Hexokinase 2 is a Key Mediator of Aerobic Glycolysis Promoting Tumour Growth in Glioblastoma Multiforme

By

Amparo Myrelle Wolf

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy,
Graduate Department of the Institute of Medical Science,
University of Toronto

© Copyright by Amparo Myrelle Wolf 2010
Hexokinase 2 is a key mediator of aerobic glycolysis promoting tumour growth in Glioblastoma Multiforme

Amparo Myrelle Wolf

Doctor of Philosophy

Institute of Medical Science

University of Toronto

2010

Abstract

Proliferating tissues, including embryonic and tumour tissues, preferentially employ aerobic glycolysis to support cell growth. This reliance on glycolysis even in the presence of oxygen, referred to as the “Warburg Effect”, may confer a proliferative, survival and invasive advantage and be exploited therapeutically. In this thesis, we demonstrate that the glycolytic enzyme Hexokinase 2 (HK2) is crucial for the “Warburg Effect” in human Glioblastoma Multiforme (GBM), the most common and therapeutically resistant malignant brain tumour. In contrast to normal brain and low-grade gliomas, GBMs exhibited a marked increase in HK2 expression, but not HK1, particularly in perinecrotic, hypoxic regions and its expression predicted poor overall survival of GBM patients. Stable loss of HK2 in GBM cells restored oxidative phosphorylation (OXPHOS)-mediated glucose metabolism, with increased oxygen consumption and decreased lactic acid production, an effect not seen with loss of glycolytic enzymes HK1 or PKM2. Furthermore, HK2 depletion resulted in decreased proliferation in vitro and in vivo and increased sensitivity to apoptotic inducers such as
radiation and chemotherapy, both common adjuvant therapies of GBMs. Intracranial xenografts of GBM cells with reduced HK2 demonstrated significantly increased survival with decreased proliferation and angiogenesis yet enhanced invasiveness. In contrast, exogenous HK2 expression in GBM cells promoted proliferation, therapeutic resistance and intracranial growth. This was dependent partly on the PI3K/AKT dependent translocation of HK2 to the mitochondrial membrane. Stable loss of glycolytic enzymes HK2, HK1 and PKM2 reduced GBM proliferation but differentially altered the PI3K/AKT/mTOR and AMPK signaling pathways, the extent to which may influence whether a cell preferentially undergoes autophagy or apoptosis as the primary mode of cell death. Collectively, targeting enzymes employed by the tumour to modulate its energy metabolism, such as HK2 in GBMs, may favourably alter its therapeutic sensitivity to radiation and both classical and novel chemotherapeutic agents.
Acknowledgements

A profound thank you to my supervisor Dr. Ab Guha who epitomizes two core values that I admire tremendously: a spirit of collaboration and openness without ego or arrogance. Although these values are not embraced by many in science and academia, one wonders how much more progress in cancer research would be achieved if they were. Despite substantial personal adversity, Dr. Guha has shown an extreme dedication and love for both neurosurgery and research. I am most thankful to have had the opportunity to observe him do both. He has had a profound influence in shaping who I wish to become, a dedicated clinician scientist and an excellent teacher who sincerely cares for the welfare of others.

I would also like to thank both current and past members of the Guha lab including Dr. Joydeep Mukherjee, Dr. Johann Micallef, Aaron Gajadhar, Sameer Agnihotri and Diana Munoz. Particularly, I am most grateful to Sameer who taught me much of my current knowledge of molecular biology, both experimentally and theoretically, which is no mean feat since I entered my PhD with very little experience in the basic sciences. Many scientific discussions (and often some rather crazy ideas) have been shared between us. As well, thank you to Diana for her help with isolating mouse embryos and for being a supportive friend during these last few years.

Thank you also to Dr. Cynthia Hawkins for her supportive nature and guidance throughout my PhD. I would also like to thank my other committee member, Dr. Andras Kapus, for his guidance and especially his enthusiasm for science.

Finally, a thank you to my parents, Nikolaus Wolf and Chantal Kieffer, and my sister Stephanie, for their patience, particularly during my many grumpy moods. They keep reminding me that the grass may appear greener on the other side, but most likely it is not. Hah!
Table of Contents

Abstract ................................................................................................................................................. ii

Acknowledgements ................................................................................................................................... iv

Table of Contents ...................................................................................................................................... v

List of Tables ........................................................................................................................................... xii

List of Figures .......................................................................................................................................... xiii

List of Appendices .................................................................................................................................... xvi

List of Abbreviations ............................................................................................................................... xvii

CHAPTER 1: Introduction to the molecular biology of astrocytomas and glycolytic metabolism .......................................................................................................................... 1

1.1 Central Hypothesis ............................................................................................................................... 1

1.2 Clinical and Pathological Overview of GBMs ................................................................................... 2

1.3 Overview of the Molecular Biology of GBMs .................................................................................. 6

1.4 The Cell of Origin of GBMs .............................................................................................................. 8

1.5 Current Targeted Therapies in GBMs ............................................................................................... 9

1.6 Glycolytic Metabolism and Oxidative Phosphorylation in the Normal Central Nervous System ...................................................................................................................... 10

1.6.1 Glycolysis .................................................................................................................................... 12

1.6.2 Hexokinase ................................................................................................................................... 16

1.6.3 Mitochondrial structure, oxidative phosphorylation and intrinsic apoptosis 20
1.7 Metabolic Remodeling and the Warburg effect in Cancer ................................. 30
  1.7.1 Introduction: The Warburg Effect ................................................................. 30
  1.7.2 Putative advantages of the Warburg effect .................................................. 31
  1.7.3 The molecular basis of the Warburg effect .................................................... 38
1.8 The Role of Hexokinase 2 in Mitochondrial Function and Growth ............... 50
  1.8.1 The HK2 gene .................................................................................................. 50
  1.8.2 Transcriptional regulation of HK2 ................................................................. 51
  1.8.3 HK2 in bioenergetics and macromolecular synthesis for proliferation ...... 52
  1.8.4 HK2 and cell survival ....................................................................................... 53
  1.8.5 HK2 and sensitization to radiation and chemotherapy .............................. 57
  1.8.6 HK2/metabolism and tumour initiation ......................................................... 58
  1.8.7 HK2 as a cancer prognostic marker ............................................................... 58
1.9 The Warburg Effect in Glioblastoma Multiforme ........................................ 59
  1.9.1 Heterogeneity in glycolytic metabolism and mitochondrial function in GBMs .............................................................................................................. 59
  1.9.2 Metabolism and oncogenic signaling pathways in GBMs ............................ 61
  1.9.3 IDH1 and IDH2 mutations in GBMs ............................................................... 63
  1.9.4 The role of HKs in GBMs ............................................................................... 64
1.10 Therapeutic Targeting of Metabolism in GBMs and Other Cancers .......... 68
1.11 Thesis Objectives .............................................................................................. 71
CHAPTER 2: Developmental profile and regulation of the glycolytic enzyme hexokinase 2 in normal brain and glioblastoma multiforme ........................................ 72

2.1 Abstract ............................................................................................................................................................................. 72
2.2 Introduction ........................................................................................................................................................................ 73
2.3 Materials and Methods .................................................................................................................................................. 76
  2.3.1 Mouse embryonic and brain samples .................................................. 76
  2.3.2 Quantitative Real-time PCR ................................................................. 76
  2.3.3 GBM cell lines and Lactate assay ........................................................ 77
  2.3.4 Western blot analysis .......................................................................... 77
  2.3.5 O₂ consumption measurements .......................................................... 78
  2.3.6 Bisulfite treatment and sequencing ...................................................... 78
  2.3.7 Statistical Analyses .............................................................................. 79
2.4 Results .............................................................................................................................................................................. 79
  2.4.1 Bioinformatic analysis of Gene Expression Omnibus ....................... 79
  2.4.2 Developmental profile of HK2 and HK1 in the mouse brain .............. 82
  2.4.3 Expression of HK2 correlates with aerobic glycolysis in GBM cell lines .. 85
  2.4.4 Methylation of HK2 promoter/intron 1 in normal adult brain and GBM cell lines that do not express HK2 ............................................................ 85
2.5 Discussion ........................................................................................................................................................................... 90

CHAPTER 3: Hexokinase 2 is a key mediator of aerobic glycolysis promoting tumor growth in glioblastoma multiforme ................................................................. 93

vii
3.1 Abstract ................................................................................................................... 93
3.2 Introduction ............................................................................................................. 93
3.3 Materials and Methods ............................................................................................ 96
  3.3.1 Human GBM samples .......................................................................................... 96
  3.3.2 Quantitative real-time PCR (qRT-PCR) .............................................................. 96
  3.3.3 Immunohistochemistry ....................................................................................... 97
  3.3.4 Cell line and siRNA treatment ........................................................................... 99
  3.3.5 Cell viability assay and BrdU incorporation assay .............................................. 99
  3.3.6 Determination of apoptosis ............................................................................... 99
  3.3.7 Generation of stable cell lines .......................................................................... 100
  3.3.8 Mitochondrial membrane permeability and immunofluorescence ............... 100
  3.3.9 Western blot analysis ....................................................................................... 101
  3.3.10 Transmission Electron Microscopy .................................................................. 101
  3.3.11 Mitochondrial fractionation and cytochrome c ELISA .................................. 101
  3.3.12 Lactate assay and oxygen consumption ......................................................... 102
  3.3.13 Hexokinase assay ........................................................................................... 102
  3.3.14 Subcutaneous GBM xenograft model .............................................................. 102
  3.3.15 Intracranial GBM model ................................................................................ 102
  3.3.16 Statistical Analyses ....................................................................................... 103
3.4 Results .................................................................................................................... 103
3.4.1 Human GBMs express HK2, correlating with poor overall survival........ 103

3.4.2 HK2 is strongly expressed in the perinecrotic region of human GBMs.... 104

3.4.3 Knockdown of HK2 decreases tumour proliferation as well as cell survival, particularly under hypoxic conditions ........................................... 108

3.4.4 Knockdown of HK2 sensitizes GBM cells to apoptosis by increasing mitochondrial membrane permeability and activating the intrinsic apoptotic pathway ........................................................................................................ 112

3.4.5 Stable loss of HK2 normalizes glycolytic metabolism ....................... 117

3.4.6 Differential role of HK2, HK1 and PKM2 on GBM metabolism and proliferation .................................................................................................................. 122

3.4.7 Mechanisms of over-expression of HK2 in GBMs............................... 125

3.4.8 Translocation of HK2, and not HK1, to the OMM after growth factor stimulation promotes GBM proliferation ............................................... 127

3.4.9 Subcutaneous growth of GBM cells is impaired when HK2 deficient..... 136

3.4.10 HK2 regulates survival and invasion in orthotopic xenograft models of GBM ................................................................................................................. 138

3.5 Discussion ..................................................................................................... 142

CHAPTER 4: Impact of glycolytic enzymes hk2, hk1 and pkm2 on proliferation and cell death in glioblastoma multiforme .......................................................... 147

4.1 Abstract ........................................................................................................ 147

4.2 Introduction .................................................................................................. 147

4.3 Materials and Methods ............................................................................... 149
4.3.1 GBM cell lines and BrdU incorporation ......................................................... 149
4.3.2 Cell cycle Propidium Iodide (PI) analysis ...................................................... 150
4.3.3 Western blot analysis ...................................................................................... 150
4.3.4 Immunofluorescence for LC3-GFP ................................................................. 150
4.3.5 Intracranial GBM model and Immunohistochemistry ...................................... 150

4.4 Results ............................................................................................................... 151

4.4.1 Transient and stable loss of glycolytic enzymes HK1, HK2 and PKM2 decreases cell proliferation under normal growth conditions ......................... 151
4.4.2 Impact on PI3K/AKT/mTOR and AMPK signaling with stable loss of HK2 and HK1 ................................................................................................. 155
4.4.3 Enhanced autophagosome accumulation with loss of HK1 and PKM2 but not HK2 ......................................................................................................... 157
4.4.4 Differential sensitivity to apoptotic inducing chemotherapeutic agents with loss of HK1, HK2 and PKM2 ................................................................. 159
4.4.5 Impact of loss of HK2, HK1 and PKM2 on in vivo intracranial growth .... 161

4.5 Discussion ........................................................................................................... 164

CHAPTER 5: Discussion and future directions ...................................................... 168

5.1 Summary and Discussion of thesis findings ..................................................... 168

5.1.1 Expression of HK2 in embryonic tissue, normal brain, low-grade astrocytomas and GBMs ......................................................................................... 168
5.1.2 Regulation of HK2 in GBMs ............................................................................ 169
5.1.3 The role of HK2 in proliferation and apoptosis in GBMs ......................... 169
5.1.4 The role of HK2 in promoting aerobic glycolysis ........................................ 169
5.1.5 Relationship between aerobic glycolysis and angiogenesis in GBMs......... 170
5.1.6 Impact of glycolytic proteins on growth factor signaling and metabolic sensing pathways .................................................................................................................. 170
5.2 Future Directions .................................................................................................................. 171
5.2.1 Impact of inhibition of HK2 in vivo tumour regression............................... 171
5.2.2 Impact of inhibition of HK2 in combination with classical and novel chemotherapies in in vivo animal models ................................................................. 171
5.2.3 Mechanisms of HK2 at the OMM ................................................................. 171
5.2.4 Mechanisms through which HK2 promotes proliferation and resistance to radiation and temozolomide................................................................. 173
5.2.5 Mechanisms underlying the association of aerobic glycolysis and angiogenesis .................................................................................................................. 174
5.2.6 Altered metabolism as a network: a systems biology approach .............. 175
5.2.7 Expression profile and functional relevance of HK2 in GBM brain tumour initiation cells (BTIC) ................................................................................................. 175
5.2.8 Epigenetic mechanisms regulating HK2 expression in GBMs............... 176
5.2.9 microRNA regulation of HK2 ................................................................. 176
5.2.10 Retrograde mitochondrial signaling in GBMs ........................................ 177
5.3 Conclusion .......................................................................................................................... 177
References ............................................................................................................................ 179
Appendices ............................................................................................................................ 207
List of Tables

Table 1-1: Kinetic and regulatory parameters of mammalian hexokinase isozymes. ...... 18

Table 1-2: HIF1α targets of glycolysis and oxidative phosphorylation. ....................... 44

Table 1-3: Summary of the role of HK2 at the OMM and interaction with the MPTP.... 55

Table 1-4: Potential metabolic targets for the treatment of cancer................................. 70

Table 2-1: Summary of normal brain adult and fetal specimens................................. 79

Table 3-1: Primary antibodies used for immunohistochemical staining ...................... 98
## List of Figures

| Figure 1-1 | Primary CNS tumors and Gliomas | 4 |
| Figure 1-2 | Molecular Pathogenesis of Primary and Secondary GBMs | 7 |
| Figure 1-3 | The Glycolytic Pathway | 14 |
| Figure 1-4 | Oxidative Phosphorylation: Krebs Cycle and the Electron Transport Chain | 15 |
| Figure 1-5 | Mitochondrial Permeability Transition Pore | 26 |
| Figure 1-6 | Metabolic Pathways Active in Proliferating Cells | 37 |
| Figure 1-7 | Decrease in interstitial pH and partial pressure of oxygen with distance from vessel walls | 39 |
| Figure 1-8 | Mechanisms of hypoxia-inducible factor 1α (HIF1α) stabilization | 43 |
| Figure 1-9 | Mining of Integrated Genome Analysis and TCGA arrays for expression of HK2 and HK1 in GBMs | 67 |
| Figure 2-1 | Gene expression of HK2 and HK1 in 8-cell and blastocyst stage of mouse preimplantation embryonic development | 81 |
| Figure 2-2 | Developmental profile of HK2, HK1, and LDHA in the mouse embryo, including CNS | 84 |
| Figure 2-3 | Expression of HK2 in GBM cell lines correlates with greater extracellular lactate and reduced oxygen consumption | 88 |
| Figure 2-4 | Epigenetic regulation of HK2 in CpG islands of intron 1 | 89 |
| Figure 3-1 | HK2 is expressed in human GBMs and is associated with decreased overall survival | 106 |
Figure 3-2: No difference in apoptotic index between “centre” and “periphery” of GBMs. ................................................................. 107

Figure 3-3: Inhibition of HK2 decreases cell viability particularly under hypoxia........ 110

Figure 3-4: Inhibition of HK2 decreases cell proliferation........................................ 111

Figure 3-5: Inhibition of HK2 sensitizes GBM cells to drug-, radiation- and hypoxia- induced apoptosis............................................................... 114

Figure 3-6: Inhibition of HK2 sensitizes GBM cells to temozolomide....................... 115

Figure 3-7: Stable loss of HK2 alters mitochondrial function, including a return to oxidative phosphorylation and increased expression of genes involved in mitochondrial biogenesis.................................................. 119

Figure 3-8: Expression of LDHA and OXPHOS proteins in Human GBM samples..... 121

Figure 3-9: Differential role of HK2, HK1 and PKM2 in GBM mitochondrial metabolism and survival................................................................. 124

Figure 3-10: Induction of HK2 with expression of Ras oncogene or loss of tumour suppressor p53. ........................................................................ 126

Figure 3-11: Increased HK2 protein stability when EGF stimulated....................... 128

Figure 3-12: GF/PI3K/AKT signaling is important for localization of HK2 but not HK1 to outer mitochondrial membrane in U343 cells............................ 131

Figure 3-13: GF/PI3K/AKT signaling is important for localization of HK2 but not HK1 to outer mitochondrial membrane in U87 cells effects.......................... 132

Figure 3-14: Both glucose phosphorylation and mitochondrial localization contribute to HK2’s growth promoting effects in GBMs over and above HK1. .............. 135

Figure 3-15: Stable loss of HK2 decreases in vivo GBM tumour growth in a subcutaneous xenograft model...................................................... 137
Figure 3-16: HK2 regulates GBM survival and invasion in an orthotopic xenograft model.

Figure 4-1: Decreased cell proliferation in U87 cells with transient loss of HK2, HK1 and PKM2 glycolytic enzymes.

Figure 4-2: Cell cycle analysis of U87 cells with stable loss of HK2, HK1 and PKM2.

Figure 4-3: Differential effects on PI3K/AKT/mTOR and AMPK signaling with depletion of HK2 and HK1.

Figure 4-4: Increased sensitivity to autophagosome accumulation with loss of HK1 and PKM2 but not HK2.

Figure 4-5: Sensitivity to apoptosis in GBM cells with stable loss of HK2 but not HK1 or PKM2.

