 33 days, \( p = 0.0005 \), Figure 3.11). Shorter Dox treatments including Dox from Day 21-end and Dox from Day 7-21 did not provide a significant survival benefit for the mice compared to control (Figure 3.11). Kaplan-Meier survival curve were generated in Prism 6 (GraphPad), and statistical significance was measured using log-rank (Mantel-Cox) test.
**Figure 3.11: Optimal timing for HK2 knockdown during GBM tumor growth.** Kaplan–Meier survival curve of mice with intracranial U87 HK2shRNA cells treated with Dox at different time points. The survival of mice with U87 tumors treated with Dox from day7-end (n=7) was significantly improved from those treated with Dox from day7-21 (n=7), day21-end (n=7) or control no Dox (n=5). Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001).
3.11 Histopathological analysis of tumors with conditional HK2 knockdown

Histopathological analysis was performed on brain sections examining pathological differences based on HK2 status. Intracranial HK2 knockdown was confirmed with immunohistochemical (IHC) staining for HK2. U87 control (No Dox) tumors had a strong HK2 staining, confirming high HK2 expression in the tumors. U87 tumors with HK2 knockdown (With Dox throughout) showed negative HK2 staining compared to controls—demonstrating successful maintenance of HK2 knockdown throughout the experiment (Figure 3.12D). The morphology of tumors varied between the U87 controls and HK2 knockdowns. Haematoxylin and eosin (H&E) staining revealed gross changes in brain pathology in HK2 knockdown tumors (Figure 3.12B) compared to U87 control (Figure 3.12A). U87 control tumors grew comparatively well-circumscribed, as previously reported 99 with no evidence of necrosis or invasion (Figure 3.12A). Tumors originated from U87 HK2 knockdown lines tended to possess three distinct regions of necrotic center, surrounded by proliferating tumor cells in the periphery and a border zone containing predominantly inflammatory cells. Therefore, for further analysis we divided the HK2 knockdown tumors based on these three distinct phenotypical zones/regions: central, border and peripheral (Figure 3.12B and D). Figure 3.12C is the close up of infiltrating neutrophils at the border region in U87 HK2 knockdown tumors. Figure 3.12D demonstrates IHC analysis in U87 control (left) tumors and U87 HK2 knockdown tumors with three distinct regions (right).

Hypoxia and necrosis/apoptosis

In U87 HK2 knockdown tumors, HIF1α staining was detected at the areas bordering the inflammatory cells, which was absent in U87 control tumors (Figure 3.12D). CAIX expression was used as a secondary marker for hypoxia and to confirm HIF1α staining. CAIX showed high level of staining at the areas bordering the inflammatory cells (similar to HIF1α staining) in U87 HK2 knockdown tumors compared to U87 control (Figure 3.12D). TUNEL staining was used to evaluate apoptosis in tumor area. TUNEL-positivity was only observed in U87 HK2 knockdown tumors in the central necrotic region (Figure 3.12D). Minimal TUNEL-staining was observed in U87 control tumors. Positive cleaved caspase 3 staining was observed at the areas bordering the inflammatory cells in U87 HK2 knockdown tumor sections (similar to HIF1α and CAIX) that were absent in U87 control tumors (Figure 3.12D).
**Tumor cellularity and proliferation**

Knockdown of HK2 in U87 tumors significantly reduced tumor cellularity by 39% compared to control no knockdown (Figure 3.13A, p < 0.0001), measured by DAPI staining in the periphery region of tumors. In addition to tumor cellularity, Ki67 staining showed that HK2 knockdown tumors had a significant reduction in cellular proliferation in the periphery region by 56% compared to control no knockdown (Figure 3.13B, p < 0.0001).