Figure 4-6: Increased survival and reduced proliferation with depletion of HK2, HK1 and PKM2 in GBM orthotopic xenograft model.
List of Appendices

Appendix 1: Image-guided sampling of representative GBM “centre” and “periphery”. .......................................................... 208

Appendix 2: REMBRANDT database: Kaplan-Meier survival plot for Glioma samples with differential HK2 gene expression. .......................................................... 210

Appendix 3: Immunoprecipitation of HK2 and LC-MS mass spectrometry in U87 GBM cells EGF stimulated. .......................................................... 212
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Anaplastic Astrocytoma</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-coA Carboxylase</td>
</tr>
<tr>
<td>ACL</td>
<td>ATP Citrate Lyase</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptotic Inducing Factor</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myelogenous Leukemia</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine Nucleotide Translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine Triphosphate</td>
</tr>
<tr>
<td>BTIC</td>
<td>Brain Tumour Initiating Cells</td>
</tr>
<tr>
<td>BTPC</td>
<td>Brain Tumour Propagating cells</td>
</tr>
<tr>
<td>BTSC</td>
<td>Brain Tumour Stem Cells</td>
</tr>
<tr>
<td>CBTRUS</td>
<td>Central Brain Tumour Registry of the United States</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine Palmitoyltransfase 1</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone Phosphate</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
</tbody>
</table>
FAS  Fatty Acid Synthase
FH   Fumarate Hydratase
FBP  Fructose 1,6-Bisphosphate
FBP2 Fructose 2,6-Bisphosphatase
FDG  Fluorodeoxyglucose
FTS  Farnesylthiosalicylic acid
GBM  Glioblastoma Multiforme
GDH  Glutamate Dehydrogenase
G6P  Glucose-6-Phosphate
G6PD Glucose-6-phosphate dehydrogenase
HIF1α Hypoxia inducible factor 1 α
HK1  Hexokinase 1
HK2  Hexokinase 2
HPRT1 Hypoxanthine Phosphoribosyltransferase 1
IDH1 Isocitrate Dehydrogenase 1
IGF  Insulin Growth Factor
IMM  Inner Mitochondrial Membrane
IMS  Intermitochondrial Space
LC3  Light chain 3
LDHA Lactate Dehydrogenase A
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGA</td>
<td>Low Grade Astrocytoma</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MGMT</td>
<td>O(^6)-methylguanine methyltransferase</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane permeability</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial Outer Membrane Permeabilization</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial Permeability Transition Pore</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic Resonance</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian Target of Rapamycin Complex 1</td>
</tr>
<tr>
<td>mtTFA</td>
<td>Mitochondrial Transcription Factor A</td>
</tr>
<tr>
<td>MXI1</td>
<td>MAX Interaction 1</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis 1</td>
</tr>
<tr>
<td>NRF1</td>
<td>Nuclear Respiratory Factor 1</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear Respiratory Factor 2</td>
</tr>
<tr>
<td>ODDDD</td>
<td>Oxygen-Dependent Degradation Domain</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer Mitochondrial Membrane</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
</tr>
<tr>
<td>PDK</td>
<td>Pyruvate Dehydrogenase Kinase</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenol Pyruvate</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFK1</td>
<td>Phosphofructo-1-kinase</td>
</tr>
<tr>
<td>PFK2</td>
<td>Phosphofructo-2-kinase</td>
</tr>
<tr>
<td>PGM</td>
<td>Phosphoglycerate Mutase</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator activated receptor γ coactivator 1α</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl Hydroxylase</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKM</td>
<td>Pyruvate Kinase Muscle</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose Phosphate Pathway</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Raptor</td>
<td>Regulatory Associated Protein of mTOR</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>R5P</td>
<td>Ribose-5-phosphate</td>
</tr>
</tbody>
</table>
SAGE  Serial Analysis of Gene Expression
SCO2  Synthesis of Cytochrome c Oxidase 2
SDH   Succinate Dehydrogenase
SOD   Superoxide Dismutase
TCA   Tricarboxylic acid
TCGA  The Cancer Genome Atlas
TK(I) Tyrosine Kinase (inhibitor)
TMZ   Temozolomide
TSC2  Tuberous Sclerosis Complex 2
UCP   Uncoupling Protein
VEGF  Vascular Endothelial Growth Factor
vHL   von Hippel-Lindau
WHO   World Health Organization
2-DG  2-Deoxyglucose
2-HG  2-Hydroxyglutarate
αKG  alpha Ketoglutarate
CHAPTER 1: INTRODUCTION TO THE MOLECULAR BIOLOGY OF ASTROCYTOMAS AND GLYCOLYTIC METABOLISM

1.1 Central Hypothesis

Glioblastoma Multiforme (GBM) is the most prevalent and malignant of adult human primary Central Nervous System (CNS) tumours. GBMs are lethal tumours due to persistent local recurrence and invasion into the surrounding brain. “Multiforme” signifies regional intra-tumoural pathological and molecular heterogeneity resulting from interactions of the tumour and stromal cells in response to tumour micro-environmental factors, notably hypoxia. This heterogeneity likely modulates many of the key biological growth properties of GBMs including altered metabolism, enhanced proliferation, cell survival, invasion and angiogenesis. GBMs are extremely resistant to apoptosis, in response to both endogenous inducers including hypoxia and nutrient deprivation, and exogenous inducers including radiation and chemotherapy. Combined, their resistance to apoptosis and invasive nature make GBMs very difficult to treat effectively.

In order to design effective targeted therapies against GBMs, it is essential to have a comprehensive understanding of the biology and signaling mechanisms that contribute to its resistance to apoptosis and invasion. Tumour cells commonly undergo a shift in glucose metabolism from oxidative phosphorylation (OXPHOS) to glycolysis and lactate formation even in the presence of oxygen, a phenomenon referred to as the “Warburg effect”. GBMs have elevated rates of glycolysis, although with regional heterogeneity, reflected on Magnetic Resonance (MR) spectroscopy and Fluorodeoxyglucose (18F) Positron Emision Tomography (FDG-PET) imaging. This glycolytic switch may confer a proliferative, survival and invasive advantage to tumour cells. The molecular basis of the Warburg effect remains elusive and may be cancer-dependent; primary mutations in metabolic enzymes, oncogenic signaling, aberrant expression of metabolic enzymes and the tumour microenvironment may all contribute to this phenotype.

This thesis is aimed at profiling the expression of the glycolytic enzyme Hexokinase 2 (HK2) in GBMs and its ensuing consequences on aerobic glycolysis, mitochondrial...
function, proliferation, and cell survival. For several reasons, HK2 may be a key player in the Warburg effect particularly in GBMs: 1) as an irreversible and rate limiting enzyme converting glucose to glucose-6-phosphate, HK2 plays an important role in controlling glycolytic flux; 2) HK2 expression is low in normal brain which predominantly expresses the normal brain isoform Hexokinase 1 (HK1); 3) HK2 is a highly regulated HK isozyme and its expression and cellular localization can be altered by many microenvironmental-associated factors including hypoxia, glucose and growth factor signaling; 4) Lastly, HK2 closely links pathways of cell survival and metabolism. In certain cell systems, HK2 has been shown to translocate to the outer mitochondrial membrane, interact with the permeability transition pore, and potentially regulate intrinsic apoptotic pathways. Taking all these reasons into account, the central hypothesis of this thesis is that HK2 is a key mediator of the Warburg effect in GBMs and affords a proliferative and cell survival advantage to tumour cells, particularly in stringent microenvironments including hypoxia. Accordingly, normalization of tumour metabolism by inhibiting HK2, in conjunction with current and emerging therapies, may well provide a novel and effective therapeutic strategy for GBM patients.

1.2 Clinical and Pathological Overview of GBMs

The incidence of primary brain and CNS tumours in the United States is estimated at 14.8 per 100,000 individuals per year (CBTRUS, 2005). Primary brain tumours are one of the top ten causes of cancer related deaths accounting for approximately 2.5% of cancer-related deaths in Canada (Canadian Cancer Society, Statistics Canada 2009). The most common primary brain tumour is glioma accounting for 33% of primary CNS tumours (Figure 1-1). Gliomas are classified according to their suspected cell of origin and extent of brain infiltration (circumscribed or diffusely infiltrating) and include astrocytomas, oligodendrogliomas, and ependymomas. The most common type of glioma is astrocytoma which accounts for about 77% of these tumours and includes GBMs (Figure 1-1).
Astrocytomas are heterogeneous in nature and may show diffuse infiltration of adjacent and distant brain structures (Kleihues et al., 2000). These neoplasms are histologically graded based on the criteria set by the World Health Organization (WHO) as grade I to grade IV. Pilocytic astrocytomas, which are benign and relatively circumscribed, are classified as Grade I tumours. Low grade diffuse astrocytomas (LGA, Grade II) are histologically characterized by moderate cellularity, mild nuclear atypia, and rare or absent mitotic figures and are moderately proliferative and invasive. Grade III astrocytomas, or anaplastic astrocytomas (AA), are characterized histologically by increased cellularity, nuclear atypia and mitotic activity. These tumours are more proliferative and infiltrative compared to grade II gliomas. GBMs, classified as grade IV gliomas, are histologically similar to AA but in addition are characterized by the presence of necrosis and glomeruloid microvascular proliferation. GBMs are significantly more proliferative, invasive and angiogenic compared to grade II and III astrocytomas (Kleihues et al., 2000). Sheet-like, hypercellular zones referred to as pseudopalisading cells are often found surrounding central necrotic regions. The presence of pseudopalisades and microvascular hyperplasia heralds the onset of aggressive growth, both of which are believed to be instrumental in malignant progression.

Figure 1-1: Primary CNS Tumours and Gliomas

Figure 1.1

(a) Primary CNS Tumours

- Glioblastoma: 17.6%
- Astrocytomas: 7.4%
- Ependymomas: 1.9%
- Oligodendro-gliomas: 3.0%
- Embryonal, including medulloblastoma: 1.5%
- Meningioma: 33.4%
- Pituitary: 12.2%
- Nerve sheath: 8.7%
- Lymphoma: 2.5%
- All other: 12.4%
- Craniopharyngioma: 0.7%

(b) Gliomas

- Glioblastoma: 54.0%
- All other astrocytomas: 9.0%
- Anaplastic astrocytoma: 6.8%
- Diffuse astrocytoma: 1.6%
- Pilocytic astrocytoma: 5.2%
- Oligodendrogliomas: 8.4%
- Ependymomas: 5.8%
- All other gliomas: 11.1%
GBMs are the most malignant and common astrocytoma accounting for approximately 54% of all gliomas (CBTRUS, 2005). GBMs are more common among men than women (male: female ratio 1.6:1) with the incidence rate increasing with age and declining again in the oldest population (CBTRUS, 2005). Although the prognosis remains poor, recent changes to treatment strategies have improved the median survival of patients with GBMs. A clinical study carried out by Stupp and colleagues found that concurrent treatment with radiation and the alkylating agent temozolomide (TMZ) followed by adjuvant TMZ increased median overall survival from 12.1 to 14.6 months compared to those patients receiving radiation alone (Stupp et al., 2005). The two-year survival rate significantly increased from 10.4% in those receiving radiation alone to 26.5% in those receiving concurrent treatment. Furthermore, a subgroup of patients who responded better to concurrent TMZ treatment was identified; patients who had silencing of the DNA repair enzyme MGMT (O\textsubscript{6}-methylguanine methyltransferase) through promoter methylation and underwent concurrent treatment as described above had a median overall survival of 21.7 months compared to 12.7 months in those patients receiving a similar treatment but with functional MGMT (Hegi et al., 2005). The two-year survival rate in patients with methylated MGMT and receiving concurrent treatment was 46% compared to 13.8% for patients receiving concurrent treatment but lacking MGMT methylation. Despite these encouraging results, GBMs still remain incurable.

The dismal prognosis of patients diagnosed with GBMs is attributed to the highly infiltrative nature of these tumours and their resistance to apoptosis. The lack of a well-circumscribed tumour precludes its complete surgical removal and recurrence, in a large majority of cases, occurs within the resection margin (Berens and Giese, 1999). Conventional radio- and chemotherapy are largely ineffective at preventing recurrence. The introduction of local treatments within the resection cavity (e.g. with implantation of Gliadel\textsuperscript{®} biodegradable biopolymers containing the alkylating agent β-chloronitrosourea) have been shown to be of limited efficacy as tumours recur at distant sites beyond the diffusion area of the drug (Berens and Giese, 1999). These conventional approaches fail therapeutically due to targeting of only proliferating cells. Identifying the signaling pathways important for resistance to apoptosis and tumour invasion will help in
the design of novel therapeutics that can be used in combination with current treatment protocols in the hope of further improving patient survival.

1.3 Overview of the Molecular Biology of GBMs

GBMs have historically been classified as primary or secondary GBMs (Figure 1-2) (Kleihues et al., 2000). Primary GBMs arise *de novo* and are more common in older patients, whereas secondary GBMs progress from a lower grade astrocytoma and usually present in a younger patient population, generally below 45 years of age. Primary and secondary GBMs share several genetic aberrations including loss of heterozygosity (LOH) of chromosome 10 and *phosphatase and tensin homolog* (*PTEN*) as well as *retinoblastoma* (*Rb*) *protein* alterations. More unique to primary GBMs is the amplification and over-expression of the epidermal growth factor receptor (*EGFR*) and *EGFR* mutants (e.g. *EGFRvIII*) as well as deletion of *CDKN2A* encoding both *p16INK4A* and *p19ARF*. Secondary GBMs, on the other hand, are most commonly characterized by over-expression of platelet derived growth factor receptor (*PDGFR*)-α and *PDGFA* as well as mutations in *p53* and LOH of 10q. Despite differences in their molecular profile, primary and secondary GBM are pathologically indistinguishable.

**Figure 1-2: Molecular Pathogenesis of Primary and Secondary GBMs.**

GBMs may arise via two pathways. Primary GBMs arise *de novo* whereas secondary GBMs progress from a lower grade astrocytoma. Primary and secondary GBMs contain similar molecular aberrations, although certain aberrations are more prevalent in one over the other. TP53, tumour protein 53; *EGFR*, epidermal growth factor receptor; *PTEN*, phosphatase and tensin homolog; LOH, loss of heterozygosity; INK4A, inhibitor kinase 4A; MDM2, mouse double minute 2; RB, retinoblastoma; *PDGFR*, platelet Derived Growth Factor Receptor. *Figure modified from World Health Organization Classification of Tumours: Pathology and Genetics: Tumours of the Nervous System* (Kleihues et al., 2000).
Figure 1.2

Astrocyte or Precursor cell

- p53 mutation (65%)
- PDGFA over-expression
- Ras & PI3K over-activation
- IDH1 mutation (75-80%)

EGFR
- amplification (50%)
- mutations (25-30%)

PTEN
mutations or no expression (80%)

Ras & PI3K overactivation (~100%)

- p53 mutation (33%)
- mdm2 overexpression (50%)
- p16 deletion (30-40%)
- LOH 10p & 10q
- pRb alterations

Grade II: Low Grade Astrocytomas

- LOH 19q (50%)
- pRb alteration (25%)

Grade III: Anaplastic Astrocytoma

- p16Ink4 deletion (19%)
- PDGFR amplification (<10%)
- PTEN mutation (5%)
- LOH 10q

Grade IV: Secondary Glioblastoma

Grade IV: Primary Glioblastoma
The Cancer Genome Atlas (TCGA) has characterized large cohorts of human GBM tumours, with associated clinical data, by performing gene expression, chromosomal copy number, LOH, methylation, and microRNA expression analyses as well as high throughput DNA sequencing and mutation analyses. Early reports from the TCGA strengthen our existing knowledge of the importance of deregulation of Rb, p53 and receptor tyrosine kinase (RTK) signaling, Phosphoinositide 3-kinase (PI3K) and Ras pathways. However, the large-scale analysis also characterized the importance of NF1 deletions and activation mutations of PIK3CA or PI3KRI among others in GBM (Cancer Genome Atlas Research, 2008). A second smaller scale study performed integrated genomic analysis of human GBMs and revealed a variety of signaling pathways and genes not previously implicated in GBM biology. One particularly interesting mutation in Isocitrate Dehydrogenase 1 (IDH1) was reported in about 12% of GBMs, particularly in secondary GBMs, which may potentially act as a more specific marker of secondary GBMs (Parsons et al., 2008).

1.4 The Cell of Origin of GBMs

Cancer arises from a series of mutations that can occur in a few initiating cells, eventually acquiring unlimited and uncontrolled proliferative ability. Two hypothetical models can explain this phenomenon: 1) the stochastic model predicts that tumours are heterogeneous but that all the cells in a tumour have a similar tumourigenic potential, although at low frequency, 2) the hierarchical model implies that only a small subpopulation of tumour stem cells can proliferate and sustain tumour growth and progression. Although originally believed to arise from mutations in differentiated astrocytes, the cell of origin of GBMs is now controversial. A recent hypothesis is that mutations or alterations in the mechanisms involved in adult neurogenesis in the subventricular zone may contribute to brain tumourgenesis. Brain tumour stem cells (BTSC, or brain tumour propagating cells, or brain tumour initiating cells) have been identified and isolated from different types of brain tumours including GBM and medulloblastomas, supporting the hierarchical model of tumour formation (Galli et al., 2004; Singh et al., 2003). BTSCs are believed to mimic normal stem cells including their ability to generate new tumours reproducing the original GBM phenotype and self-renewal capacity. However, the de-differentiation hypothesis
suggests that differentiated cells can be reprogrammed to an earlier progenitor form upon expression of specific proteins and that this may be the cell of origin and not mutations in a normal stem cell. A major limitation in BTSC research is the lack of specific antigenic and molecular markers. Theoretically, the development of agents that could selectively target and inhibit BTSCs and their propagation potential may well reduce or eliminate tumour growth and recurrence.

1.5 Current Targeted Therapies in GBMs

Currently the standard of care of patients with GBMs is surgical resection of the tumour followed by radiation and temozolomide, originally referred to as the Stupp regimen (Stupp et al., 2005). However, novel targeted therapies are currently undergoing clinical trials to determine if they may be more effective in prolonging survival of GBM patients than the standard of care. Because aberrations in EGFR play an important role in the pathogenesis of a variety of neoplasm, including GBMs, there is considerable interest in developing targeted therapies against this receptor. Several small molecule inhibitors have been designed against the tyrosine kinase domain of the EGFR/EGFRvIII kinase domain. Two Tyrosine Kinase Inhibitors (TKIs) have been used in pre-clinical and clinical trials in glioma patients including erlotinib (OSI-774, Tarceva) and gefitinib (ZD1839, Iressa). In vitro results in cell lines expressing EGFRvIII showed that high levels of gefitinib were needed to inhibit EGFRvIII signaling. On the other hand, low levels of gefitinib conferred a growth advantage to EGFRvIII expressing cells (Lassman et al., 2006). Erlotinib only showed modest effects in a lung cancer mouse model harbouring EGFRvIII mutation. Despite these preclinical findings, these agents have been used in several clinical trials, either alone or in combination with radiotherapy or chemotherapy. The results of these clinical studies have been disappointing. Overall, these agents are ineffective at inhibiting phosphorylation of EGFR and downstream targets such as AKT/Protein Kinase B (PKB) and Extracellular signal-regulated kinase (ERK) (Lassman et al., 2006).

In an attempt to understand the molecular basis of response to TKIs, 47 patients with recurrent malignant gliomas treated with either erlotinib or gefitinib were analyzed
Positive clinical response was associated with co-expression of PTEN and EGFRvIII. Patients that lacked PTEN expression did not respond well to treatment. Considering 70-80% of GBM patients lack PTEN, this raises serious doubts of the effectiveness of these therapies. These results were confirmed in vitro, where they showed that the co-expression of PTEN and EGFRvIII sensitized U87 GBM cells to erlotinib. Similarly, using GBM xenografts derived from human GBM explants with differing PTEN status and EGFR aberrations, another group showed that mutation in the extracellular domain of wtEGFR in the presence of PTEN was necessary to confer sensitivity to erlotinib (Sarkaria et al., 2006). However, two xenografts both expressing EGFRvIII and PTEN responded differently to TKI treatment, where one xenograft was sensitive and the other was not. Their results suggest that EGFR amplification and mutation combined with PTEN expression is necessary but not sufficient in conferring sensitivity to erlotinib (Sarkaria et al., 2006). The dismal results of the clinical studies to date provide a strong rationale for the necessity of combinatorial therapeutics. One study combined rapamycin treatment, an inhibitor of mammalian target of rapamycin (mTOR) downstream of PI3K/AKT, with erlotinib (Wang et al., 2006). Using U87EGFRvIII and U87 mouse xenograft models which are deficient in PTEN, they showed that the combination of rapamycin and erlotinib resulted in decreased tumour growth with an additive effect of inhibiting downstream PI3K signaling pathways. The combination of TKIs (targeting EGFR, ERBB3, MET, PDGFRα) was reported to decrease glioma cell survival and anchorage-independent growth compared to the use of any single inhibitor, in a PTEN-independent fashion (Stommel et al., 2007). These results emphasize the co-activation of multiple RTKs in gliomas and thus the limited efficacy of inhibiting just one RTK.

1.6 Glycolytic Metabolism and Oxidative Phosphorylation in the Normal Central Nervous System

The normal CNS has a very high metabolic rate, receiving 15% of cardiac output, and consuming approximately 20% of oxygen and 25% of total body glucose utilization,
despite accounting for only 2% of whole body weight. No substrate can adequately replace glucose for brain energy metabolism. Lactate and pyruvate can sustain synaptic activity; however, they cannot cross the blood-brain barrier and thus cannot substitute plasma glucose for brain function. Only under conditions of starvation or diabetes are ketone bodies (D-3-hydroxybutyrate, acetoacetate) utilized by the brain as alternative energy sources.

Changes in regional brain energy metabolism can be studied in humans using techniques including FDG-PET and MRS. At the cellular level, neurons require high levels of ATP to maintain normal function including ionic gradients across cell membranes and for neurotransmission (for review, see (Kann and Kovacs, 2007)). However, other cell types including glial cells and endothelial cells also consume energy in addition to playing an active role in the flux of energy substrates to neurons. Astrocytes have specific functions with respect to metabolism and homeostasis including 1) maintaining extracellular K+ homeostasis via energy-dependent Na+,K+ATPase, 2) neurotransmitter (glutamate) uptake from extracellular space/synaptic cleft, 3) glycogen storage. In vitro studies suggest metabolic compartmentalization and trafficking between astrocytes and neurons. It has been suggested that astrocytes take up glucose and metabolize it primarily to lactate, which is then released by the cell and taken up by neurons via monocarboxylate transporters (Ames, 2000). This may occur predominantly only under certain conditions such as: 1) intercapillary regions with low pO₂, 2) segregation of glycolytic enzymes in a single cell, e.g. when there is compartmentalization of specific ion channels or ATPases. Other intermediates released by astrocytes include pyruvate, a-ketoglutarate, citrate and malate (Ames, 2000). However, there exist many inherent difficulties and experimental obstacles in studying brain metabolism, both at the regional and cellular levels, which has hindered progress in this field including: 1) energy-related reactions proceed so quickly that quenching is required when harvesting specimens that reflect in vivo conditions, 2) there is difficulty in measuring rates rather than levels, and 3) there are limitations in spatial and temporal resolution.
1.6.1 Glycolysis

Glycolysis (Embden-Meyerhoff pathway) is the metabolism of glucose to pyruvate and then lactate. It results in the net production of 2 mol ATP/mol of glucose as well as the generation of NAD$^+$ upon conversion of pyruvate to lactate (Figure 1-3). Alternatively, pyruvate can enter the mitochondria and undergo oxidative phosphorylation (OXPHOS) via the Tricarboxylic acid cycle (TCA, citric acid cycle, Krebs cycle) and electron transport chain (ETC) and produce ~36 mol ATP/ mol of glucose (Figure 1-4).

Glycolysis can be viewed as a universal metabolic pathway that occurs, with variations, in nearly all organisms both aerobic and anaerobic. It is comprised of a sequence of ten reactions involving ten intermediate compounds, with one step having two intermediate compounds. There exist three rate-limiting irreversible steps in the glycolytic pathway, influencing the overall kinetics: 1) HK, 2) phosphofructokinase (PFK), and 3) pyruvate kinase (PK). The rate of glycolytic flux is controlled by different mechanisms including substrate availability, enzyme concentrations, allosteric effectors and covalent modification on regulatory enzymes.
Figure 1-3: The Glycolytic Pathway.

Steps of the glycolytic pathway, ranging from the entry of glucose into the cell by glucose transporter (GLUT), conversion to glucose-6-phosphate by Hexokinase, to the generation of lactate by the enzyme lactate dehydrogenase (LDH).

Figure 1-4: Oxidative Phosphorylation: the Krebs Cycle and Electron Transport chain.

Pyruvate enters into the mitochondria and is catabolized by the Krebs cycle to generate reducing equivalents FADH$_2$ and NADH. Electrons are then transferred across the ETC to eventually generate a total of ~36 mol ATP/ mol of glucose.
1.6.2 Hexokinase

HK catalyzes the irreversible conversion of glucose to glucose-6-phosphate (G6P) thereby entrapping G6P inside the cell for commitment to either the glycolytic pathway for ATP generation or into pentose-phosphate pathway (PPP) to be used in biosynthetic reactions. This reaction consumes ATP and helps keep the intracellular concentration of glucose low, promoting continuous transport of glucose into the cell through glucose transporters.

\[
\text{Glucose + Mg \cdot ATP } \rightarrow \text{Glucose-6-Phosphate + Mg \cdot ADP}
\]

1.6.2.1 HK isozymes and tissue specificity

There exist four mammalian HK isozymes, designated HK1-4, whose expression differ across tissues and were originally identified by ion exchange chromatography (Gonzalez et al., 1964) or electrophoresis (Katzen and Schimke, 1965). See Table 1-1 for a comparison of the different HK isoforms and their regulatory mechanisms (Robey and Hay, 2006; Wilson, 2003). HK1, HK2 and HK3 have a 250-fold lower Km for glucose and are inhibited by their product G6P, unlike glucokinase (HK4, GK), which has a much lower affinity to glucose and is insensitive to product inhibition. HK1 to 3 have a mass of 100 kDa and are believed to have arisen from duplication and fusion of an ancestral GK isoform (50 kDa). HK1 and HK2 can localize to the outer mitochondrial membrane (OMM), the extent of which may vary across tissue types (Wilson, 2003). Localization of HK1 and HK2 to the mitochondria has been attributed to a 21 amino acid sequence at their N termini forming a hydrophobic α helix necessary for insertion into the hydrophobic core of the OMM (Wilson, 2003). Binding of HK1 and HK2 isozymes to mitochondria can be reversed by the product G6P. G6P acts as an allosteric inhibitor of HK1 and 2 and competes with ATP for binding to the enzymes. HK3 has traditionally been reported within the perinuclear compartment. GK is found within the cytosol of liver and pancreatic tissue (Wilson, 2003). In HK1 and HK3, only the C-terminal half has retained catalytic activity while both N and C terminal halves are active in HK2 and are sensitive to inhibition by G6P (Ardehali et al., 1996). There has also been a report of functional interaction between the C and N terminal halves of HK2 since glucose binding
by the N terminal half causes increased sensitivity to G6P regulation (at lower concentrations) in the C terminal half (Ardehali et al., 1996).

The existence of different HK isozymes may be necessary for multiple reasons including: 1) the HK isozymes may differ in their catalytic and/or regulatory properties, making them more suitable for specific metabolic roles, 2) transcriptional regulation of the isozymes may result in tissue specificity and distinct responses to altered metabolic status (e.g. hormonal effects) and 3) differences in subcellular location of the isozymes may result in compartmentalization of glucose metabolism and channeling of G6P to particular metabolic pathways (Wilson, 2003).

Differentiating HK1 and HK2 function has been suggested based on temperature differences and heat lability, where HK1 can function at higher temperatures (stable at 45°C) but not HK2. However, this technique has been refuted for differentiating HK isozyme activity, which still remains difficult to achieve in crude extracts or subcellular fractions (Wilson, 1998).
Table 1-1: Kinetic and regulatory parameters of mammalian hexokinase isozymes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HK1</th>
<th>HK2</th>
<th>HK3</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human gene locus</td>
<td>10q22</td>
<td>2p13</td>
<td>5q35.2</td>
<td>7p15.1-3</td>
</tr>
<tr>
<td>MW (kDa)</td>
<td>~100</td>
<td>~100</td>
<td>~100</td>
<td>~50</td>
</tr>
<tr>
<td>Km glucose (mM)</td>
<td>0.03</td>
<td>0.3</td>
<td>0.003</td>
<td>6</td>
</tr>
<tr>
<td>Km ATP (mM)</td>
<td>0.5</td>
<td>0.7</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Ki G6P</td>
<td>0.02</td>
<td>0.02</td>
<td>0.1</td>
<td></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>
<tr>
<td>Major tissue expression</td>
<td>Brain, kidney</td>
<td>Muscle, adipose</td>
<td>Lung, spleen</td>
<td>Pancreas, liver</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>No</td>
<td>Yes</td>
<td>?</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.6.2.2 Hexokinase 1

The HK1 gene, located at human chromosome 10q, encodes a ubiquitous form of HK which localizes to the outer membrane of mitochondria. HK1 is the predominant HK found in the CNS and is also referred to as normal brain HK. Mutations in this gene have been associated with hemolytic anemia due to hexokinase deficiency (Rijksen et al., 1983). Alternative splicing of this gene results in five transcript variants encoding different isoforms, some of which are tissue-specific. A sixth transcript variant has been described, but due to the presence of several stop codons, it is not thought to encode a protein. Evidence suggests that the association of HK1 to the mitochondria, with preferential use of intra-mitochondrial generated ATP, results in close coupling of glucose phosphorylation and OXPHOS (reviewed in Wilson, 2003). This mechanism ensures coordination of glucose phosphorylation with TCA cycle and ETC, avoiding the buildup of potentially toxic lactate. The extent of HK binding to mitochondria depends on the cell type, with highly glycolytic brain cells having a larger percentage of mitochondrion-bound HK1 than cells from other organs.

1.6.2.3 Hexokinase 2

The human HK2 gene (also called HKII, HXKII, muscle form hexokinase) is located on chromosome 2p13 and is comprised of 18 exons encoding a protein of 917 amino acids. Most normal mammalian tissues express little HK2 with those from muscle, adipocytes and lung expressing low but significant levels (Wilson, 1995; Wilson, 2003). In skeletal muscle, nearly 60% of HK2 is bound to mitochondria (Chang et al., 1996).

1.6.2.4 Differential role of Hexokinase 2 and Hexokinase 1 in tissues

The response of HK isoymes to inorganic phosphate (Pi) may provide a clue to their different physiological roles (Wilson, 1995). Only for HK1 does Pi antagonize G6P inhibition. Pi is inhibitory to HK2 and does not antagonize G6P and rather adds to its inhibition (reviewed in Wilson, 1995). An increase in cellular Pi from hydrolysis of high energy phosphate compounds and a decrease in cellular G6P due to increased flux through downstream PFK reaction generally occur during periods of increased energy demand and increased glycolytic metabolism (e.g. as in brain) (Lowry et al., 1964). The
resulting increase in the $[\text{Pi}]/[\text{G6P}]$ ratio increases HK1 activity, leading to the suggestion that HK1 functions primarily in a catabolic role, metabolizing glucose with the primary aim of generating ATP (Wilson, 1995). Consistent with this hypothesis is the ubiquitous expression of HK1 in virtually all mammalian tissues and its particular strong expression in the brain, a tissue known to rely heavily on glycolytic metabolism to sustain energy. In contrast, HK2 expression is confined primarily to insulin-sensitive tissues such as skeletal muscle and adipose tissue. The response of HK2 to G6P and Pi may make it more suitable to an anabolic role either shunting G6P through the pentose phosphate pathway (PPP) generating NAPDH or for glycogen synthesis (Sebastian et al., 2000). However this remains an unsubstantiated hypothesis.

1.6.3 Mitochondrial structure, oxidative phosphorylation and intrinsic apoptosis

Mitochondria are found in eukaryotic cells, where they make up as much as 10% of the cell volume and consume approximately 98% of the oxygen delivered to the cell. They are pleomorphic organelles with structural variations depending on cell type, cell-cycle stage and intracellular metabolic state. One key function of mitochondria is to produce energy (ATP) through OXPHOS and lipid oxidation. Several other metabolic functions are performed by mitochondria including steroid hormone and porphyrin synthesis, lipid metabolism, interconversion of amino acids, urea cycle, intracellular $\text{Ca}^{2+}$ homeostasis, the generation of free radicals, and intrinsic apoptotic cell death (for extensive review see (Duchen, 2004)).

1.6.3.1 Mitochondrial genetics and structure

Mitochondria are believed to have evolved from a bacterial progenitor. The primary evidence supporting this is the presence of mitochondrial DNA, independent of nuclear DNA, consisting of a circular 16.6kb double stranded DNA similar to primitive bacteria. Mitochondrial DNA encodes 13 polypeptides, consisting of only a portion of the proteins of the respiratory chain. The remaining proteins are encoding by nuclear genes. Mitochondrial DNA also codes for two rRNAs and 22 tRNAs which are needed to synthesize the 13 proteins (Duchen, 2004).
The abundance of mitochondria varies with cellular energy level and is a function of cell type, cell-cycle stage and proliferative state. The morphology of mitochondria is highly variable. In dividing cells, the organelle can switch between a fragmented morphology with many ovoid-shaped mitochondria (classical appearance) and a reticulum in which the organelle is a single, multi-branched structure. The cell cycle– and metabolic state– dependent changes in mitochondrial morphology are controlled by a set of proteins that cause fission and fusion of the organelle mass. Mutations in these proteins are the cause of several human diseases, often producing lactic acidemia, exercise intolerance or neurological disorders. Mitochondrial morphology is also controlled by cytoskeletal elements, including actin filaments and microtubules. In nondividing tissue, overall mitochondrial morphology is very cell dependent. There is also emerging evidence of functionally significant heterogeneity of mitochondrial forms within individual cells. Two types of mitochondria have been suggested to exist. The interfibrillar mitochondria (appear round and dense on electron microscopy (EM)) and the sarcolemmal mitochondria (underneath plasma membrane, lighter on EM). These two types of mitochondria may express different levels of enzymes and respond differently to stresses including ischemia/reperfusion (Lesnefsky and Hoppel, 2003).

Mitochondria have two membranes, an outer and an inner membrane, that likely have contact sites or form junctional complexes. The OMM is permeable to ions and small molecules, likely in a regulated fashion (MW < 1500 Da). Important pro- and anti-apoptotic proteins interact at the OMM including Bax, Bcl-2, among others. The inner mitochondrial membrane (IMM) is impermeable, forming the major barrier between the cytosol and mitochondrial matrix, but has ion channels and transporters including Ca\(^{2+}\) uniporter, K\(^{+}\) (ATP-dependent) channel, and Na\(^{+}\)/Ca\(^{2+}\) exchanger. Most importantly, the IMM contains the enzymes of the ETC linking the mitochondrial matrix to the intermembrane space (IMS). The IMM forms multiple infoldings that are collectively referred to as cristae. The structure of cristae has been found to vary extensively with the tissue type, although the functional impact of these differences is not clear. Between the OMM and IMM is the IMS which contains enzymes including creatine kinase and holocytochrome c as well as pro-apoptogenic proteins (reviewed in (Duchen, 2004)).
1.6.3.2 Citric acid cycle and the electron transport chain (ETC)

The enzymes of the TCA break down acetyl CoA, derived from pyruvate, fatty acid and amino acid breakdown to generate CO2 and reduced NADH and FADH$_2$ (Figure 1-4). The ETC consists of complex I (NADH ubiquinone reductase), complex II (succinate dehydrogenase), complex III (uniquinol cytochrome c oxidoreductase and complex IV (Cytochrome c oxidase). Only complex II is entirely encoded by nuclear DNA while the other complexes are encoding by a mixture of nuclear and mitochondrial DNA. Electrons are transferred from NADH and FADH$_2$ formed during TCA to Complex I and II, respectively. Electrons are transferred from these complexes to ubisemiquinone, shuttling to Complex III. Cytochrome c then shuttles electrons to Complex IV. A proton motive force is generated from the transfer of electrons down the electron transport chain. Protons move from the mitochondrial matrix to the IMS of the mitochondrion, creating a higher concentration of positively charged particles, resulting in an electrochemical gradient composed of both a pH gradient (H$^+$ ion concentration difference) and an electrical gradient (voltage difference due to separation in charge). Complex I, III and IV pump protons from the matrix to the IMS generating a potential difference of 150-180mV (negative to the cytosol). This mitochondrial membrane potential (MMP) provides the driving force for the F$_1$F$_0$.ATP synthase complex V to generate ATP and for cytosolic Ca$^{2+}$ to accumulate in the matrix via the Ca$^{2+}$ uniporter. The translocation of protons back into the matrix by F$_1$F$_0$.ATP synthase (tending to depolarize mitochondrial potential) drives the enzyme that phosphorylates ADP and releases ATP (Figure 1-4). Respiratory uncoupling drugs (e.g. FCCP, CCCP) or heat will shuttle protons across membrane into the matrix and dissipate the proton gradient, resulting in a compensatory increase in respiratory rate. Inhibition of the proton flux through ATP synthase inhibitors (e.g. oligomycin) increases the membrane potential and slows respiration.

1.6.3.3 Permeability transition pore and intrinsic mitochondrial apoptosis

The mitochondrial permeability transition pore (MPTP) is a multi-protein complex found at contact sites between the IMM and OMM (Zoratti and Szabo, 1994). Research over the past years has uncovered a central role for MPTP in both normal respiration and transport
of metabolites as well as in intrinsic apoptotic cell death. Several studies have attempted to determine the protein composition of the MPTP. Various soluble and membrane proteins including the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), peripheral benzodiazepine receptor (PBR), creatine kinase (CK), HK and/or cyclophilin D (CypD) have been implicated in forming part of this complex (Figure 1-5) (Brenner and Grimm, 2006). The precise molecular composition of the MPTP remains a debate, and may actually vary as a function of the cellular differentiation or activation state (Kroemer et al., 2007).

VDAC is a 30-kDa β-barrel protein spanning the outer membrane of the mitochondria, allowing the transport of many solutes and metabolites in and out of the IMS (Mannella et al., 1992). VDAC mediates ATP and ADP translocation across the outer mitochondrial membrane with the ANT mediating transport across the inner membrane (Figure 1-5). Thus, one of the basic functions of MPTP is to regulate the fluxes of ADP/ATP between the matrix, the IMS and the cytosol. In response to the energetic demands, ADP and ATP can be used by adenylate kinase (AK), CK and/or HK to phosphorylate their respective substrates. Furthermore, the opening of MPTP allows an influx of solutes with molecular masses of <1,500 Da (protons, water, solutes) into the matrix and can result in the abrupt dissipation of the mitochondrial membrane potential. Therefore, the imposed opening of MPTP (depolarization) or its prolonged closure (hyperpolarization) may be deleterious to the cell. ADP pools, the pH within the matrix, the mitochondrial membrane potential $\psi_m$, the redox state and a variety of chemotherapeutic agents (e.g. cyclosporin A) can influence the function of the MPTP (Figure 1-5) (Brenner and Grimm, 2006).

The involvement of MPTP in apoptosis was established primarily from pharmacological studies on isolated mitochondria and from measurements of the $\psi_m$ in cells by flow cytometry. Once permeability transition is commenced, depolarized mitochondria undergo a massive entry of water resulting in matrix swelling, remodeling of cristae and local rupture of the OMM (Vander Heiden et al., 1997). Induction of mitochondrial outer membrane permeabilization (MOMP) is accompanied by the release of proapoptotic proteins into the cytosol, such as cytochrome c and apoptosis-inducing factor (AIF) (Kroemer et al., 2007). Cytochrome c then binds and activates the caspase activator
Apaf1, which in turn cleaves pro-caspase 9 to yield active caspase 9. Caspase 9 then initiates a caspase cascade resulting in activation of the executioner caspases (Salvesen and Dixit, 1997).