**Tumor vascularity**

Tumor vessels were visualized with CD31 staining. The CD31 staining was significantly decreased in the tumors with HK2 knockdown in the periphery region away from the necrotic core compared to U87 control tumors (Figure 3.14A). There was 55% reduction in microvascular density (MVD) (p = 0.0033), accompanied with 61% increased in microvascular diameter (p = 0.0001) in U87 HK2 knockdown tumor sections relative to U87 control tumors (Figure 3.14B).
Figure 3.12: Histopathological analysis of U87 tumors +/- HK2 knockdown. Representative brain sections from U87 control tumors (A) and U87 HK2 knockdown tumors (B). U87 HK2 knockdown tumor sections were divided into three regions of center (black box), periphery (green box) and border (red box) (B). Close up of infiltrating neutrophils at the border region of U87 HK2 knockdown tumors (C). Histopathological analysis in U87 control and HK2 knockdown tumors (D). Color-coded division of HK2 knockdown tumors with black box for central region, green box for peripheral region and red box for the border.
D

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Figure 3.13: Changes in proliferation and cellularity with conditional HK2 knockdown. Ki67 (E) and DAPI (F) quantifications for U87 control and the peripheral region of U87 HK2 knockdown tumor sections. Bars are normalized to U87 control. Bars represent relative mean values ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and **** p < 0.0001).
Figure 3.14: Changes in vascular morphology with conditional HK2 knockdown.

Immunohistochemical (IHC) staining with CD31 in U87 control and the periphery region of U87 HK2 knockdown tumor sections (A). Quantifications of microvascular density (B, left) and microvascular diameter (B, right). Bars represent mean values ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; and ***, p < 0.001).
3.12 Reduction in vessel permeability parameter $K_{\text{trans}}$

Three weeks after implantation of U87 HK2 knockdown and U87 control tumors, mice were subjected to MR imaging. Mice with U87 HK2 knockdown tumors showed smaller tumors compared to control, in both dynamic contrast-enhanced (DCE) MR images (Figure 3.15A, top frame) and T1-weighted with contrast (T1w) MR images (Figure 3.15A, bottom frame). We were able to measure $K_{\text{trans}}$ values for each tumor, which is an imaging biomarker of response to changes in the vasculature indicating a combination of both flow and permeability of the vessel walls\textsuperscript{192,193}. $K_{\text{trans}}$ was reduced in U87 HK2 knockdown tumors compared to control (Figure 3.15B, $p = 0.0047$). Data were analyzed for significance by standard Student’s t-test. A value of $p < 0.05$ was considered significant.
Figure 3.15: MRI of U87 xenograft tumors +/- HK2 knockdown. Mice with U87 tumors +/- HK2 knockdown (A) were imaged by MRI three weeks post-implantation. Representative dynamic contrast-enhanced (DCE) MR image (A, top frame) and T1-weighted (A, T1w) MR images (bottom frame) of mice with tumor. Vascular permeability was measured using $K_{\text{trans}}$ values calculated from brain scans (B). Bars represent mean values ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001).
3.13 The synergistic effect of loss of HK2 along with RAD and/or TMZ in vivo

To evaluate the therapeutic response of tumor xenografts with HK2 knockdown, mice with intracranial injection of U87 HK2shRNA were randomized to Dox (HK2 knockdown) and no Dox (control) groups at day 7 post-implantation. The mice were screened using BLI 7 days post-implantation prior to Dox randomization to assess tumor presence and mice without tumors or smaller than average BLI signals were excluded from the study. 14 days post-implantation mice with established tumors were randomized to one of four groups

1) TMZ alone (66 mg/kg daily × 3 days)
2) RAD alone (2 Gy daily × 3 days)
3) Concomitant RAD/TMZ
4) Control (no treatment)

Survival analysis revealed that median survival of the mice with conditional HK2 knockdown was significantly increased compared to control group (44.5 days vs. 36 days, p < 0.0001, Figure 3.17A). The median survival for mice receiving RAD treatment combined with HK2 knockdown was further increased compared to control RAD without HK2 knockdown (52 days vs. 43 days, p = 0.0004, Figure 3.16). There was no associated survival benefit in TMZ monotherapy groups in respect to HK2 knockdown (85.5 days vs. 85 days, p = 0.5202, Figure 3.16). The biggest effect was observed in the combination triple regimen arm. The concurrent RAD/TMZ with HK2 knockdown was significantly more effective at prolonging survival of the mice compared to RAD/TMZ control (114 days vs 75 days, p = 0.0004, Figure 3.17A). 5 mice were alive at day 118 post-implantation in RAD/TMZ with HK2 knockdown group (survival proportion = 38%) (Figure 3.17A). These mice were sacrificed at day 118 to assess tumor pathology. 2 mice out of 5 were found to have residual tumors. Kaplan-Meier survival curves were generated in Prism 6 (GraphPad), and statistical significance was measured using log-rank (Mantel-Cox) test.