However the sequence of events resulting in MOMP are unclear. In addition to proteins of the Bcl-2 family (e.g. Bax, Bak), MOMP is found to be regulated by proteins of the permeability transition pore complex (Brenner and Grimm, 2006), proteins that affect mitochondrial dynamics (fusion and fission) (Herzig and Martinou, 2008) and even transcription factors (e.g. p53) that can translocate from the nucleus to mitochondria to stimulate MOMP (Moll et al., 2006). Different mechanisms or order of events have been implicated in initiating intrinsic apoptosis. Hyperpolarization of mitochondria and closure of MPTP may precede eventual mitochondrial depolarization and MOMP followed by release of pro-apoptotic factors. MPTP induction has also been reported to occur with dissipation of MMP. However, MOMP may occur independent of the MPTP, relying on Bax and/or Bak interaction with the OM channel including VDAC (reviewed in (Brenner and Grimm, 2006).

Complete or partial OXPHOS defects can also induce apoptosis resistance. Inhibition of the respiratory chain has been shown to suppress the activation of the proapoptotic proteins Bax and Bak (Tomiyama et al., 2006). Thus, severe OXPHOS defects that occur in some cancers, might result in a blockade of MMP and hence inhibition of apoptosis. OXPHOS defects may also reduce generation of reactive oxygen species (ROS) in mitochondria, thereby abrogating their proapoptotic activity. Cells that lack mitochondrial DNA (p0) and hence have limited OXPHOS are resistant against various apoptotic inducers by provoking futile redox cycles in mitochondria (Kroemer et al., 2007). Therefore, a state of deficient OXPHOS might automatically compromise the intrinsic pathway of apoptosis through a variety of distinct mechanisms.
Figure 1-5: Mitochondrial Permeability Transition Pore.

The MPTP has been affiliated with proteins including VDAC, ANT, Cyclophilin D, HK, CK, PBR and the Bcl family of proteins. Many signals can converge on the mitochondrion to regulate MPTP. In response to calcium, adenine nucleotide depletion, chemotherapeutics and pro-oxidant agents, membrane permeabilization can result from the opening of MPTP and lead to the release of proapoptotic factors into the cytosol. In contrast, many agents (Cyclosporin A, bongkrekic acid, Mg2+, high ADP/ATP) or conditions (pH < 7) promote the closure of the MPTP.
Opening of PTP
Ca\(^{2+}\)
Pro-oxidant agents
ATP/ADP depletion
Chemotherapeutics

Closure of PTP
pH < 7
Mg\(^{2+}\)
Cyclosporin A
high ATP/ADP

Figure 1-5
1.6.3.4 Mitochondrial biogenesis and dynamics

Each cell may have thousands of copies of mtDNA, following population genetics rather than Mendelian genetics. In some cells, mitochondria form networks that are continuously remodeling, while in other cells, they are relatively more fixed (e.g. muscle). More recent studies are aimed at deciphering the roles of fusion proteins (Mitofusin 1, 2 and Opa1) and dynamic related protein (drp1) in the regulation of mitochondrial fusion and fission, respectively, and their association with cell cycle events, OXPHOS, autophagy and apoptosis (Herzig and Martinou, 2008). Mitochondrial replication requires the close coordination of mitochondrial DNA replication as well as protein synthesis by genes both nuclear and mitochondrially encoded. The transcription factor Peroxisome proliferator activated receptor γ coactivator 1α (PGC1α) is believed to play an essential role in synchronizing these processes by regulating gene expression of transcription factors Nuclear respiratory factors 1 and 2 (NRF1, NRF2), and mitochondrial transcription factor 1 (Tfam or mtTFA) (Rohas et al., 2007). The regulation of PGC1α is under active investigation but its activation has been associated partly with sustained rise in intracellular calcium acting on CaMKinase IV, AMPK signaling as well as p38 MAP Kinase (Lin et al., 2005).

1.6.3.5 Oxidative stress

A consequence of OXPHOS is the generation of unpaired electrons that interact with oxygen to generate superoxide ions, which are highly reactive free radicals. These are converted to other ROS including hydroxyl ions (OH⁻) and H₂O₂. Oxidative stress arising from the production of ROS can result in lipid peroxidation, enzyme inhibition, DNA damage, and alterations in signaling cascades including inflammatory cascades (Duchen, 2004). The sites of free radical generation remain contentious although both complexes I and III are likely important sources. The leak of electrons seems to increase with increases in mitochondrial potential and decrease with mitochondrial depolarization.

Mitochondria possess a variety of anti-oxidant defenses including: 1) a high concentration of glutathione, a variant of superoxide dismutase (SOD), 2) high concentration of catalase, to remove harmful peroxide produced by SOD (Duchen, 2004).
It has also been hypothesized that mitochondria have evolved mechanisms to regulate the rate of ROS production through feedback loops regulating the expression of uncoupling proteins (UCP), proteins implicated implicated in thermogenesis, particularly UCP1.

1.6.3.6 Autophagy

In addition to apoptosis, cells can undergo autophagy, the process of lysosomal degradation of cytosolic contents. There are different types of autophagy including macroautophagy (within a double membrane vesicle), microautophagy and chaperone induced autophagy. Various proteins have been implicated in macroautophagy including beclin1, atg5, PI3K class III protein Vsp34, LC3 and nutrient sensing via mammalian Target of Rapamycin (mTOR). Autophagy is an evolutionary conserved catabolic process whereby cells self-digest intracellular organelles promoting cell survival particularly under conditions of starvation or metabolic stress; however, when allowed to go to completion, autophagy may be a means of achieving cell death (Edinger and Thompson, 2003; Levine, 2005). The mechanisms of induction of autophagy are still not clear. Mitochondria are important regulators of both apoptosis and autophagy. ROS are believed to play a role in triggering either apoptosis or autophagy, depending on cellular context. It has been hypothesized that in cells resistant to apoptosis, they may preferentially undergo autophagy (Mathew et al., 2007).

1.6.3.7 Techniques for assessing mitochondrial structure and physiology

Mitochondrial structure and function can be studied using a variety of different techniques. Below is a short description of a subset of these experimental paradigms.

*In vitro slice preparations of mitochondria:* Electrophysiological experiments can be performed on purified VDAC or other MPTP proteins reconstituted into planar lipid bilayers.

*Mitochondrial fractionation:* The isolation and enrichment of mitochondria from cells can be achieved using approaches including differential centrifugation, aimed either at isolating a highly pure fraction or at maintaining functional integrity.
Electron Microscopy: Transmission EM can allow for the non-quantitative visualization of structure of the inner and outer membranes and cristae folding.

Fluorescent dyes of mitochondrial function: There exist many different fluorescent dyes to assist in studying mitochondrial activity, localization and abundance.

Mitotracker probes: These are cell-permeant mitochondria-selective dyes that contain a mildly thiol-reactive chloromethyl moiety, which assists in keeping the dye associated with the mitochondria after fixation.

MitoSOX Red: This is live-cell permeant dye that selectively targets mitochondria. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide, but not by other ROS, and exhibits bright red fluorescence upon binding to nucleic acids (excitation/emission maxima = 510/580 nm). Oxidation of the probe is prevented by superoxide dismutase.

Rhodamine 123 and derivative TMRE: Rhodamine 123 and Tetramethylrhodamine, ethyl ester (TMRE) are cell-permeant, cationic, red-orange fluorescent dyes readily sequestered by active mitochondria. Staining with these dyes provides a straightforward method of determining mitochondrial membrane potential.

JC-1: JC-1 is cationic cyanine dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration dependent formation of red fluorescent J-aggregates. JC-1 is reported to be more specific for mitochondrial versus plasma membrane potential, and more consistent in its response to depolarization than other cationic dyes including rhodamine 123. The ratio of green to red fluorescence is reported to be unaffected by other factors including mitochondrial size, shape and density, which can interfere with single-component fluorescence signals. This allows for comparative measurements of membrane potential and determination of the percentage of mitochondria within a population responding to a stimulus. The most widely implemented application of JC-1 is for detection of
mitochondrial depolarization occurring in the early stages of apoptosis (Smiley et al., 1991).

**Calcein AM:** This acetoxymethyl (AM) ester of calcein is a colorless and nonfluorescent esterase substrate which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, calcein AM is cleaved by intracellular esterases to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein is quenched by the addition of CoCl$_2$, while the fluorescence from the mitochondrial calcein is maintained. Conditions that trigger opening of the MPTP (e.g. Ca$^{2+}$ ionophores), results in subsequent loss of mitochondrial calcein fluorescence.

**NAO:** This dye is well retained in the mitochondria of live cells for days, making it useful to follow mitochondria over long periods of time. The mitochondrial uptake of this metachromatic dye is reported independent of membrane potential, binding to cardiolipin in all mitochondria and making it a useful marker for mitochondrial mass.

### 1.7 Metabolic Remodeling and the Warburg effect in Cancer

#### 1.7.1 Introduction: The Warburg Effect

A major metabolic change within solid tumours was identified by Otto Warburg in the early 1920s (Warburg, 1956). Warburg noticed that normal tissues use glycolysis to generate about 10% of the cells’ total ATP with the mitochondria generating the remaining 90%. However, in some tumour tissues, more than 50% of ATP can be generated from glycolysis, even under aerobic conditions in which the mitochondria should be functioning normally. Warburg reported that tumour cells have to consume more glucose and upregulate glycolysis in order to generate more ATP. Accordingly, tumour cells generate more lactate, the product of anaerobic glycolysis, even under
aerobic conditions, a process thus referred to as aerobic glycolysis or the “Warburg Effect”.

Upregulated glycolysis and increased expression of glycolytic enzymes including LDHA have been reported in a variety of cancers and found to correlate with increased tumour aggressiveness, metastasis and poor patient prognosis (Langbein et al., 2006b; Schwickert et al., 1995; Walenta et al., 1997). Of note, although aerobic glycolysis is now considered characteristic of cancer, not all cancers necessarily display the Warburg effect. For example, human mensenchymal stem cells are reported to rely more on OXPHOS for energy production when transformed (Funes et al., 2007). However, it is not only solid cancers that undergo the Warburg phenomenon; leukemia and other hematological cancers have also been demonstrated to favour aerobic glycolysis (Samudio et al., 2008).

The almost universal phenotype of increased glucose metabolism in cancer is exploited radiologically by Positron Emission Tomography (PET) using 2-deoxyglucose labeled with the radioemitter $^{18}\text{F}$. FDG-PET can assist with disease diagnosis, staging and restaging as well as monitoring response to therapy in a variety of oncologic settings (Kelloff et al., 2005). Clinical trials in breast cancer among others have shown the potential of FDG-PET in assessing early response to therapy and clinical outcome (Kelloff et al., 2005). Non-invasive MR techniques such as H-MR spectroscopy (MRS) can measure the presence of lactate in tissue, the product of anaerobic glycolysis. Levels of lactate and Choline/N acetyl aspartate (NAA) ratios on H-MRS have also been correlated to grade and potential recurrence of tumours (Lemort et al., 2007).

### 1.7.2 Putative advantages of the Warburg effect

The Warburg effect has been suggested to provide a selective proliferative and survival advantage to tumour cells within their microenvironment. The following is a more detailed account of potential advantages attributed to the Warburg effect.
1.7.2.1 Bioenergetic demands

Accelerated glycolysis may ensure that ATP levels meet the demands of highly proliferating tumour cells, particularly in a hypoxic environment. As much as 50% of the ATP produced in some tumour cells can be derived from glycolysis in contrast to normal cells where this value is less than 10% (Nakashima et al., 1984). Under hypoxic conditions, the already elevated glycolytic rate can be doubled or more (Weinhouse, 1972). Thus, it was originally supposed that the predominant advantage of the Warburg effect was enhanced glycolytic flux resulting in significantly faster production of ATP per mole of glucose. However under non-stressful conditions when resources are not scarce, ATP would be more efficiently provided by OXPHOS, which suggests other potentially more important advantages to the Warburg effect. Overall, evidence suggests that no matter the extent of cell division of tumour cells, they maintain high ATP to ADP ratios as well as NADH/NAD+, supporting that ATP is never limiting in these cells (Christofk et al., 2008a; Deberardinis et al., 2008a).

1.7.2.2 Survival and regulation of apoptosis

Increased glucose uptake and glycolysis may provide a survival advantage to tumour cells via different yet interrelated mechanisms.

a) The glycolytic phenotype is associated with enhanced mitochondrial membrane polarization (i.e. hyperpolarized) and resistance to MMP (Chen, 1988; Kroemer et al., 2007).

b) Glycolysis has been found to protect from apoptosis by rendering tumour cells independent of growth factors (Plas and Thompson, 2002).

c) Complete and even partial loss of OXPHOS can induce resistance to apoptosis by suppressing the activation of pro-apoptotic proteins Bax and Bak, important mediators of MMP (Tomiyama et al., 2006).

d) Because ROS are natural by-products of mitochondrial function, inhibition of OXPHOS by generation of lactate may protect cancer cells from oxidative stress (Brand and Hermfisse, 1997). Cells that lack mitochondrial DNA (p⁰ cells) are
resistant against a variety of apoptotic inducers (Kroemer et al., 2007). Loss of the p53 regulated TIGAR enzyme has been shown to result in enhanced apoptosis, most likely due to increases in ROS (Bensaad et al., 2006).

e) Enhanced formation of G6P can result in greater flux into the PPP and generate NAPDH which ensures the cell’s antioxidant defenses and protects the cells from ROS (Bensaad et al., 2006; Feron, 2009).

f) Glycolytic enzymes including HK2 can localize to the OMM and interact with the MPTP thereby regulating intrinsic apoptotic pathway (Gottlob et al., 2001; Majewski et al., 2004a).

g) Cells dependent on glycolysis can better adapt to conditions of fluctuating oxygen levels resulting from altered vascularity (Gatenby and Gillies, 2004).

1.7.2.3 Enhanced invasion and suppressed immune response

By favouring aerobic glycolysis, tumour cells generate bicarboxic and lactic acids, which are exported from the cell. This renders the extracellular environment more acidic, potentially favouring tumour invasion by pH-dependent activation of cathepsins and metalloproteinases (Swietach et al., 2007) and suppressing anti-cancer immune effectors such as T cells (Fischer et al., 2007). It has also been proposed that extracellular lactate may be taken up by stromal cells via monocarboxylate transporters (MCT1, MCT2 which co-transport H⁺ with lactate) and reverted back to pyruvate thereby altering the NAD+/NADH ratio. The pyruvate can then be used for OXPHOS in stromal cells or exported out to refuel tumour cells thereby sustaining cancer cell survival (Feron, 2009; Koukourakis et al., 2006).

1.7.2.4 Substrates for biosynthetic/anabolic pathways

In order for tumour cells to proliferate, they require nucleotides, fatty acids, membrane lipids, and proteins in addition to ATP. Tumour cells may alter their metabolism in order to assist in the synthesis of biosynthetic precursors (Deberardinis et al., 2008b). Along with increased glutaminolysis, accelerated glycolysis can provide a constant supply of metabolic intermediates necessary for macromolecule biosynthesis and cell growth and
proliferation (Vander Heiden et al., 2001). Two major biosynthetic activities required by proliferating cells is the production of ribose-5-phosphate (R5P) for nucleotide biosynthesis and fatty acids for lipid synthesis (Figure 1-6).

To generate R5P, tumour cells divert carbon from glycolysis into the oxidative or non-oxidative arm of the PPP. Tight regulation at different steps of glycolysis including the enzymes PFK1 (by TIGAR), PGM (by p53) and PKM2 can result in accumulation of substrates and diversion of carbon towards R5P. Expression of dimeric PKM2 can result in accumulation of F-1,6-BP which suppresses glucose-6-phosphate dehydrogenase (G6PD), thus enhancing flux into the non-oxidative arm of the PPP. Furthermore, the non-oxidative arm of PPP seems to be more important for tumour cells based on higher expression and activity of transketolase (TKTL1) found to correlate with rate of tumour growth in some cancers (Langbein et al., 2006a). A further level of regulation includes the ratio of NADP+:NADPH in cells. When it increases, the body realizes it needs to produce more NADPH (an important reducing agent for several reactions like fatty acid synthesis). This will cause the G6P to be dehydrogenated by G6PD. This reversible reaction is the initial step of the PPP, which generates the useful cofactor NADPH as well as ribulose-5-phosphate, a carbon source for the synthesis of other molecules. G6P can also be used to generate glycogen stores.

Tumour cells also use glucose to assist in generation of fatty acids. Tumour cells employ fatty acids to alter membrane-targeted proteins in cell signaling and for de novo membrane synthesis. Perturbations in enzymes involved in lipid synthesis have also been shown to inhibit tumourigenesis. Depletion of ATP citrate lyase (ACL), the enzyme that converts citrate into the lipid precursor cytosolic acetyl-coA, results in decreased tumour growth (Hatzivassiliou et al., 2005). Acetyl-coA carboxylase (ACC) and fatty acid synthase (FAS) are upregulated in many cancers and also play a role in tumourigenesis (Menendez and Lupu, 2007; Wang et al., 2005). FAS synthesizes long chain fatty acids (e.g. palmitate) from malonyl-coA and NAPDH. The PI3K/AKT/mTOR pathway stimulates expression of these lipogenic genes ACL, ACC and FAS by increasing nuclear localization of the transcription factor sterol response element binding protein-1 (SREBP-1) (Porstmann et al., 2005). In addition to enhanced glucose uptake and growth factor
signaling, fatty acid synthesis also requires two supporting pathways: generation of NADPH and anaplerosis. Anaplerotic reactions are those that form intermediates of the TCA cycle, to replenish TCA intermediates that have been extracted for biosynthesis (cataplerotic reactions). The export of mitochondrial citrate for fatty acid synthesis requires the replacement of oxaloacetate (OAA) by anaplerosis. Glutaminolysis, the metabolism of glutamine to lactate, is another important metabolic pathway in tumour cells which can help replenish TCA intermediates and generate NAPDH. The rate of consumption of glutamine in tumour cells is above and beyond that required for protein synthesis (Reitzer et al., 1979). The first step of glutaminolysis is the deamidation of glutamine to glutamate via the enzyme glutaminase (GLS). Glutamine-derived α-ketoglutarate (α-KG) is the major source of OAA in some cells. NMR spectroscopy in human GBM cells demonstrated via $^{13}$C labeling that glutamine contributes to the bulk of anaplerotic carbon to the TCA cycle (Deberardinis et al., 2007; Portais et al., 1996). Glutaminolysis also results in export of glutamine-derived malate for NAPDH production by malic enzyme. Glutamine metabolism as an anaplerotic precursor and source of NAPDH results in secretion of a large quantity of glutamine-derived carbon and nitrogen as lactate and alanine. Other intermediates of glycolysis feed into anabolic reactions including dihydroxyacetone phosphate (DHAP) for triacylglyceride and phospholipids synthesis and pyruvate for alanine and malate synthesis.

A large amount of NAPDH is required for synthesis of fatty acids (e.g. 16-carbon fatty acid palmitate requires 14 molecules of NAPDH). Sources of NAPDH include G6PD from the oxidative arm of PPP, cytoplasmic malic enzyme ($ME1$ gene) which converts malate to pyruvate and is a SREBP-1 target, and IDH1 and 2 (cytosolic IDH1, mitochondrial IDH2). The relative importance of these enzymes in tumour growth is only now being investigated. Furthermore, whether these biosynthetic pathways fluctuate with the stage of cell cycle also remains unanswered. Of note, aerobic glycolysis does not always reflect cell growth because conditions that stabilize HIF1α can stimulate aerobic glycolysis while suppressing biosynthetic pathways (Lum et al., 2007a).
**Figure 1-6: Metabolic Pathways Active in Proliferating Cells.**

Interconnection of glycolysis, oxidative phosphorylation, the pentose phosphate pathway, and glutamine metabolism and regulation by known oncogenes and tumour suppressor genes in proliferating cells. NADPH production and acetyl-CoA flux to the cytosol for lipid synthesis are favoured in proliferating cells. Activation of growth factor receptors leads to both tyrosine kinase signaling and PI3K activation. Via AKT, PI3K activation stimulates glucose uptake and flux through the early part of glycolysis. Tyrosine kinase signaling negatively regulates flux through the later steps of glycolysis, making glycolytic intermediates available for macromolecular synthesis as well as supporting NADPH production. Myc has been found to promote glutamine metabolism, also resulting in NADPH production. LKB1/AMPK signaling and p53 decrease metabolic flux through glycolysis in response to cell stress.
1.7.3 The molecular basis of the Warburg effect

The molecular basis of the Warburg effect is currently under intense investigation. It is likely to be cancer specific and involve interplay between oncogenic signaling, the tumour microenvironment, and metabolic enzymes. Altered expression of enzymes controlling metabolic flux rates may preferentially promote a glycolytic phenotype.

1.7.3.1 The Tumour Microenvironment

Altered glycolytic metabolism is most commonly associated with the physiological stresses that occur in a solid tumour environment, most recognizably the presence of hypoxia. Other physiologically relevant stresses include acidosis, nutrient deprivation, and increased interstitial pressure. All of the above are either directly or indirectly a result of altered vascularity or vascular supply. As the tumour expands and grows, a diffusion-limited gradient of oxygen, nutrients and increased waste products is formed around blood vessels. Tumour interstitial pH and the partial pressure of oxygen (pO2) decrease with distance from a vessel wall, and with exact distances varying with cancer type (Figure 1-7) (Gatenby and Gillies, 2004).

Figure 1-7: Decrease in interstitial pH and partial pressure of oxygen with distance from vessel walls.

Although dependent on the type of cancer, a diffusion-limited gradient of oxygen, nutrients and waste products is formed around blood vessels as tumours grow. This results in the formation of gradient within the tumor microenvironment (grey circle) of well-oxygenated, perfused and normal pH near functional vessels (red line) and poorly oxygenated and low pH at distances far from the vasculature.
Figure 1-7

<table>
<thead>
<tr>
<th>pH</th>
<th>7.3</th>
<th>7.0</th>
<th>6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO2 (mmHg)</td>
<td>14</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Distance (um)

0  100  200  300  400
The process of oxygen diffusion and consumption was originally modeled by Krogh in 1919. He showed that oxygen concentrations decreased with distance from a capillary, and that oxygenated cells were limited to a distance of less than 150um from vessels (Krogh, 1919). Krogh’s original results were validated by others both empirically and experimentally. Subsequent studies verified that partial pressures of O2 are near zero at distances of 100um from a vessel (Dewhirst et al., 1994; Helmlinger et al., 1997). Further studies have measured variability in oxygen levels, and more specifically oxic-hypoxic cycles in tumours that can range in minutes, hours or days (Gatenby and Gillies, 2004). Changes over days likely reflect vascular remodeling and neo-angiogenesis due Vascular endothelial growth factor (VEGF) induction needed for new vessels (Gilead and Neeman, 1999; Patan et al., 2001). Periodic hypoxia likely results in selection of cells in which anaerobic glycolysis is constitutively upregulated in order to survive low oxygen periods (Gatenby and Gillies, 2004).

One important adaptation to tumour hypoxia is the stabilization of the transcription factors Hypoxia Inducible Factor 1-alpha (HIF1α). HIF1 is a heterodimeric transcription factor consisting of HIF1α (or HIF2α) as the O2 sensing units and HIF1β (ARNT), which is constitutively expressed. HIF1α is rapidly degraded in well-oxygenated tissues via ubiquitin-mediated degradation due to the presence of an amino acid sequence known as the oxygen-dependent degradation domain (ODDD) located at the centre of the protein. Prolyl hydroxylases (PHD), which require O2 as a substrate, hydroxylate Prolines 402 and 564 of the ODDD of HIF1α allowing it to be recognized by vHL acting as an E3 ligase leading to ubiquitination of HIF1α. If vHL is lost, as in renal cancer, then HIF1α is constitutively stable even under normoxia (Maxwell et al., 1999). Furthermore, Asparagine hydroxylation at A803 in the C-terminal transactivation domain by Factor Inhibiting HIF1α (FIH) has been shown to regulate HIF1α activity but not stability (Denko, 2008) (Figure 1-8). However, HIF1α stability and expression can be regulated by other factors than O2 including oncogene activation or loss of tumour suppressors. HIF1α has been shown to accumulate with activation of oncogenic Ras, SRC, and PI3K signaling or with loss of tumour suppressor including VHL and PTEN, even under normoxic conditions (Denko, 2008). Increased expression of metabolites of OXPHOS including succinate and fumarate, as well as the generation of free radicals can also
stabilize HIF1α (Chandel et al., 2000; Denko, 2008). However, HIF1α levels are still closely coupled to oxygen levels seen by heterogeneity of expression within the tumour microenvironment, with increased expression in hypoxic regions (Wiesener et al., 2001; Zhong et al., 1999).

When HIF1α is stabilized, it shifts to the nucleus where it interacts with HIF1β and CBP/p300 and can bind to the promoter elements of genes containing Hypoxia Response Elements (HRE) (Figure 1-8). The consensus sequence for the HRE consists of 5’-RCGTG-3’. This is very similar to the consensus sequence of Myc. One overall repercussion of HIF1 activation is the upregulation of glycolysis and inhibition of mitochondrial function. HIF1α activation leads to increased expression of genes involved in glucose entry (GLUT1, 3) and many enzymes of glycolysis, as well as increased expression of proteins that inhibit OXPHOS including pyruvate dehydrogenase kinase (PDK1) (Kim et al., 2006; Papandreou et al., 2006) which inhibits pyruvate dehydrogenase (PDH), and MAX interaction 1 (MXI1) (Zhang et al., 2007). HIF1 can also favour adaptation of mitochondria to hypoxic conditions by transactivating COX subunit 4-2 and LON protease, a protease that degrades COX4-1 (Fukuda et al., 2007). See Table 1-2 for a list of glycolytic and mitochondrial associated HIF1α targets.

In addition to metabolic remodeling, HIF1α plays an important role in angiogenesis and enhanced delivery of oxygen to tissues by upregulating VEGF and erythropoietin (Bergers and Benjamin, 2003). However, these newly formed blood vessels are often disorganized and may not adequately deliver oxygen and nutrients, resulting in spatial and temporal fluctuations in oxygen levels.

However, cancer cells have been reported to use glycolytic metabolism even before the exposure to hypoxic conditions (Christofk et al., 2008a; Nolop et al., 1987). Furthermore, leukemic cells are highly glycolytic yet reside in the well-oxygenated bloodstream (Elstrom et al., 2004; Gottschalk et al., 2004). This suggests that the Warburg effect is not totally dependent on upregulation of HIF1α or the presence of hypoxia, which may be a later occurring phenomenon after the switch to aerobic glycolysis, and that other
mechanisms, including metabolic changes induced by oncogene activation or loss of tumour suppressors, may also be important.

**Figure 1-8: Mechanisms of hypoxia-inducible factor 1α (HIF1α) stabilization.**

The classical O₂ sensing pathway is through O₂-dependent hydroxylation of P402 and/or P564 on HIF1α. Three PHD enzymes mediate recognition of the vHL–elongins complex and ubiquitination of HIF1α and hence targeting for proteasomal degradation (red pathway). Oncogenic activation, associated with activation of the Ras–Raf–MAPK and PI3K/PTEN/AKT (PI3K), can also cause HIF1α accumulation. TCA cycle intermediates such as succinate and fumarate and ROS can inhibit the activity of PHDs, thereby also stabilizing HIF1α (green pathway). Stabilized HIF1α associates with HIF1β (ARNT), which binds to cognate HREs in target genes.
Figure 1-8

- **Hypoxia**: O2, αKG
- **Succinate**: CO2, succinate
- **ROS**: 

The diagram illustrates the interaction between HIF1α and HIF1B, regulated by PHDs, vHL, and proteasome-mediated degradation. HIF1α is regulated by Ubiquitination and FIH. CBP/p300 and HRE are involved in the regulation of HIF1-target gene expression.
Table 1-2: HIF1α targets of glycolysis and oxidative phosphorylation.

<table>
<thead>
<tr>
<th>HIF1 target</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1, 3</td>
<td>Glucose entry</td>
<td>Gleadle et al., 1997</td>
</tr>
<tr>
<td>HK2</td>
<td>Glucose phosphorylation</td>
<td>Mathupala et al., 2001</td>
</tr>
<tr>
<td>PGI, PFK1, aldolase, TPI, GAPDH, PGK, PGM, enolase, PK, PFKFB1-4</td>
<td>Glycolysis</td>
<td>Semenza et al., 2004</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate generation</td>
<td>Firth et al., 1995</td>
</tr>
<tr>
<td>MCT4</td>
<td>Lactate transporter</td>
<td>Ullah et al., 2006</td>
</tr>
<tr>
<td>PDK1, MXI1</td>
<td>Decreased mitochondrial activity</td>
<td>Kim et al., 2006; Papandreou et al, 2006</td>
</tr>
<tr>
<td>COX4I2, LON protease</td>
<td>Increased O2 consumption in hypoxia</td>
<td>Fukuda et al., 2007</td>
</tr>
</tbody>
</table>
1.7.3.2 Mutations in metabolic enzymes

Mutations of metabolic enzymes encoded at the nuclear DNA or mitochondrial DNA level may play an important role in establishing the Warburg effect in certain cancer types. Mitochondrial DNA mutations may arise as a result of tumour progression (Brandon et al., 2006) or potentially contribute to tumour initiation. Mutations in genes encoding the inner mitochondrial membrane protein succinate dehydrogenase (SDH) and the mitochondrial matrix protein fumarate hydratase (FH) contribute to transformation in malignant pheochromocytomas and leiomyomata respectively (Denko, 2008; Gottlieb and Tomlinson, 2005). Loss of function mutations in FH or SDH subunits (SDHB, SDHC, SDHD) result in accumulation of their substrates fumarate and succinate. Accumulation of these intermediates interferes with functioning of the enzyme α-ketoglutarate dehydrogenase, which converts α-ketoglutarate and O₂ to succinate and CO₂. Since α-ketoglutarate is a cofactor of PHDs, a decrease in this intermediate via accumulation of succinate has been shown to cause HIF1α to be stabilized in normoxic cells. Pyruvate and lactate, also 2-oxoacids, have also been implicated in stabilizing HIF1α suggesting a novel feedforward mechanism (Lu et al., 2005). These cancer syndromes illustrate that primary mitochondrial dysfunctions can contribute to metabolic and oncogenic changes. More recently, mutations in the IDH1 enzyme was identified in gliomas and acute myeloid leukemias (Mardis et al., 2009; Yan et al., 2009), although the significance of this mutation is under active investigation.

1.7.3.3 Growth Factor/PI3K/AKT signaling in metabolism

Many metabolic enzymes and energy-sensing metabolic pathways are closely linked to growth regulatory pathways. Glycolysis has been reported regulated by oncogenic signaling pathways including Ras (Dang and Semenza, 1999; Ramanathan et al., 2005), PI3K/AKT (Manning and Cantley, 2007), and myc (Kim et al., 2007) (Figure 1-6).

AKT has multiple effects on metabolism (Plas and Thompson, 2005). It can stimulate glucose uptake by increasing expression of glucose transporter (GLUT1) and induce the translocation of GLUT4 to the plasma membrane. AKT also stimulates glycolysis by the activating phosphorylation of 6-phosphofructo-2-kinase (PFK2) (Manning and Cantley,
Although the detailed mechanisms remain unclear, AKT promotes translocation of HK2 to the OMM (Pastorino et al., 2005). PI3K/AKT activation also promotes fatty acid synthesis through phosphorylation of ATP citrate lyase (ACL) and inhibits fatty acid B-oxidation by downregulating the carnitine palmitoyltransferase 1a (CPT1a) transporter (Manning and Cantley, 2007). AKT over-expressing cancer cells have been found to be dependent on glucose for survival (Elstrom et al., 2004), believed to result from constitutive AKT’s suppression of fatty acid oxidation (Buzzai et al., 2005). However, it has also been demonstrated that constitutive AKT can result in enhanced sensitization to oxidative stress resulting in cellular apoptosis (Nogueira et al., 2008).

Of therapeutic relevance, dietary restriction has been shown in some cancers to be an effective way of reducing tumour growth in the preclinical setting (Kalaany and Sabatini, 2009). However, this was found to be only in the context of normal or hyperactive growth factor signaling via the PI3K/AKT pathway. Cancer cells that carry mutations in PI3K enzyme or PI3K pathway, resulting in constitutive PI3K activation, were found to be resistant to dietary restriction. For example, glioma cells in which PTEN is frequently deleted were refractory to dietary restriction (Kalaany and Sabatini, 2009). The positive effects of dietary restriction in some cancers are believed to result from reduced levels of IGF1 and insulin, measurable in the plasma. Dietary restriction may exert pro-apoptotic effects mediated via FOXO1 activation (Kalaany and Sabatini, 2009).

### 1.7.3.4 Loss of the tumour suppressor p53

Inactivation of p53 may favour the Warburg phenomenon via multiple mechanisms. Loss of p53 has been associated with the glycolytic phenotype via its effects on the OXPHOS protein Synthesis of Cytochrome c Oxidase 2 (SCO2), positively regulated by p53 and required for the assembly of the COX complex (Matoba et al., 2006). As well, p53 negatively regulates the glycolytic enzyme phosphoglycerate mutase (PGM), which catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate (Kondoh et al., 2005) (Figure 1-6). The conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (FBP) by phosphofructokinase 1 (PFK1) is the second irreversible step of glycolysis. This step is strongly regulated allosterically by fructose-2,6-bisphosphate, which is the product of the dual enzyme PFK2 that has two catalytic centres: 1) PFK2 generates fructose-2,6-
bisphosphate from fructose-6-phosphate. Fructose-2,6-bisphosphate is a strong activator of PFK1, needed for the conversion of fructose-6-phosphate to FBP and thereby stimulates glycolysis. 2) The fructose-2,6-bisphosphatase (FBP2) enzyme catalyses the opposite reaction of fructose-2,6-bisphosphate to fructose-6-phosphate, thereby decreasing glycolysis. When Ser32 is phosphorylated, the negative charge causes the conformation of the enzyme to favor the FBP2 activity (Bensaad et al., 2006). When it is not phosphorylated, PFK2's activity is favored. TIGAR (TP53-induced glycolysis and apoptosis regulator), molecularly similar to the bisphosphatase domain of PFK2, is induced by p53 and inhibits glycolysis by inhibiting fructose-2,6-bisphosphate, necessary for stimulation of glycolysis at PFK1 step and inhibition of gluconeogenesis (Bensaad et al., 2006). This results in the redirection of glucose towards PPP and NADPH production. As mentioned above, AKT also stimulates glycolysis at this step by the activating phosphorylation of PFK2 (Manning and Cantley, 2007).

1.7.3.5 AMPK signaling in cancer metabolism

AMPK acts as an intracellular energy sensor that is activated in low energy states by increased AMP levels. AMP levels are elevated by stress conditions such as glucose deprivation and hypoxia resulting in allosteric activation of AMPK to maintain energy balance within the cell (Shackelford and Shaw, 2009). AMPK serves as a metabolic checkpoint in the cell, arresting cell growth in conditions of low intracellular ATP via multiple mechanisms including p53 activation (Jones et al., 2005). A central mitogenic pathway suppressed by AMPK is mammalian Target of Rapamycin complex 1 (mTORC1) pathway, which is inhibited through AMPK phosphorylation of tuberous sclerosis complex 2 (TSC2) and regulatory associated protein of mTOR (raptor). Under glucose-deprived conditions, AMPK will inhibit fatty acid synthesis and promote fatty acid oxidation (Shackelford and Shaw, 2009). Of note, fatty acid oxidation stimulated by AMPK can rescue the glucose dependence of AKT cells as seen by increased cell viability when stimulating fatty acid oxidation under glucose deprivation (Buzzai et al., 2005). These results reinforce the important balance and duality between two key signaling molecules in metabolism: AKT and AMPK. Drugs targeting AMPK, including metformin or AICAR require careful investigations for their effects on tumour growth, as
tumour cells may be able to adapt to their effects on cell cycle and metabolism and result in sustained growth via fatty acid breakdown (Shackelford and Shaw, 2009). AMPK may also modulate glycolytic flux through phosphorylation of PFK2 isoforms, particularly PFKFB3 (Almeida et al., 2004; Bando et al., 2005).

### 1.7.3.6 Role of specific glycolytic enzymes in the Warburg effect

Specific enzymes of the glycolytic pathway have been implicated in establishing the Warburg effect in cancers and are described briefly in this section. Mechanisms underlying a role of specific metabolic enzymes in the Warburg effect may include primary mutations (described above), altered isoform expression profile and altered regulation or function of metabolic enzymes secondary to oncogenic signaling pathways or the tumour microenvironment.

*Pyruvate kinase M2*

PKM is the last irreversible step of glycolysis, catalyzing the reaction of phosphoenolpyruvate (PEP) to pyruvate. Humans contain two PK genes (*PKLR and PKM2*) and four PK isozymes (L, R, M1 and M2). PK-L and PK-R proteins are expressed from alternative *PKLR* promoters in gluconeogenic tissues (liver) and erythrocytes, respectively. PKM1 and PKM2 are alternatively spliced transcripts of the *PKM2* gene. PKM1 is primarily expressed in the brain and muscle while PKM2 is found in proliferating tissues including embryonic tissue and tumour cells (Altenberg and Greulich, 2004a). Cantley and colleagues reported the preferential expression of the embryonic PKM2 splice variant, rather than the adult PKM1 variant, in a panel of cancer cell lines (Christofk et al., 2008a). The tumour specific PKM2 is believed to oscillate between an active tetramer and a less active dimer, regulated by phospho tyrosine binding, leading to the release of its allosteric activator FBP, although the details of this regulation have yet to be deciphered (Christofk et al., 2008b; Mazurek et al., 2005). The decreased activity of the PKM dimer may result in shunting of upstream glycolytic intermediates into biosynthetic pathway, including lipid synthesis, and therefore may not be as efficient in generating ATP but generated precursors (Christofk et al., 2008b). Furthermore, it is not clear how tumour cells generate lactate if glucose is shuttled into
biosynthetic pathways, although anaplerotic reactions may be crucial, including glutaminolysis in which glutamine is metabolized by the citric acid cycle producing NAPDH for lipid synthesis and oxaloacetate for replenishment of Krebs cycle intermediate (Deberardinis et al., 2007).

*Lactate Dehydrogenase A*

Inhibition of LDHA, the final enzyme in glycolysis generating lactate from pyruvate and replenishing NAD+, has been shown to inhibit aerobic glycolysis, by reverting back to OXPHOS and preventing tumour progression of breast cancer cell lines and transformed Rat1a fibroblasts (Fantin et al., 2006; Shim et al., 1997).