In order to replicate the combination treatment arm, mice were injected with U87 cells with conditional HK2shRNA construct and were randomized to 4 groups of:

1) U87 control (No Dox)
2) U87 HK2 knockdown (With Dox)

3) U87 RAD/TMZ (No Dox)

4) U87 RAD/TMZ HK2 knockdown (With Dox)

**Figure 3.17B** shows a rain plot corresponding to the total number of days mice survived in each group, combined with numbers from previous *in vivo* experiments. Analysis of survival days confirmed our previous results that HK2 knockdown confers a survival benefit to the mice (**Figure 3.17B, p = 0.0074**). Moreover, mice that received triple regimen of RAD/TMZ with HK2 knockdown survived substantially longer compared to mice that received RAD/TMZ alone (**Figure 3.17B, p < 0.0001**). On the plot, each dot represents a mouse. Differences between groups were examined using ANOVA followed by Tukey's post hoc test. A value of *p* < 0.05 was considered significant.
Figure 3.16: Survival benefit in radiation and temozolomide monotherapy in respect to HK2 knockdown. Kaplan–Meier survival curve showing percent survival of mice bearing U87 tumors randomized to Dox (HK2 knockdown) (n=10) or No Dox (control) (n=11) and treated with RAD alone (n=6) and TMZ alone (n=6 No Dox and n=10 With Dox). Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001, and ****, p < 0.0001).
Figure 3.17: Survival benefit in combination regimen of radiation (RAD) and temozolomide (TMZ) in respect to HK2 knockdown. Kaplan–Meier survival curve showing percent survival of mice bearing U87 tumors randomized to With Dox (HK2 knockdown) (n=11) or No Dox (control) (n=10) and treated with combined RAD and TMZ (n=8 No dox and n=12 With Dox). Rain plot shows number of the days mice survived in U87 With Dox (n=18), U87 No Dox (control) (n=18), U87 RAD/TMZ No Dox (n=13) and U87 RAD/TMZ With Dox (n=19) groups – each dot represents a mouse on the rain plot (B). Asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001, and ****, p < 0.0001).
3.14 Histopathological analysis of treated tumors

Since combination regimens resulted in a profound therapeutic response in mice, histological analysis was performed in concomitant RAD/TMZ group +/- HK2 knockdown. H&E staining revealed that the tumors were smaller in combination RAD/TMZ group regardless of HK2 knockdown. Tumors did not show significant necrosis, however some tumor sections in RAD/TMZ with HK2 knockdown showed small necrotic regions with accumulation of inflammatory cells compared to control (Figure 3.18). The persistence of HK2 knockdown was determined by IHC for HK2, where no staining was observed in tumor sections with HK2 knockdown (Figure 3.18). Ki67 staining showed that proliferation was significantly reduced in U87 RAD/TMZ with HK2 knockdown by 56% compared to U87 RAD/TMZ alone (Figure 3.19A, p < 0.0001). DAPI staining showed 21% reduction in cellularity in U87 RAD/TMZ with HK2 knockdown compared with RAD/TMZ alone (Figure 3.19B, p = 0.0061). HIF1α and CAIX staining was detected in both treatment groups regardless of HK2 knockdown (Figure 3.18). TUNEL-positive cells were only present in the necrotic regions of U87 RAD/TMZ with HK2 knockdown tumors (Figure 3.18). Positive cleaved caspase 3 staining was observed in the same areas that stained for TUNEL (Figure 3.17A).

The CD31 staining was significantly decreased in tumors with triple regimen of RAD/TMZ HK2 knockdown compared to control RAD/TMZ alone (Figure 3.20A and B). There was 32% reduction in microvascular density (MVD) in U87 RAD/TMZ HK2 knockdown tumor sections relative to control tumors (Figure 3.20B, p = 0.0259). Changes in microvascular diameter were not significant in treatment groups +/- HK2 knockdown (Figure 3.20B).
Figure 3.18: Histopathological analysis in U87 tumors with combination regimen of radiation (RAD) and temozolomide (TMZ) in respect to HK2 knockdown. Representative tumor sections of U87 RAD/TMZ (A, left) and U87 RAD/TMZ HK2 knockdown (A, right).
Figure 3.19: Changes in proliferation and cellularity with HK2 knockdown in treated tumors. Ki67 (A) and DAPI (B) quantifications for tumor sections—bars are normalized to U87 control. Bars represent relative mean values ± SD, and asterisks denote a significant difference (* indicates p < 0.05; **, p < 0.01; ***, p < 0.001; and **** p < 0.0001).
Figure 3.20: Changes in vascular morphology with HK2 knockdown in treated tumors. Immunohistochemical (IHC) staining with CD31 in U87 RAD/TMZ treatment +/- HK2 knockdown (A). Quantifications of microvascular density (B, left) and microvascular diameter (B, right). Bars represent mean values ± SD, and asterisks denote a significant difference (* indicates $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).
Chapter 4: Discussion, Conclusion and Future Directions