*Pyruvate dehydrogenase kinase*

PDH is a mitochondrial multi-enzyme complex that catalyzes the oxidative decarboxylation of pyruvate, whose enzymatic activity is regulated by phosphorylation/dephosphorylation cycle. The mitochondrial matrix protein PDK (PDK1 to 4) is an important inhibitor of OXPHOS via its phosphorylation of the E1 alpha subunit of PDH. HIF1α transactivates PDK1 resulting in decreased conversion of pyruvate to acetyl-coA and compromising OXPHOS (Kim et al., 2006). Treatment of cancer cells with the small molecule inhibitor of PDK dichloroacetate (DCA), currently employed for the treatment of congenital lactic acidosis, was found to activate OXPHOS and promote apoptosis. DCA is believed to sensitize to apoptosis via two mechanisms: 1) enhanced flux of electrons through the ETC resulting in greater depolarization of mitochondrial membrane (usually hyperpolarized in tumour cells) and enhanced release of apoptotic cytochrome c; 2) OXPHOS function generates greater ROS, upregulating voltage-dependent K+ channel leading to an efflux of K+ and activation of caspases (Bonnet et al., 2007).
1.8 The Role of Hexokinase 2 in Mitochondrial Function and Growth

Significant evidence is accruing that the glycolytic enzyme HK2 plays a pivotal role in promoting growth and survival in highly malignant tumour cells including hepatocellular carcinomas (Pedersen et al., 2002), pancreatic cancer (Lyshchik et al., 2007) and gastric carcinoma (Rho et al., 2007), among others. HK isozymes from tumours exhibiting a strong glycolytic phenotype including hepatocellular carcinoma have been cloned and sequenced and found to correspond to insulin-sensitive HK2, found normally in muscle and adipose tissue in low amounts (Thelen and Wilson, 1991). HK1, found at high levels in brain, breast, kidney and retinal tissue is also present in some highly glycolytic tumours but at potentially much lower levels than HK2 (Pedersen et al., 2002). HK2 enhancement in cancers is possible via different mechanisms including gene amplification, increased gene expression, increased translation, post-translational modification and regulation by protein-protein interactions or microRNAs. This section will summarize the existing literature on HK2’s role in cell proliferation and survival as well as its regulation at the transcriptional and protein levels.

1.8.1 The HK2 gene

Sequence analysis of the HK2 gene and promoter from normal hepatocytes and highly glycolytic hepatomas revealed less than 1% difference [GenBank U19605, AY082375] (Pedersen et al., 2002). Tumour cell promoters and the normal cell counterpart have well defined TATA and CAAT boxes for precise transcription initiation (Mathupala et al., 1995). Amplification of the HK2 gene may play a part in its over-expression in malignant hepatomas (Rempel et al., 1996) although amplification has not yet been reported in other cancers.

1.8.1.1 HK2 knockout mouse model

HK2 deficient mice are embryonic lethal, indicating that HK2 is irreplaceable by the other HK isoforms and reinforcing its important role in embryonic development (Heikkinen et al., 1999). Embryonic death at approximately E7.5 of the HK2+/− mice
supports a vital role of HK2 during or after the blastocyst stage but before organogenesis, probably during early gastrulation (Heikkinen et al., 1999). HK2+/- mice were viable and exhibited approximately 50% of the normal levels of HK2 mRNA and activity in adipose, heart, and skeletal muscle. HK2+/- mice showed no phenotypic changes with respect to insulin action or glucose tolerance (Heikkinen et al., 1999). Total HK activity has been reported to increase during compaction when the eight-cell embryo forms a blastocyst (Saito et al., 1994). The timing of this event corresponds to the switch from pyruvate- to glucose-based metabolism in embryos (Gardner and Leese, 1988). This early stage of embryonic development is associated with a highly proliferative state, and thus reliance on aerobic glycolysis may assist in the generation of biosynthetic precursors necessary for cellular proliferation.

1.8.2 Transcriptional regulation of HK2

HK2 is the most regulated form of HK, particularly at the transcriptional level (Lee and Pedersen, 2003; Malkki et al., 1997). Promoter activation likely plays an essential role in HK2 overexpression in cancer cells. Its selective over-expression in contrast to other HK isozymes may be related to its unique response element organization within the proximal region of its promoter. The 4.3 Kbp HK2 promoter has response elements for many molecules that play an important role in tumour growth including insulin growth factor (IGF), glucagon, glucose, cAMP, the phorbol ester TPA, among others (Mathupala et al., 1995; Mathupala et al., 2001). Both wild-type and mutant p53 were reported to activate the HK2 promoter (Mathupala et al., 1997a). HK2 contains HRE’s in its promoter thereby regulated by HIF1α (Denko, 2008; Mathupala et al., 2001). The greatest activation of HK2 in hepatoma cell lines has been reported under hypoxic conditions in the presence of glucose (Mathupala et al., 2001). Both proximal and distal promoter regions were found to be important for this activation via HIF1α. In contrast, although HK1 has a putative HRE, its response under hypoxia is diminished relative to that of HK2 (Kim et al., 2007; Mathupala et al., 2001). C-myc has also been reported to increase transcription of HK2, often in combination with HIF1α, via interaction at its consensus sequences (E-box) on the HK2 promoter (Kim et al., 2007; Kim et al., 2004). Altogether,
the HK2 promoter seems ideally adapted to provide an enhanced response to microenvironmental stimuli, resulting in enhanced HK2 synthesis.

Other potential mechanisms resulting in HK2 over-expression include promoter methylation/demethylation and enhanced mRNA stability. There is some preliminary evidence for both of these events occurring but their relative contribution to HK2 over-expression has yet to be determined (Mathupala et al., 1997b). Bisulfite sequencing of the HK2 promoter in a hepatocyte cell line revealed 18 methylated CpG sites within CpG islands encompassing the transcription start site (-350bp to +781bp) but none in a hepatoma cell line (Goel et al., 2003).

1.8.3 HK2 in bioenergetics and macromolecular synthesis for proliferation

HK2 has both C and N terminal catalytic sites. In contrast, the N terminal of HK1 serves as a regulatory domain while the C terminal has both catalytic and regulatory function (Wilson, 2003). Therefore, it makes intrinsic sense for tumour cells to upregulate the HK isoform with the greatest catalytic potential. Furthermore, the preferential expression of HK2 over HK1 may also be due to their differential regulation at the transcriptional level, protein level and subcellular localization.

The mitochondrial localization of HK2 and HK1 has been extensively studied and is known to be dependent on the first 23 residues in its N terminal hydrophobic tail (Wilson, 2003). The percent of HK that localizes to the mitochondria appears to vary with tissue type and between HK2 and HK1 isoforms, although the exact implications of this is not clear (Pedersen et al., 2002). Binding of HK2 at the OMM is believed to yield many bioenergetic advantages resulting in enhanced glycolytic flux: 1) it reduces product inhibition by G6P (Bustamante and Pedersen, 1977), 2) it yields preferred access to ATP generated by the mitochondria and released by VDAC/porin (Arora and Pedersen, 1988), and 3) it protects against its proteolytic degradation (Rose and Warms, 1982). For HK2 to have greater access to ATP from the mitochondria, this requires cooperation and potentially interaction with at least two other mitochondrial proteins in addition to
VDAC: ANT that transports ATP to HK2/VDAC and the inner mitochondrial protein ATP synthase (complex V) that generates ATP.

Over-expression of HK2 along with its localization to the mitochondria can result in rapid production of G6P, which is a major source of carbon for biosynthetic pathways assisting in proliferation. Although the regulatory mechanisms remain unclear, excess G6P may be shuttled into the PPP to generate ribose for nucleotide synthesis, it can assist in amino acid synthesis, as well as it can undergo glycolysis and enter a truncated TCA cycle for fatty acid synthesis.

The fact that tissues not normally expressing HK2 start expressing it suggests that it must be providing additional advantages that HK1 cannot accommodate. Furthermore, HK2’s advantages may encompass more than just an increase in total hexokinase activity. It has been hypothesized that HK2 compared to HK1 may be shuttling towards more anabolic functions of the cell including PPP and glycogen synthesis (rather than catabolic breakdown of glucose to ATP via OXPHOS), particularly in proliferative tissues. This hypothesis set forth by Wilson is based primarily on the different enzyme kinetics and regulation of HK2 and HK1 with respect to the product Pi. As mentioned earlier, the inhibitory effects of Pi are found to be additive to those of G6P for HK2, while Pi negates G6P inhibition for HK1. Thus, tissues that require a lot of ATP including brain cells will have some G6P and a lot of Pi resulting in greater HK1 activity but less HK2 activity. However, in cancer tissues, incorporation of nutrients into the biomass to assist in proliferation of new cells may be more important than efficient ATP generation. This hypothesis remains to be directly tested.

In addition to a biosynthetic and a proliferative advantage, HK2 expression in cancer cells has been shown to have a direct effect on cell survival via its role at the OMM and interaction with the MTP.

1.8.4 HK2 and cell survival

A greater percentage of HK2 is localized to the mitochondria in transformed cells compared to their normal counterpart (Pedersen et al., 2002). Further \textit{in vitro} and \textit{in vivo}
studies have demonstrated that mitochondrial HKs play a clear role in protecting cells against mitochondrially regulated apoptosis as reported in Rat1a fibroblasts (Gottlob et al., 2001), HeLa cells (Pastorino et al., 2002), NIH-3T3 cells (Fanciulli et al., 1994), WEHI 7.1 cells (Sade et al., 2004) and lung epithelial tumour cells (A549) under hyperoxia (Ahmad et al., 2002).

The survival effects attributed to HK2 may result from one or the combination of the following: a) inhibition of MPTP and cytochrome c release via interaction at VDAC, b) inhibition of OXPHOS which can sensitize cells to Bax-induced apoptosis, c) inhibition of OXPHOS thereby reducing generation of ROS and oxidative stress, and d) increase in NAPDH formation via the PPP resulting in increased anti-oxidant function.

### 1.8.4.1 HK2 and interaction with the permeability transition pore

Focusing on HK’s association with VDAC at the OMM, there are two potential scenarios explaining how HK2 may be exerting its cell survival effects: 1) HK2 competes with binding of pro-apoptotic proteins including Bax/Bak at VDAC and interferes with formation of a conduit and permeability of the OMM necessary for the release of apoptogenic molecules (e.g. cytochrome c) from the IMS; and/or 2) HK2/VDAC interaction could transmit molecular changes to proteins of the IMM (ANT, cyclophilin D) and alter MPTP opening/closure. Opening of the MPTP by loss of HK2 could elicit depolarization, matrix swelling, and consequently cristae unfolding and breaches in the OMM that are pervious to proteins. In support of the first hypothesis, Pastorino and colleagues showed that HK2 binding to the mitochondria results in resistance to Bax-induced release of cytochrome c and apoptosis in HeLa cells (Pastorino et al., 2002). Since the opening of MPTP is reported to be independent of Bax (Majewski et al., 2004a), these early results by Pastorino et al. suggest that mitochondrial HK2’s effects are independent of MPTP. More recently, Pastorino et al. (2005) showed that GSK-3β induced the dissociation of HK2 from the mitochondria by phosphorylating VDAC, thereby promoting apoptosis. Both AKT and GSK3B are known to localize to the mitochondria under certain conditions; GSK3B is phosphorylated by AKT rendering it inactive. There is also evidence in the literature to suggest that HK localization is dependent on intracellular pH (Miccoli et al., 1996).
Other research supports the second proposed mechanism where dissociation of HK from the mitochondria alters MPTP, followed by reduced mitochondrial metabolite exchange culminating in mitochondrial swelling and cell death (Majewski et al., 2004a). PKCε was reported to phosphorylate VDAC in cardiac cells and result in significantly decreased MPTP opening, thereby promoting cell survival (Baines et al., 2003). Chiara and colleagues demonstrated that detachment of HK2 from the mitochondria using clotrimazole or HK2 N-terminal peptide resulted in increased MPTP and cell death but that this surprisingly was not dependent on interactions with VDAC (Chiara et al., 2008). See Table 1-3 for a summary of HK2’s role at the mitochondria with respect to interaction with VDAC and MPTP. Overall, it remains controversial whether mitochondrial binding of HK2 opens, closes or is independent of MPTP and/or VDAC and Bax.

Table 1-3: Summary of the role of HK2 at the OMM and interaction with the MPTP.

Table summarizing literature findings with respect to the role of HK2 in cell survival at the OMM.

<table>
<thead>
<tr>
<th>Author/reference</th>
<th>Bax dependent?</th>
<th>MPTP dependent</th>
<th>VDAC dependent</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gottlob, 2001</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Rat1a fibroblast</td>
</tr>
<tr>
<td>Pastorino, 2002</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>HeLa</td>
</tr>
<tr>
<td>Majewski, 2004</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>MEF, Rat1a fibroblast</td>
</tr>
<tr>
<td>Chiara, 2008</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>HeLa, Jurkat cells</td>
</tr>
</tbody>
</table>
1.8.4.2 HK2 kinase activity in cell survival

Sun and colleagues demonstrated using mutant constructs of HK2 and HK1, including N-terminal truncated (no localization to mitochondria) and mutant kinase constructs, that both glucose phosphorylation and mitochondrial binding are required for the full protective effects of HK1 and HK2 in response to H$_2$O$_2$ treatment (Sun et al., 2008). This would suggest that the cell survival effects of HK are dependent on the presence of glucose (i.e. glucose phosphorylation) and its mitochondrial localization. Furthermore, they showed that HK2 promotes phosphorylation of VDAC by PKC$_\varepsilon$, previously associated with closure of the PTP (Baines et al., 2003).

The presence of glucose on its own may favour HK2’s interaction with the mitochondria, an effect that is reversed upon glucose deprivation (Gottlob et al., 2001). Gottlob et al. showed that the antiapoptotic activity of AKT, unlike that of Bcl-X or Bcl-2, requires the first, rate-limiting step of glucose metabolism catalyzed by HK. Interestingly, their findings demonstrate that 2DG, which is a nonmetabolizable but phosphorylatable form of glucose, is sufficient to confer protection in their model, in which cell death was caused by UV irradiation combined with serum deprivation. However, the nonphosphorylatable and nonmetabolizable form of glucose, 5-thioglucose, could not confer this protection (Gottlob et al., 2001). Other studies have supported the role of the PI3K/AKT pathway in the cell survival effects of HK2 by localizing HK2 to the mitochondria (Majewski et al., 2004b). Miyamoto and colleagues showed in cardiomyocytes that AKT phosphorylates HK2 directly, promoting its mitochondrial localization (Miyamoto et al., 2008), although these results have yet to be reproduced in a cancer setting. One study found that HK2 can be induced in primary brainstem neurons with chronic inhibition of GSK3 providing a protective effect to the mitochondrial inhibitor rotenone (Gimenez-Cassina et al., 2009). Chronic inhibition of GSK3 led to increased lactate production associated with both increased expression of HK2 and its localization to the mitochondria. Of importance, they saw no changes in expression or localization of HK1 (Gimenez-Cassina et al., 2009).
1.8.4.3 Anti-oxidant role of HK2

The anti-oxidant effects of mitochondrial HKs may be exerted potentially through a couple of mechanisms: 1) Increased shuttling to the PPP resulting in NAPDH production, which is required to generate the reduced form of glutathione, a major intracellular defense against damage mediated by ROS. 2) Direct interaction with mitochondria and impact on ADP recycling and mitochondrial function (da Silva et al., 2004).

Mitochondrial ROS generation is associated with elevated mitochondrial membrane depolarization ($\Delta \psi$) (Korshunov et al., 1997), which can trigger early events of apoptosis (Fujimura et al., 1999). Mitochondrial bound HK was found to specifically decrease production of $H_2O_2$ and thus mitochondrial function. Using HK-depleted mitochondria, incubation with a yeast HK isoform that is unable to bind to VDAC resulted in much less efficient reduction in rate of $H_2O_2$ generation (da Silva et al., 2004). The effects of mitochondrial HK on ROS generation were found to be dependent on proper kinase activity as well as interaction with VDAC, maintaining a reduced state of mitochondrial membrane depolarization by promoting ADP cycling with the F1F0 ATP complex and VDAC/ANT exchanger (da Silva et al., 2004).

1.8.5 HK2 and sensitization to radiation and chemotherapy

Disruption of the binding of HK2 to VDAC has been shown previously to potentiate chemotherapy-induced cytotoxicity in certain cancer cells. Inhibition of AKT activity has been reported to potentiate chemotherapeutic cytotoxicity (e.g. doxorubicin, paclitaxel) via effects on GSK3$\beta$ activation and release of HK2 from the mitochondria in HeLa cells (Pastorino et al., 2005). Furthermore, specific inhibition of HK2 binding to mitochondria using a N-terminal HK2 peptide, even upon GSK3$\beta$ inhibition, also potentiated the cytotoxicity to chemotherapeutic agents in HeLa cells (Pastorino et al., 2005). Studies looking at the effect of loss of HK2 in sensitizing GBM cells to apoptosis are limited.

Drugs that broadly target hexokinase activity and other metabolic processes have also been shown to be effective in combination with chemotherapies or specific genetic backgrounds. For example, 2DG in combination with wild-type p53 over-expression was reported to enhance cytotoxicity in human prostate cancer cells via oxidative stress
(Ahmad et al., 2008). Similarly, 2DG in combination with cisplatin was reported to be more effective in head and neck cancer (Simons et al., 2007). 2DG was also reported to act synergistically with specific chemotherapeutic agents, particularly those causing DNA damage, in causing cell death in breast cancer cells (Zhang and Aft, 2009). 3-bromopyruvate, a lactate/pyruvate analog that inhibits hexokinase selectively, depletes ATP and induces cell death, was reported to potentiate the effects of low dose platinums in colon cancer cells independent of p53 status (Ihrlund et al., 2008).

1.8.6 HK2/metabolism and tumour initiation

Although metabolic remodeling is conventionally associated with tumour progression and adaptation to the tumour microenvironment, there is evidence that alterations in metabolic enzymes may play an important role in tumour initiation. Enhanced glycolytic flux and mitochondrial HK activity were found to be early events in transformation of chicken embryo fibroblasts using a Rous sarcoma virus mutant (Singh et al., 1974). Recent results from high throughout screens of GBM samples revealed mutations in the enzyme IDH1 in a large percentage of low grade gliomas and secondary GBMs, suggesting mutation of this enzyme is important for the initiation of these tumours (Parsons et al., 2008; Yan et al., 2009).

1.8.7 HK2 as a cancer prognostic marker

HK2 has been found to predict poor patient outcome in hepatocellular carcinoma (Peng et al., 2008b), pancreatic cancer (Lyshchik et al., 2007) and gastric carcinoma (Rho et al., 2007). Few articles have looked at the expression of HK2 in GBMs and whether it is prognostic. More recently, a study by David Louis’s group found that HK2 gene expression in perinecrotic cells isolated by laser capture microdissection was a negative prognostic indicator of overall outcome in GBM patients (Dong et al., 2005).

Although HK2 provides many advantages similarly attributed to the Warburg effect, such as promoting proliferation and survival, there is very little literature exploring the role of HK2 in establishing or promoting the Warburg effect, particularly for GBMs.
1.9 The Warburg Effect in Glioblastoma Multiforme

The preferential glycolytic phenotype is present across many cancer types (Altenberg and Greulich, 2004b) and has been reported in brain tumours as well (Elstrom et al., 2004; Floridi et al., 1989; Macbeth and Bekesi, 1962; Oudard et al., 1996). The most poorly differentiated tumours have been shown to exhibit the fastest proliferation and the highest glycolytic rates (Pedersen, 1978). GBMs are reported to upregulate glycolysis more than 3 times that of normal brain tissue, measured by increased lactate:pyruvate ratio (Oudard et al., 1996).