4.1 HK2 inhibition as an attractive therapeutic approach

The clinical prognosis for GBM patients is still extremely poor despite surgery and radiochemotherapy. In the last 20 years, concurrent use of temozolomide (TMZ) with radiation therapy has been the only advancement in the management of GBM patients, providing only a 16% increase in patient survival up to 2 years post-diagnosis (from 10% to 26%)\textsuperscript{30}. This incremental improvement in patient survival rationalizes integration of therapeutic approaches that combine novel targeted therapies with standard treatments such as radiochemotherapy.

In the last decade, refocusing research on altered tumor metabolism has established an intimate link between cancer-specific metabolic anomalies and the principal hallmarks of cancer\textsuperscript{58}. This link between cancer and metabolism has led to the emergence of therapeutic concepts aimed at inhibiting cancer-specific metabolic programs or drivers. Now it is becoming increasingly apparent that the metabolic shift towards aerobic glycolysis is a critical mechanism by which tumor cells including GBMs proliferate and adapt to their unfavorable microenvironment. Among different metabolic anomalies, HK2 is emerging as a major hexokinase isoform overexpressed in tumor tissue that facilitates metabolic reprogramming towards the Warburg effect in many cancers including GBMs\textsuperscript{99,123,124,159}.

Amongst the many glycolytic enzymes, HK2 represents a potential therapeutic target in GBMs for several reasons. HK2 is differentially expressed between GBMs and normal brain or low grade astrocytoma\textsuperscript{99}, in addition, disrupting glycolysis though HK2 inhibition has shown anti-cancer effects not only by reversing the metabolic reprogramming and limiting anabolic reactions but also by inducing radio-chemo sensitization and disrupting growth and redox balance of the cell. Since HK1 (the brain HK) is the most abundant HK isoform in the brain and neurons\textsuperscript{106-108}, pharmacological inhibition of HK2 may act selectively on tumor cells to minimize systemic toxicity and side effect toward normal brain tissue. To date, there has been no reports of pre-clinical or clinical combination of glycolysis inhibitors such as HK2 inhibitors and radiation plus-TMZ in GBMs. Moreover, no isozyme specific inhibitors of HK2 have been developed to
precisely evaluate its therapeutic effect\textsuperscript{60}. Thus, we propose that selective inhibition of HK2 (with tissue and molecular specificity) may serve as an attractive addition to the current anti-cancer regimens in GBMs.

### 4.1.1 Specific HK2 inhibitors

Anti-glycolytic-based therapeutic strategies have focused on targeting glycolytic enzymes and dysregulated metabolism and have shown encouraging anti-tumor effects by altering cellular energy metabolism, cellular survival and most importantly have shown radio-chemo sensitization effects. Agents that inhibit glycolysis through HK inhibition or HK-mitochondrial detachment in combination with conventional radiochemotherapy have shown striking cytotoxicity effects and generated excitement about the possibility of using these agents in clinical settings\textsuperscript{71,132}. Various glycolytic inhibitors and broad-spectrum HK inhibitors such 2DG and 3-BrP have been shown to be effective as potential anticancer drugs when used in conjunction with chemotherapeutic drugs (cisplatin and BCNU), radiotherapy or in specific genetic backgrounds\textsuperscript{194,185,161,187}. However, these agents are non-specific inhibitors and show global hexokinase inhibition (HK1, HK2, HK3, HK4 and GAPDH) and thus the specific impact of HK2 inhibition is not entirely known. In addition, the systemic toxicity limits the clinical application of these compounds as primary therapeutics. Given our data and those of the literature demonstrate the pivotal role of HK2 in GBM growth and response to radiochemotherapy, there is a strong rational to support the development and implementation of therapeutic agents that specifically target HK2 isoform.