Using stereotactic microdialysis, one study measured products of glucose metabolism and glutamate and glycerol levels, reflecting neuronal function, in adjacent brain and GBM tissues identified by contrast enhancing on MRI and pathologically verified (Tabatabaei et al., 2008). They measured values at baseline and after 5 days (total 10 Gray of radiotherapy). Baseline levels of glucose (2.9 mM brain vs 1.17 mM tumour) and pyruvate (146uM vs 107uM) were lower in tumour compared to adjacent brain. The ratio of lactate/pyruvate was higher in tumour tissue compared to adjacent brain. There were no significant changes reported in glycolytic metabolites after 5 days of radiotherapy (Tabatabaei et al., 2008).

The molecular basis of aerobic glycolysis likely varies across cancers depending on the genetic background and the tumour microenvironment. Only in the past year have advances in the molecular basis of altered metabolism been made in GBMs with the identification of mutant IDH1 (and IDH2 to a lesser extent) particularly in low grade and secondary GBMs. The drivers of this switch in primary GBMs are still under investigation.

1.9.1 Heterogeneity in glycolytic metabolism and mitochondrial function in GBMs

Decreased mitochondrial numbers, altered mitochondrial structure and decreased expression of mitochondrial genes have been reported in GBMs along with other cancers (Dmitrenko et al., 2005; Ristow, 2006; Tani et al., 1971). However, other reports
demonstrate large variability in GBMs with respect to mitochondrial respiration and glucose dependency (Griguer et al., 2005; Lichtor and Dohrmann, 1986; Turcotte et al., 2002). Using cell lines derived from human GBM tissue and human GBM xenografts, regional heterogeneity of oxygen consumption in GBMs was prevalent; furthermore, modulation of mitochondrial respiration was found to be an important component of the ability of cells to survive hypoxic conditions (Allalunis-Turner et al., 1999; Franko et al., 1998; Parliament et al., 1997). Mutations at the mitochondrial DNA level have also been theorized to promote glioma tumourigenesis (Shidara et al., 2005).

GBMs are pathologically heterogeneous tumours with regions of pseudopalisading perinecrotic cells under moderate levels of hypoxia (pO2 = 2.5-5%) and infiltrating regions within normal brain under relative normoxia (pO2 = 10%) (Evans et al., 2004). HIF1α levels are stabilized within perinecrotic pseudopalisading cells (Brat et al., 2004; Kaur et al., 2005) but have also been reported stabilized under normoxic conditions in GBMs due to PTEN loss and enhanced mTOR signaling (Denko, 2008). Associated with increased HIF1α is upregulation of many of the enzymes of glycolysis (Denko, 2008). There is also burgeoning research investigating the cross-talk between stromal cells and tumour cells with respect to their metabolism. Although not specific to GBMs, there is a strong contingent of research supporting the generation of lactate from stromal tissues that can then be imported into tumour cells by over-expressed lactate transporters and then used as fuel for metabolism (Pavlides et al., 2009). It has been hypothesized that lactate produced by anaerobic glycolysis within hypoxic regions can be used as an oxidative substrate in another region where oxygen supply is higher (Griguer et al., 2005). In glycolytic dependent GBM cell lines, the LDHB isoform, converting lactate to pyruvate, was found to be expressed to a greater degree than LDHA, which would be expected if extracellular lactate were to fuel cell activities (Griguer et al., 2005). Interestingly, in a recent report comparing the expression profile of primary breast cancers with unlinked brain metastasis, HK2 was found to be consistently upregulated in the brain metastatic tumours. This confirms that there are important microenvironmental influences or interactions perhaps unique to the brain that may be promoting HK2 expression and tumour growth (Palmieri et al., 2009).
Although glycolysis is often associated with hypoxic conditions, gliomas are known to exhibit high rates of aerobic glycolysis, resulting in increased lactic acid production, even in the presence of normal oxygen levels (Mangiardi and Yodice, 1990; Ziegler et al., 2001). This suggests that not only the microenvironment is playing a role in enhanced glycolytic rates but that other factors are contributing as well.

### 1.9.2 Metabolism and oncogenic signaling pathways in GBMs

Growth factor signaling also plays an important role in promoting the Warburg effect, particularly in GBMs. As mentioned previously, the PTEN/PI3K/AKT signaling pathway has multiple effects on glycolysis including enhanced glucose uptake (upregulation and translocation of GLUTs to plasma membrane) and increased glycolytic flux by regulating mitochondrial HK and PFK (Gottlob et al., 2001; Manning and Cantley, 2007). Glucose withdrawal induces extensive apoptosis in GBM cells but not in normal astrocytes (“glucose addicted”) (Jelluma et al., 2006). ATP levels are sustained with glucose withdrawal due to the cell’s compensatory elevation of fatty acid oxidation and respiration. However, this results in increased oxidative stress generated in the mitochondrial respiratory chain and ensuing GBM cell death (Jelluma et al., 2006). GBM cells’ addiction to glucose and the rate of aerobic glycolysis is attributed to AKT activity (Elstrom et al., 2004). Constitutive AKT in GBM cells results in resistance to the in vivo growth suppressive effects of dietary restriction but sensitization if AKT is hyperactivated but not constitutive (Kalaany and Sabatini, 2009). The ability of AKT to enhance the rate of glycolysis is also due in part to its ability to promote the expression of glycolytic enzymes through HIFα (Lum et al., 2007b; Semenza et al., 1994).

Other growth factor signaling pathways including Ras/Raf/MAPK have also been implicated in glucose metabolism in GBM cells. Although oncogenic Ras mutations are not prevalent in human gliomas, studies have revealed abundant presence of Ras-GTP in these tumours (Guha et al., 1997), partly arising from deletions of NF1 tumour suppressor in a subset of GBMs (Cancer Genome Atlas Research, 2008) and from enhanced growth factor signaling downstream of EGFR, VEGF and PDGF. Use of the Ras inhibitor trans-
farnesylthiosalicylic acid (FTS) resulted in decreased expression of HIF1α and shut down of glycolysis, resulting in energy crisis and cell death (Blum et al., 2005).

It is important to note that in addition to glucose metabolism, glutamine metabolism is strongly associated with the Warburg effect as well. Glucose consumption is believed to promote tumour cell proliferation via the rapid synthesis of macromolecules including lipids, proteins, and nucleotides and the secretion of lactate. However, using 13C NMR spectroscopy, it was shown that GBM cells, particularly driven by oncogenic myc, undergo glutamine metabolism at a high rate as well, resulting in 1) conversion of glutamine to lactate (glutaminolysis) with rapid production of NADPH sufficient enough to support fatty acid synthesis, 2) synthesis of anaplerotic oxaloacetate for continued TCA cycle function and suppression of pyruvate carboxylation despite adequate mitochondrial pyruvate metabolism (Deberardinis et al., 2007). Glutamine catabolism was accompanied by secretion of alanine and ammonia, such that most of the amino groups from glutamine were lost from the cell rather than employed for nucleotide synthesis, protein synthesis or glucosamine, with which it is traditionally associated. To summarize, glutamine metabolism provides a carbon source that assists the cell in using glucose-derived carbon and TCA cycle intermediates as biosynthetic precursors particularly for fatty acid synthesis (Deberardinis et al., 2008a; Deberardinis et al., 2007). The close coordination and integration of glucose metabolism, glutamine metabolism, and oncogenic signaling in GBM cells was further recognized in a recent study showing that glucose deprivation does not affect net glutamine utilization but elicits a switch in utilization of glutamine to produce OAA, pyruvate, and acetyl-CoA (rather than just OAA) thereby maintaining the TCA cycle function in the absence of glucose. This was associated with a large increase in the activity of glutamate dehydrogenase (GDH). The effect on GDH from glucose withdrawal could be mimicked with the glycolytic inhibitor 2DG and reversed with a pyruvate analogue. Furthermore, inhibition of Akt signaling similarly increased GDH activity whereas overexpression of Akt suppressed it, suggesting that Akt indirectly regulates GDH through its effects on glucose metabolism (Yang et al., 2009). Inhibition of GDH activity limits the use of glutamine carbon in the TCA cycle resulting in increased sensitization of GBM cells to glucose withdrawal (Yang et al., 2009).
In addition to the importance of growth factor signaling and constitutive AKT in promoting aerobic glycolysis in GBMs, more recently was the identification of mutations in the IDH1 and IDH2 metabolic enzymes, providing a novel molecular mechanism for the Warburg effect in at least a subset of GBMs.

1.9.3 IDH1 and IDH2 mutations in GBMs

Mutation of the IDH1 and IDH2 metabolic enzymes has been found to be a very frequent and early genetic alteration in astrocytomas and oligodendrogliomas (Parsons et al., 2008; Yan et al., 2009), and more recently in Acute Myelogenous Leukemia (AML) (Mardis et al., 2009). This mutation was identified at residue R132 in IDH1 (and analogous R172 in IDH2) located within the isocitrate (substrate) binding site, and is commonly mutated to histidine (R172H). The somatic mutation of R132 residue has been reported to occur in greater than 80% of grades II and III astrocytomas and oligodendrogliomas as well as secondary GBMs that develop from these lower grade lesions (Balss et al., 2008; Hartmann et al., 2009; Ichimura et al., 2009; Parsons et al., 2008; Watanabe et al., 2009; Yan et al., 2009). Mutation analysis of IDH2 revealed somatic mutations at the analogous residue R172 with most mutations occurring in tumours that lacked IDH1 mutations (Hartmann et al., 2009; Yan et al., 2009). This mutation occurs in only a single allele (i.e. heterozygotic). Watanabe and colleagues reported that IDH1 mutations always preceded acquisition of TP53 mutations (in astrocytomas) and loss of 1p/19q (in oligodendrogliomas). These results suggest that IDH mutations are early genetic events from a cell of origin that can give rise to both astrocytes and oligodendrocytes. Clinically, patients with IDH mutations are significantly younger and have a median overall survival of 31 months compared to 15 months in patients with wild-type IDH (Yan et al., 2009). However, IDH mutations have not been reported in pediatric GBMs. Multivariate analysis confirmed that IDH1 mutations are independent favourable prognostic indicator even when adjusting for age, MGMT status, genomic profile and treatment (Sanson et al., 2009). As a whole, it appears that IDH1 and IDH2 mutations may define a specific subtype of disease with a specific pattern of genetic mutations (e.g. TP53) not seen in primary GBMs (e.g. PTEN, EGFR, CDKN2A/CDKN2B).
*IDH1* mutation was found to impair the ability of IDH1 to convert isocitrate to αKG (Yan et al., 2009; Zhao et al., 2009). This was shown to result in decreased generation of the product αKG and subsequent stabilization of HIF1α (Zhao et al., 2009). It was then later shown that *IDH1* mutations result in the new ability of the enzyme to catalyse the NADPH-dependent reduction of αKG to R(-)-2-hydroxyglutarate (2HG) (Dang et al., 2009). 2-HG metabolite levels were found to be elevated in glioma specimens (Dang et al., 2009). A few mechanisms have been proposed regarding the tumourigenic potential of a gain of function ability of IDH1 to produce 2HG: 1) increased ROS levels due to decreased NADPH, 2) toxicity from competitively inhibiting αKG and glutamate using enzymes including a) transaminases necessary for utilization of glutamate nitrogen for amino and nucleic acid biosynthesis, b) prolyl hydroxylases that regulate HIF1α. To date, it remains unclear how 2HG accumulation promotes the Warburg effect in GBMs. Clinically, 2HG levels (potentially in serum) may help identify patients with IDH1 mutations, known to differ in prognosis. These findings may also provide another therapeutic approach by targeting 2HG production and potentially slowing down the conversion of a low-grade glioma to GBM.

Mutations in IDH1 and IDH2 provide a potential molecular basis of altered metabolism in low-grade gliomas and secondary GBMs. However, more than 90% of GBMs are primary GBMs and the molecular basis of the Warburg effect in this subset of GBMs remains to be elucidated. The dysregulation of specific metabolic enzymes including HK2 may play an important role in promoting aerobic glycolysis in this subset of GBMs.

### 1.9.3.1 The role of HKs in GBMs

It was previously reported that a high percentage of GBMs do not express normal brain HK1 since it is located on 10q22 and may be lost in a subset of GBM patients that have LOH/deletion of 10q (Oudard et al., 1996). This same group attempted to elucidate the reasons for glycolytic deviation commonly found in brain tumours by measuring total HK activity, mitochondria-HK binding, OXPHOS and mitochondrial ultrastructure in 4 human xenografted gliomas (Oudard et al., 1997). They reported that lactate:pyruvate ratios were increased 3-4 fold, yet HK activity was 2-4 fold lower than that of normal rat
brain tissue. The mitochondrial HK fraction varied considerably across the four xenografts and represented 9 to 69% of the total HK of normal rat brain. The mitochondrial respiratory activity, assessed by polarography and spectrophotometry, was within the normal range. However, they reported that mitochondrial “content” of gliomas was lower than in the rat normal brain tissue, as revealed by the markedly decreased expression of cytochrome c oxidase and citrate synthase. Electron microscopy studies revealed profound alterations of mitochondrial ultrastructure, namely of cristae and matrix densities. However, this study only examined 4 xenografts and did not quantify their electron microscopy studies. They concluded from their results that, because of the very low content of normally functioning mitochondria (despite reports that respiration remained unchanged), gliomas shift their energy metabolism towards a high-level glycolysis to maintain cellular ATP supply.

The studies of Oudard and colleagues did not look at the expression of other HK isoforms including HK2 in GBMs. Few studies have explored HK2 expression in GBMs, forming part of this thesis’ objectives. However, over the course of this PhD, several high throughput screens have been conducted and whose data are accessible. These resources include 1) An integrated genome analysis of 22 human GBMs including sequencing of 20,661 protein coding genes, determination of the presence of amplifications and deletions using high-density oligonucleotide arrays, and measuring gene expression analyses using Serial Analysis of Gene Expression (SAGE) (Parsons et al., 2008), 2) Results from gene expression profiling of large set of GBMs by the TCGA. These resources were mined and the relative expression of HK2 and HK1 are reported in Figure 1-9. Noteworthy, Oudard and colleagues reported that total HK activity was reduced in their glioma xenografts compared to normal brain. This implies that expression of HK2 in GBMs may be advantageous over and above general HK activity. This thesis will attempt to explore the functional relevance of expression of HK2 in GBMs and whether it is of prognostic value.
Figure 1-9: Mining of Integrated Genome Analysis and TCGA arrays for expression of HK2 and HK1 in GBMs.

a) Data mining of results of SAGE analysis performed on 16 GBM xenografts and 2 normal brains. Data is accessible online through the Science website (Parsons et al., 2008). b) Mining of TCGA datasets (2 different microarray platforms) demonstrates increased expression of HK2 in more than 60% GBM samples while HK1 tends to be decreased relative to normal brain (data accessed March 2010). The TCGA portal data browser is accessible online at cancergenome.nih.gov.
### a

<table>
<thead>
<tr>
<th>Tumor</th>
<th>HK1</th>
<th>Fold Diff</th>
<th>HK2</th>
<th>Fold Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB1</td>
<td>95</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>NB2</td>
<td>144</td>
<td></td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Br01X</td>
<td>195</td>
<td>1.64</td>
<td>147</td>
<td>5.88</td>
</tr>
<tr>
<td>Br02X</td>
<td>74</td>
<td>0.62</td>
<td>171</td>
<td>6.84</td>
</tr>
<tr>
<td>Br05X</td>
<td>60</td>
<td>0.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Br07X</td>
<td>84</td>
<td>0.70</td>
<td>60</td>
<td>2.4</td>
</tr>
<tr>
<td>Br10PT</td>
<td>2</td>
<td>0.02</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Br13X</td>
<td>44</td>
<td>0.37</td>
<td>315</td>
<td>12.6</td>
</tr>
<tr>
<td>Br14X</td>
<td>26</td>
<td>0.22</td>
<td>11</td>
<td>0.44</td>
</tr>
<tr>
<td>Br15X</td>
<td>24</td>
<td>0.20</td>
<td>242</td>
<td>9.68</td>
</tr>
<tr>
<td>Br16X</td>
<td>20</td>
<td>0.17</td>
<td>21</td>
<td>0.84</td>
</tr>
<tr>
<td>Br17X</td>
<td>31</td>
<td>0.26</td>
<td>11</td>
<td>0.44</td>
</tr>
<tr>
<td>Br18X</td>
<td>21</td>
<td>0.18</td>
<td>96</td>
<td>3.84</td>
</tr>
<tr>
<td>Br19X</td>
<td>6</td>
<td>0.05</td>
<td>118</td>
<td>4.72</td>
</tr>
<tr>
<td>Br20PT</td>
<td>12</td>
<td>0.10</td>
<td>124</td>
<td>4.96</td>
</tr>
<tr>
<td>Br23PT</td>
<td>23</td>
<td>0.19</td>
<td>122</td>
<td>4.88</td>
</tr>
<tr>
<td>Br25X</td>
<td>24</td>
<td>0.20</td>
<td>34</td>
<td>1.36</td>
</tr>
<tr>
<td>Br26X</td>
<td>21</td>
<td>0.18</td>
<td>72</td>
<td>2.88</td>
</tr>
</tbody>
</table>

~ 0.35 ~4

### b

**Ratio of tumor samples with 2 fold greater HK2 expression relative to normal brain:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>broad.mit.edu</th>
<th>unc.edu</th>
<th>Gene Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>143/236</td>
<td>163/266</td>
<td>Chr 2: 74913289 - 74973989</td>
</tr>
</tbody>
</table>

**Ratio of tumour samples with 2 fold decreased HK1 expression relative to normal brain:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>broad.mit.edu</th>
<th>unc.edu</th>
<th>Gene Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK1</td>
<td>206/236</td>
<td>216/266</td>
<td>Chr 10: 70699761 - 70831643</td>
</tr>
</tbody>
</table>
1.10 Therapeutic Targeting of Metabolism in GBMs and Other Cancers

Due to the infiltrative nature of GBMs, complete surgical removal of tumour is not possible. Consequently, GBMs inevitably recur. For therapies to be effective, this would necessitate targeting both proliferating and invading cells with minimal toxicity to normal brain. Targeting the metabolic reprogramming of cells and particularly their requirement for aerobic glycolysis may constitute of such an approach.

Increasing tumourigenicity was demonstrated to correlate with greater sensitivity to glycolytic inhibition (Ramanathan et al., 2005). Any number of steps in glycolytic metabolism can act as potential therapeutic targets in cancer with varying extent of side effects. Table 1-4 lists various drugs that target metabolic enzymes and have been investigated for their potential effectiveness in the treatment of various cancers. The higher potential across the inner mitochondrial membrane of carcinoma cells serves as a selective driving force for the accumulation of toxic delocalized lipophilic cations like dequalinium, MKT-077, and F16 (Fantin and Leder, 2006; Modica-Napolitano and Aprille, 2001).