### 4.2 The role of HK2 in modulating metabolism and sensitization to radiochemotherapy

Results from this thesis demonstrate that in GBM cell lines, HK2 functions optimally to shunt glucose toward glycolysis and to promote the Warburg phenotype (Figure 3.3). Conditional inhibition of HK2 by shRNA disrupts energy homeostasis and leads to attenuation of aerobic glycolysis by reducing lactate production and increasing oxygen consumption rate of GBM cells (Figure 3.3). Our \textit{in vitro} results indicate that conditional HK2 knockdown impairs GBM cell viability and clonogenic potential under hypoxic conditions. In addition, conditional HK2 knockdown sensitizes GBM cells to cytotoxic therapies of RAD and/or TMZ with subsequent reduction in cell viability (Figure 3.5), colongentic potential (Figure 3.8), and enhances the
DNA damage (Figure 3.7) under hypoxic conditions. Most notably, inhibition of glycolysis through HK2 knockdown is most effective in promoting radio-chemo sensitization under low oxygen conditions (1% O₂). While in normoxic conditions, loss of HK2 provided GBM cells with minimal sensitization to RAD and/or TMZ treatments (Figure 3.4 and 3.6). This might be due to several reasons. First, under standard culture conditions glutamine is another carbon source besides glucose that is present in the media and in the instances where glucose cannot be metabolized by glycolysis, glutamine is an alternative substrate that can be catabolized (glutaminolysis) to provide fuel and biosynthetic requirements for the cells. Second, when oxygen is present, intact mitochondria can contribute to cellular bioenergetics by utilizing oxygen to produce enough ATP to compensate for the lack of glycolysis. However, in hypoxic conditions due to reduced oxygen availability, the mitochondrial contribution to cellular bioenergetics would be minimal. Thus, inhibition of glycolysis in combination with oxygen-deprivation will act as a “dual hit” to preferentially induce cell death (apoptosis or necrosis)⁶⁰. On the same line, it has been shown that GBM and breast cancer cells are more sensitive to HK inhibitors under hypoxia, and HK inhibitors impair proliferation and ATP production only under hypoxia or glucose limiting culture conditions¹⁵⁹,¹⁹⁵.

In addition, we have utilized an inducible HK2shRNA system for conditional HK2 knockdown and with this technique the cells might not have had enough time to adapt to the cellular conditions, compared to a constitutive gene silencing. However, the conditional approach to the knockdown is an essential part of our experimental design since we were able to temporally regulate HK2’s expression in established tumors. Previous results from our laboratory indicated that GBM cell lines with constitutive HK2 knockdown do not show sufficient intracranial growth⁹⁹, so would not be suitable for therapeutic purposes. Thus, in order to delineate HK2’s role in GBM tumor progression (rather than establishment) and determine its potential as a therapeutic target, it is imperative to use an inducible system.

### 4.3 The role of HK2 in tumor growth and response to therapy

In order to examine the effects of HK2 on in vivo tumor growth, we generated intracranial GBM models with an inducible HK2 suppression, which allowed us to regulate HK2 expression levels at different stages of tumor growth and also prevented adaptive modification of cells to constant HK2 knockdown. We showed that conditional HK2 knockdown reduces the growth rate of
intracranial GBM xenografts and prolongs the median survival time compared to control with high HK2 expression (Figure 3.10, p < 0.0001). Since we used a doxycycline-inducible system, we were able to control HK2 suppression at different stages of tumor growth (Figure 3.11) and we anticipated that modulating HK2 levels at different stages of GBM growth would vary the pattern of tumor growth and in turn yield differential survival benefits. Our data confirmed our postulate as mice that were administered Dox throughout the experiment (from day 7 to the end) – had loss of HK2 throughout all stages of GBM growth– had the most significant increase in the median survival time relative to control no Dox (Figure 3.11, p = 0.0005). When Dox delivery was stopped at day 21, 57 % of the mice in this group expired early by day 34 and the median survival (34 days) was similar to control no Dox (33 days) –suggesting that the survival advantage of HK2 knockdown was mostly lost (Figure 3.11, p = 0.2254). Similarly, HK2 knockdown at late stages (from day 21)– allowing HK2 to be present at early stages of tumor take– did not provide a significant survival advantage for the mice (median survival of 34 days). Thus, based on our temporal studies it appears that loss of HK2 is optimal when present at early stages of tumor development and continued throughout all stages of tumor progression in GBM.

Since concomitant radiochemotherapy has been well established in the clinic for GBM patients, new treatment strategies should compare with the efficacy of that protocol. In this regard, our in vivo combinatorial therapy results demonstrate for the first time that mice that received the triple regimen of HK2 knockdown with concomitant radiochemotherapy survived significantly longer compared to mice with the same treatment regimen but without HK2 knockdown (114 days vs 75 days, p = 0.0004, Figure 3.17A). Addition of HK2 knockdown to fractionated RAD alone or radiochemotherapy markedly increased the antitumor effect of the standard treatment regimens, where 5 of 13 (38%) mice were alive on day 118 in HK2 knockdown radiochemotherapy group, whereas all of the 9 control mice in radiochemotherapy group without HK2 knockdown were expired by day 84 post-implantation (Figure 3.17A). From 5 mice that were alive at the end-point, only 2 showed residual tumor on histology.

Our in vivo data confirms our hypothesis that altering tumor metabolism through selective HK2 knockdown may potentiate the anti-tumor activity of standard cytotoxic therapies in GBM orthotopic mouse models. The precise mechanism underlying radio-chemo sensitization of glycolytic inhibition is complex and requires further investigation. Some of contributing factors reported in the literature have been further reduction in ATP production, induction of oxidative
stress and attenuation of DNA repair\textsuperscript{179-181}. Altogether, these data support the notion that combining standard anticancer agents with targeted therapies are expected to have the greatest efficacy by broadening the scope of pathways inhibited, targeting tumor cells with heterogeneous tumorigenic potential\textsuperscript{159,195} and/or lessening the mechanism of resistance\textsuperscript{36}.

### 4.4 Remodeling of GBM tumors upon HK2 knockdown

It has been suggested that tumor oxygenation is very sensitive to changes in oxygen consumption rate of the cells \textit{in vivo}\textsuperscript{196}. Since inhibition of HK2 increases the oxygen consumption rate of GBM cells \textit{in vitro} (\textbf{Figure 3.3A and B}), we postulate that during the course of tumor development, HK2 intervention would increase oxygen consumption, decrease tumor oxygenation and in turn enhance the hypoxic fraction of the tumors. Indeed, histological evaluations demonstrated that intracranial GBM tumors with HK2 knockdown have increased necrosis, increased areas of hypoxic fraction, presence of infiltrating inflammatory cells as well as reduced vascularization (\textbf{Figure 3.12}). Histologically, U87 HK2 knockdown tumors had prominent and large regions of necrosis in the central portion of the tumors that was surrounded by infiltrating inflammatory cells including neutrophils –presumably recruited to remove dead cells in the necrotic region (\textbf{Figure 3.12B and C}). We assessed the hypoxia index in the tumor area by HIF1\(\alpha\) staining. In addition, CAIX staining was used to confirm HIF1\(\alpha\) expression and the extent of hypoxia. CAIX is considered to be a much more reliable endogenous biomarker of hypoxia since its protein is much more stable under normoxia or during processing compared to HIF1\(\alpha\). High spatial correlations between HIF1\(\alpha\) and CAIX staining was observed, with their distribution in the tumor tissues adjacent to the necrotic areas (border), while no expression was seen in normal brain or U87 control tumors (no Dox) (\textbf{Figure 3.12D}). CAIX staining was more extensive in peripheral region of knockdown tumor encompassing a larger area compared to HIF\(\alpha\) staining (\textbf{Figure 3.12D}).

Furthermore, IHC staining with an endothelial marker CD31 and measuring microvascular density (MVD) demonstrated that HK2 knockdown leads to a significant reduction in the MVD (\textbf{Figure 3.14A and B, }\(p = 0.0001\)), however this decrease in MVD was evident predominantly in the periphery region, as characterized by areas of proliferating and higher density viable cells surrounding the region of necrosis. Along with the decrease in MVD, there is evidence of an increase in microvascular diameter in the peripheral region suggesting a compensatory change in
the vascular dynamics in comparison to U87 control tumors (no Dox) (Figure 3.14A and B, p = 0.0033). In addition, we were able to use contrast-enhanced MRI to measure global $K_{\text{trans}}$ values, which reflect the rate of the movement of contrast agent from intravascular to the extravascular space. $K_{\text{trans}}$ is an imaging biomarker of response to changes in the vasculature—indicating a combination of both flow and permeability of the vessel walls. $K_{\text{trans}}$ was reduced in U87 HK2 knockdown tumors relative to control, which could suggest a reduction in vascular permeability (Figure 3.15B, p = 0.0047).

The phenotypical and histopathological alterations seen as a consequence of HK2 suppression, such as presence of necrosis, hypoxia and inflammatory infiltration suggests that the increase in the oxygen consumption rate of the cells induced by HK2 loss, decreases oxygen availability and enhances tumor hypoxia in vivo. This hypoxic microenvironment in combination with reduced vascularity or microvascular function contributes to GBM remodeling towards necrosis. However, whether disrupted/attenuated vasculature morphology is the cause or consequence of necrosis is unclear.

### 4.5 Remodeling of GBM tumors with HK2 knockdown in combination with radiochemotherapy

Our in vivo triple regimen results indicate that combining HK2 knockdown with concomitant radiochemotherapy significantly increases the antitumor effect of the standard treatments in U87 xenografts (Figure 3.17A). The histologic findings showed that radiochemotherapy treated tumors were smaller relative to control U87 +/- HK2 knockdown tumors and showed minimal necrosis. However, small number of treated tumors with HK2 knockdown had necrotic foci with accumulation of inflammatory cells. Similarly, HIF1α and CAIX staining was detected in high levels in both treatment groups regardless of HK2 status (Figure 3.18). We postulate that less necrosis in tumors of treated animals compared to U87 HK2 knockdown alone may be because tumors that received radiochemotherapy had lower growth rate along with smaller tumor size, and thus the metabolic demands of the tumors were better met even with HK2 knockdown. Conversely, tumor growth rate in HK2 knockdown alone outstripped the oxygen and nutrition demand and resulted in necrosis. The histological findings in regards to Ki67/DAPI staining supports this postulate that treated tumors—with and without HK2 knockdown—had a significant
reduction in proliferation and cellularity compared to U87 control (no knockdown) (Figure 3.19).

Our data showed extensive vascular changes in treated tumors with significant reduction in MVD in the concurrent radiochemotherapy group with HK2 knockdown compared to radiochemotherapy alone (Figure 3.20 A and B p = 0.0259). However, loss of HK2 did not show an additive increase in vascular diameter in treated tumors (Figure 3.20B). This could suggest that the vessels had gained their maximum dilation upon concurrent treatments (RAD+TMZ) and were insensitive to further alteration by HK2. Overall, vascular adaptation is necessary to meet the metabolic demand of the tumors during growth or in response to microenvironmental stressors such as hypoxia, metabolic scarcity or cytotoxic therapeutics.

4.6 Conclusion

Nearly all cancer cells regardless of their genetic background rely on changes in metabolism and reorganization of metabolic pathways to support growth and survival, thus targeting metabolism holds significant potential as an effective therapeutic strategy in various cancer types. The major challenge of directly targeting metabolic pathways are obtaining high selectivity and to eliminate unwanted toxicity caused by off-target effects. Recently, there has been resurgence of interest in targeting tumor-specific metabolic enzymes such as HK2 for cancer therapy, since these enzymes are amenable to target with small molecular inhibitors. Collectively, this thesis explores how targeting tumor metabolism through HK2 inhibition is not only able to act as a facilitator of metabolic homeostasis but also as a therapeutic target in combination with radiochemotherapy in GBMs. Since GBM tumors show strong resistance to multimodal therapies, there is a growing clinical demand for incorporation of targeted therapies such as ones aiming at tumor metabolism. We anticipate that targeting key metabolic enzymes involved in modulating the Warburg effect would provide a unique paradigm for the management of brain tumors—which are highly metabolic– and will be instrumental in optimizing existing treatment modalities to inhibit tumor growth and/or invasion in GBMs.

Metabolic adaptation in GBMs can be influenced by various microenvironmental factors such as dynamic oxygenation, oxidative stress or acidity. The specific role of these microenvironmental factors and their interactions with inherent molecular/genetic variability in GBMs should be
taken into consideration during treatment, therapy stratification and therapeutic resistance. Recently, different GBM subtypes with distinct molecular signatures including differential HK2 expression have been recognized (Figure 1.6), which suggest that effective metabolism-based therapy would be promising for GBMs if patients were segregated based on tumor subtypes with high or low metabolic profile. Since GBM is one of the highly vascularized tumors, it is of interest to elucidate how changes in HK2 expression could regulate tumor vascularization, tissue perfusion or angiogenesis. In addition, disseminating the role of HK2 in regulating metabolic genes or important transcription factors under different metabolic conditions is crucial to understand glycolytically-converted tumor behavior. Our work has mostly focused on HK2 and its downstream effect – however one question that remains to be answered is what are the key upstream regulators of HK2 that may be amenable to target pharmaceutically? Altogether, the key to understand metabolic reprogramming and to take advantage of it therapeutically lies in the determination of not only key enzymatic targets but also genomic, proteomic, epigenetic and metabolomics profile of tumors in different stages of tumor growth or recurrence.

4.7 Future experimental directions

1) In vivo work presented in this thesis and discussed above supports a role for HK2 as a radio-chemo sensitizer in U87 xenograft models. It is necessary, however, to complement these in vivo data with a second xenograft line preferably GS2 cell lines. Since U87 xenograft models grow as well-circumscribed tumors and do not accurately recapitulate GBM’s infiltrative nature, it would be worthwhile to utilize glioma derived stem cell lines (GSCs) for in vivo studies since these cell lines have been shown to recapitulate GBM pathology and to retain invasive intracranial phenotype. A potential therapeutic milestone could be reached by understanding the metabolic signature of GSCs and the interplay between metabolic enzymes, microenvironmental stressors (hypoxia) and cytotoxic therapies.

2) Our next set of experiments will be geared towards using MR imaging in mice with HK2 knockdown tumors to closely monitor tumor growth, volume, vascular changes and oxygenation after interference with metabolic enzymes including HK2. MRI is a versatile non-invasive imaging modality that can be used to evaluate changes in tumor volume, metabolites such as lactate and glutamine and measure biomarkers of response to changes in the vasculature such as $K_{\text{trans}}$ and $K^\text{ep}$. These real-time studies would provide a better indication as to how and at what
stages HK2 knockdown affects tumor growth, oxygenation, metabolism and vascularization. These data may help answer unresolved questions of how and why metabolic deprivation through HK2 knockdown induces necrosis and hypoxia.

3) We are interested in elucidating the molecular mechanism by which conditional HK2 knockdown coordinates metabolic reprogramming. Microarray analysis of established GBM cell lines and GSCs with conditional HK2 knockdown would reveal gene signatures and networks associated with HK2. Microarray results can be used to determine changes in signaling pathways and cellular functions and can help us understand how loss of HK2 affects metabolism and modifies the cellular response to cytotoxic therapies. In addition, we are interested in combining this genomic analysis with metabolomics analysis–looking at metabolites such as glucose, glutamine, amino acids and fatty acids– in GBM tumors with variable HK2 levels to acquire a more global picture of genomic/metabolic adaptation under low HK2 levels.

4) Our knowledge of microRNA-mediated mechanisms that regulate GSCs is limited. Towards this, we are interested in investigating the global microRNA-regulated mechanisms that may help maintain and/or establish metabolic status of GSCs. These experiments will give us insights into the significance of microRNAs in modulating GSCs’ metabolic status, cancer-associated aerobic glycolysis or their sensitivity to metabolic inhibition.


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Appendix

Appendix 1: *In vivo* response to Dox administration. *In vivo* 2Photon Laser Microscopy imaging for U87 control and U87 HK2 knockdown mice with xenograft models generated in an intracranial window chamber. Representative images from Day 1, Day 3 and Day 7 post-Dox administration. Blue is CD31 staining for the vessels, green is bone marrow derived cells (BMDCs) and red is U87 HK2shRNA cells. By Day 3 post-Dox, red cells can be visualized in U87 HK2 knockdown group suggesting that the transgene is on (HK2shRNA), while the red signal is absence in the control group.