Evidence suggests that inhibitors of glucose uptake and/or metabolism may have particular therapeutic efficacy in cells over-expressing AKT, as in GBMs. Since constitutive AKT results in glucose dependency and inability to employ other resources for energy such as fatty acid synthesis (Buzzai et al., 2005; Elstrom et al., 2004), these cells will be particularly sensitized to glucose inhibitors while sparing non-transformed proliferating cells and quiescent cells that have normal ability to use alternative substrates. 2-deoxyglucose (2DG) antagonizes the high glucose dependency of tumours by partially inhibiting glucose uptake and preventing metabolism of G6P. It has been evaluated in vitro as a potential novel chemotherapeutic approach with effectiveness in breast and head and neck cancers (Aft et al., 2002; Simons et al., 2007). 3-bromopyruvate (3Br) is a non-metabolizable analog of pyruvate, which inhibits mitochondrial binding of HK2 (and likely HK1) and mitochondrial ATPase function (Ko et al., 2001). It has been found effective in inducing apoptosis in hepatocellular carcinomas in vitro and in vivo.
Lonidamine, which targets the interaction of HK with mitochondria, was reported to potentiate chemotherapy in recurrent malignant gliomas and in brain metastasis although it does not appear as effective as a radiation enhancer. Lonidamine is not specific to HK2 and the side effects reported included myalgias, testicular pain and ototoxicity with no serious organ toxicity or myelosuppression (Schiffer et al., 1991). Anti-fungals including bifanazole and clotrimazole have been shown to selectively detach HK2 from the mitochondrial membrane without affecting its kinase activity (Majewski et al., 2004a; Pastorino et al., 2002). However, nonspecific respiratory side effects have been described for clotrimazole (Chiara et al., 2008). Selective targeting of HK2 may have dual effects on cells by inhibiting glycolysis and promoting OXPHOS as well as dissociating the anti-apoptotic interaction between HK2 and VDAC. Newer agents are being developed to interfere more selectively the interaction between HK2 and VDAC (e.g. methyl jasmonate) (Goldin et al., 2008), however these are still in the preclinical setting. To date, no specific inhibitor of HK2 has been developed.

The results of this thesis support the belief that HK2 may be a particularly attractive target in GBMs with little toxicity since it has limited expression in normal brain. This will require selective targeting of HK2, without effects on the normal brain isoform HK1. Furthermore, the combination of inhibiting HK2 along with classical and novel chemotherapeutic agents may prove the most effective.
Table 1-4: Potential metabolic targets for the treatment of cancer.

This table lists drugs and their targets that have been investigated for their effectiveness in various cancer settings along with accompanying references.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-deoxyglucose</td>
<td>Glucose uptake</td>
<td>Inhibition</td>
<td>Simons et al. 2007</td>
</tr>
<tr>
<td>3-bromopyruvate</td>
<td>HK1, HK2</td>
<td>Inhibition</td>
<td>Kim et al., 2007, 2008,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pedersen et al. 2007</td>
</tr>
<tr>
<td>Lonidamine</td>
<td>HK1, HK2</td>
<td>Translocation to mitochondria</td>
<td>Schiffer et al., 1991</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>HK1, HK2</td>
<td>Translocation to mitochondria</td>
<td>Pastorino et al., 2002</td>
</tr>
<tr>
<td>Methyl jasmonate</td>
<td>HK1, HK2</td>
<td>inhibit HK2/VDAC interaction</td>
<td>Goldin et al., 2008</td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>PDK</td>
<td>Inhibition</td>
<td>Bonnet et al., 2007</td>
</tr>
<tr>
<td>somatostatin, TT-232</td>
<td>PKM2</td>
<td>Translocation to nucleus</td>
<td>Stetak et al., 2007</td>
</tr>
<tr>
<td>SB-2039990</td>
<td>ACL</td>
<td>Inhibition</td>
<td>Hatzivassillou et al., 2005</td>
</tr>
<tr>
<td>Soraphen A</td>
<td>ACC</td>
<td>Inhibition</td>
<td>Beckers et al., 2007</td>
</tr>
<tr>
<td>Cerulen, C57</td>
<td>FASN</td>
<td>Inhibition</td>
<td>Wang et al., 2005</td>
</tr>
<tr>
<td>MN58b</td>
<td>Choline Kinase</td>
<td>Inhibition</td>
<td>Al-Haffar et al., 2009</td>
</tr>
<tr>
<td>Echinomycin</td>
<td>HIF1</td>
<td>Inhibition of DNA binding</td>
<td>Kong et al., 2005</td>
</tr>
<tr>
<td>N-acetylcysteine, Vit C</td>
<td>ROS</td>
<td>Inhibition, reduce HIF1</td>
<td>Gao et al., 2007</td>
</tr>
<tr>
<td>Tirapazamine</td>
<td>Hypoxia</td>
<td>Cytotoxic in hypoxic cells</td>
<td>Britzel &amp; Esclamado, 2006</td>
</tr>
<tr>
<td>MKT-077, F16</td>
<td>Mitochondria</td>
<td>Toxic lipophilic cations</td>
<td>Fantin et al., 2006, Modica-</td>
</tr>
<tr>
<td>Cariporide</td>
<td>Na+/H+</td>
<td>Inhibition</td>
<td>Pouyssegur et al., 2006</td>
</tr>
<tr>
<td></td>
<td>exchanger</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3705</td>
<td>Bicarb/Cl-</td>
<td>Inhibition</td>
<td>Pouyssegur et al., 2006</td>
</tr>
<tr>
<td></td>
<td>exchanger</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-cyano-4-OH-cinnamate</td>
<td>MCT1</td>
<td>Inhibition</td>
<td>Pouyssegur et al., 2006</td>
</tr>
<tr>
<td>Sulfonamide indisulam</td>
<td>CA9, CA12</td>
<td>Inhibition</td>
<td>Thiry et al., 2006</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>ATP synthase</td>
<td>Inhibition</td>
<td>Chi et al., 2007</td>
</tr>
<tr>
<td>Metformin</td>
<td>AMPK</td>
<td>Activator</td>
<td>Evans et al., 2006</td>
</tr>
<tr>
<td>2-aminobicyclo-heptane</td>
<td>LAT</td>
<td>Inhibition</td>
<td>Nawshiro et al., 2006</td>
</tr>
</tbody>
</table>
1.11 Thesis Objectives

The overall hypothesis of this thesis is that HK2 is a key mediator of the Warburg effect in GBMs, thereby providing a proliferative and survival advantage to tumour cells, particularly within harsher microenvironments. A corollary of this hypothesis is that HK2 has a central role in resistance of GBMs to standard therapies including radiation and chemotherapy. The following three objectives were addressed in this thesis, relating to the expression, regulation and function of HK2 in GBMs.

**Objective 1:** To determine the expression profile of HK2, including regional heterogeneity in expression, in normal brain, low grade astrocytomas and highly proliferative tissues including GBMs and fetal tissue. Furthermore, we wished to determine the prognostic significance of HK2 in GBMs.

**Objective 2:** To examine whether there is a role of epigenetic regulation of HK2 in fetal brain, adult normal brain and GBMs.

**Objective 3:** To determine the impact of loss or gain of HK2 in GBM cells on tumor metabolism, proliferation, survival, *in vivo* growth and sensitivity to apoptosis.

**Objective 4:** To explore mechanisms of HK2’s growth promoting effects and impact on metabolism, in contrast to alterations of other glycolytic enzymes HK1 or PKM2.

Results from this thesis provide novel insights into the role of HK2 in aerobic glycolysis, proliferation and cell survival in GBMs.
2.1 Abstract

Proliferating embryonic and tumour tissues are thought to rely on aerobic glycolysis, or the metabolism of glucose to lactate under oxygenated conditions, to assist in the synthesis of biosynthetic precursors necessary for proliferation. The preference for aerobic glycolysis may be mediated by the expression of specific metabolic enzymes or isoforms. Mammalian Hexokinases HK2 and HK1 are 100kDa proteins that phosphorylate glucose to glucose-6-phosphate as the first step of the glycolytic pathway. In normal adult tissues, HK1 is ubiquitously expressed but is particularly prominent in the brain and kidney. HK2 is generally expressed at low levels within adipose tissue and skeletal tissue and negligently in normal brain. In this study, we wished to determine the ontogeny of these HK isoforms within the developing embryo and post-natal Central Nervous System (CNS) and how their expression relates to the extent of aerobic glycolysis in Glioblastoma Multiforme (GBM), a highly aggressive malignant brain tumour. Mining of existing published microarray data found expression of HK1 in 1, 2, 4 cell stage but a switch to stronger expression of HK2 in the blastocyst stage, previously reported to rely heavily on aerobic glycolysis. By means of quantitative RT-PCR on tissues extracted at E8.0, E10.5, E15.5, postnatal day 1, day 20 and 2 months, we demonstrate an inverse temporal relationship in which HK2 was strongest at early embryonic timepoints while HK1 expression increased with CNS maturation. Using a panel of GBM cell lines with differing levels of aerobic glycolysis, we demonstrate that HK2 but not HK1 expression was higher in GBM cells that had low \( O_2 \) consumption and high extracellular lactate levels, supporting the association between HK2 and aerobic glycolysis. As HK2 expression is nearly silent in adult brain but expressed in embryonic tissue and GBM cells, we hypothesized that DNA methylation/demethylation events may be playing important in its regulation. Adult normal human brain and GBM cell lines that had no HK2 expression were found to be methylated at CpG islands within intron 1 on
bisulfite sequencing in contrast to fetal brain and HK2-expressing GBM cells. The degree of methylation correlated with transcript expression in GBM cell lines. 5-aza-2-deoxycytidine treatment of U343 GBM cells, which do not express HK2, resulted in restoration of HK2 transcript expression, supporting its regulation epigenetically. Overall, our results demonstrate that the expression of the HK2 isoform, in contrast to HK1, may be particularly important in tissues relying on aerobic glycolysis for proliferation including embryonic tissue and GBM cells.

2.2 Introduction

Embryonic development is characterized by increased metabolic demand necessary for sustained growth, differentiation, and metabolism of fetal tissues. Developing embryonic tissues are believed to rely on aerobic glycolysis, or the metabolism of glucose to lactate under oxygenated conditions, to assist in the synthesis of biosynthetic precursors necessary for proliferation (Gardner and Leese, 1988; Ramalho-Santos et al., 2009). Similarly, proliferating tumour cells have been hypothesized to alter their metabolism and expression profile back to a more embryonic state (Christofk et al., 2008a; Vander Heiden et al., 2009). Expression of specific enzyme isoforms found in the developing embryo but repressed in normal adult tissue are re-expressed in proliferating tumours. It was demonstrated that proliferating tumour tissues including breast cancer cells express the embryonic PKM2 splice isoform, and not the adult PKM1 isoform, and that this was associated with a greater dependence on aerobic glycolysis, or the metabolism of glucose to lactate, rather than oxidative phosphorylation (OXPHOS)(Christofk et al., 2008a; Mazurek et al., 2005). Otto Warburg first reported more than a half century ago that tumour cells rely on aerobic glycolysis rather than OXPHOS to generate ATP, a phenomenon referred to as the “Warburg effect” (Warburg, 1956). This phenotype may provide several advantages to tumour cells including promoting a state of apoptosis resistance (Kroemer and Pouyssegur, 2008; Plas and Thompson, 2002) and increased invasive ability (Stern et al., 2002). Furthermore, it may assist in the generation of precursors including nucleic acids and fatty acids essential for proliferation of tumour cells, similar to embryonic tissue (Vander Heiden et al., 2009).
Glioblastoma Multiforme (GBM) is the most aggressive adult primary brain tumour that remains resistant to standard therapeutic strategies (Kleihues et al., 2000). These tumours are very heterogeneous pathologically but have regions dependent on glycolysis with the generation of lactate (Di Costanzo et al., 2008; Oudard et al., 1996; Tabatabaei et al., 2008). The signaling pathways and metabolic enzymes necessary for the dependence on aerobic glycolysis are only now being investigated. Mutations of the IDH1 and IDH2 metabolic enzymes have been found to be a very frequent and early genetic alteration in astrocytomas and oligodendrogliomas and secondary GBMs arising from their progression (Parsons et al., 2008; Yan et al., 2009). However, more than 90% of GBMs are primary GBMs and the molecular basis of the Warburg effect in this subset of GBMs remains to be elucidated. A better understanding of the enzymes necessary for embryonic development and aerobic glycolysis may shed light on similarly essential enzymes in GBM tumourigenesis. The glycolytic enzyme Hexokinase (HK) catalyzes the first step in glucose metabolism, which is the ATP-dependent conversion of glucose to glucose-6-phosphate. This reaction serves as the entry point for glucose into glycolysis, glycogen synthesis, and the pentose phosphate pathway. Four isoforms of HK have been identified in mammalian tissues including HK1–3 (100 kDa) and HK4 or glucokinase (50 kDa) (Wilson, 1995; Wilson, 2003). The HKs can be distinguished from each other on the basis of their tissue distributions, substrate affinities, and different responses to hormonal, dietary, and metabolic stimuli (Wilson, 1995). Both HK1 and HK2 have been shown to bind to the outer mitochondrial membrane. HK2 is the most regulated HK and predominantly expressed at low levels in adult adipose tissue and skeletal tissue and only negligently in normal brain. HK1 is present in most tissues but is especially prominent within the brain and kidney (Wilson, 2003). The expression and tissue specific distribution of HK isoforms may be dependent on the developmental and metabolic state of the organism (Girard et al., 1992; Postic et al., 1994). Few studies have profiled the expression of HKs during embryonic and post-natal development. One study demonstrated a maturation-dependent change in its cellular localization and expression of HK1 in the rat (Coerver et al., 1998). During gestation, HK1 expression was present in mitotic cells of the germinal matrix. As the cerebrum and cerebellum mature, HK1 was found to localize to nerve cell bodies, and finally to white matter after 5 weeks of
postnatal development (Coerver et al., 1998). This was hypothesized to coincide with the development of neurological competence including brain myelination and nerve tract growth. However, there is a paucity of research profiling the expression of the HK2 isoform in the developing mouse embryo including the Central Nervous System (CNS). HK2 deficient mice are embryonic lethal, indicating that HK2 is irreplaceable by the other HK isoforms and necessary for embryonic development (Heikkinen et al., 1999). Lethality at E7.5 of the HK2<sup>−/−</sup> mice supports a vital role of HK2 during or after the blastocyst stage but before organogenesis, potentially during early gastrulation (Heikkinen et al., 1999). It has been reported that total HK activity increases during compaction when the eight-cell embryo forms a blastocyst (Saito et al., 1994). This event corresponds to the switch from pyruvate- to glucose-based metabolism in embryos (Gardner and Leese, 1988).

We hypothesize that expression of the HK2 isoform is important for early embryonic and fetal brain development as well as GBM tumour growth, favouring aerobic glycolysis and thereby promoting proliferation. Since HK2 expression is negligent in normal adult brain, we also hypothesize that HK2 may be epigenetically silenced. To address this, we performed quantitative real-time RT-PCR (qRT-PCR) of HK2 and HK1 in mouse embryos ranging from E8.0, E10.5, E15.5, postnatal day 1 (P0), day 20 (preweaning) and 2 months. An inverse temporal relationship was evident with HK2 strongest at early embryonic timepoints while HK1 expression was lowest early on and increased expression with brain maturation. Furthermore, we investigated the expression of HK2 and HK1 in GBM cell lines with varying dependence on aerobic glycolysis. Overall, our results demonstrate that the HK2 isoform may be particularly important in tissues relying on aerobic glycolysis including embryonic tissues and GBMs. Bisulfite sequencing of fetal and adult brain and GBM cell lines supports HK2’s regulation at the epigenetic level.
2.3 Materials and Methods

2.3.1 Mouse embryonic and brain samples

C57BL/6 mice were utilized for this study, in accordance with the guidelines established by the Canadian Council for Animal Care for the care and use of laboratory animals. Pregnant mice were sacrificed at different embryonic timepoints including E8.0, E10.5, and E15.5. In order to extract embryos, mice were anesthetized and sacrificed, the uterus cut open and embryos extracted from gestational sac in cold PBS. Whole embryos were extracted at E8.0 while the developing forebrain was isolated at E10.5 and the whole brain at E15.5. For post-natal developmental timepoints including day 1 (P0), day 20 (preweaning) and 2 months, mice were anesthetized, perfused with cold PBS and then brains were extracted followed immediately by either flash freezing or formalin fixation/paraffin embedding. Excluding E8.0 in which embryos were pooled together, three embryos/brains were analyzed for each timepoint.

2.3.2 Quantitative Real-time PCR

Total RNA was extracted from mouse tissue using RNAeasy kit (Qiagen) and RNA quantity and purity verified by spectrophotometry. cDNA was synthesized from 100ng of total RNA using the Quantitect RT kit (Qiagen). QRT-PCR was performed on cDNA template using SYGR green (Invitrogen) and StepOnePlus Real-time PCR system (Applied BioSystems). PCR conditions were incubation at 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 58°C. Melting curve analysis was performed to verify specificity of PCR products. Data was analyzed using the ΔΔCt method and relative quantitative values were generated. The housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) was employed as reference gene. The relative mRNA level of genes of interest during ontogeny was determined by comparison to the adult mRNA level after adjustment to HPRT1. Samples were run in duplicates and repeated in three independent experiments. Efficiency of primers was calculated from serial fivefold dilutions of mouse adult brain cDNA and calculated as \[\text{Efficiency} = (10^{-1/slope}) -1\] and were found to be near 100% for all primers. The
following mouse specific primers were employed: HK1 forward 5’-CACCGGCAGATTGAGGAAAC-3’ and reverse 5’-CTCAGCCCCATTTCCATCTCT-3’, HK2 forward 5’- GGAACCCAGCTGTTTGACCA-3’ and reverse 5’- CAGGGGAACGAGAAGGTGAAA-3’, HPRT1 forward 5’-TCAGTCAACGGGGACATAAAA-3’ and reverse 5’-GGGGCTGTACTGCTTAAC-3’, LDHA forward 5’-TGTCTCCAGCAAGACTACTGT-3’ and reverse 5’-GACTGTACTTGACAATGGGGA-3’.

2.3.3 GBM cell lines and Lactate assay

GBM and NHA cell lines were obtained from the American Type Culture Collection (Manassas, VA) while immortalized NHAs were generously provided by Dr. Russ Pieper (UCSF). All cell lines were maintained in DMEM containing 10% FBS and antibiotics at 37°C, 5% CO2 in a humidified chamber. The lactate assay was performed using the Eton Bioscience lactate assay kit, according to manufacturer’s protocol, and read at 420nm using a visible plate reader (Microdynamics). Lactate results were normalized to cell numbers.

2.3.4 Western blot analysis

Cell lines were lysed in modified PLC lysis buffer supplemented with protease and phosphatase inhibitors (Sigma). Protein concentration was determined using the BCA assay (Pierce Chemical Co., Rockford, IL). Protein lysates were separated on SDS-PAGE gels (either 10 or 12.5% gels) and transferred onto PVDF membrane (NEN Research Products/Du Pont, Boston, MA) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were probed overnight with the following antibodies: B actin (Sigma-Aldrich, 1:20,000), HK2 (Cell Signaling, 1:1000), HK1 (Cell Signaling, 1:1000). Membranes were washed the next day and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (BioRad, Hercules, CA). Protein bands were visualized with Chemiluminescence Reagent Plus (PerkinElmer Las, Inc. Boston, MA).
2.3.5 O₂ consumption measurements

O₂ consumption was measured using the Instech model 110 Fiber optic oxygen monitor (Instech, PA, USA). Cells were trypsinized, counted and 5 million cells were resuspended in 500uL of media, warmed at 37°C. Measurements were made in triplicates in three independent experiments and reported as nmol O₂/million cells/min.

2.3.6 Bisulfite treatment and sequencing

DNA was isolated from cell lines using a DNeasy Blood & Tissue Kit (Qiagen). Total normal human adult brain DNA and normal fetal brain DNA were generously provided by Dr. Michael Taylor (Hospital for Sick Children, Toronto). DNA was bisulfite treated and purified using the EZ DNA methylation kit (Zymogen, BaseClear, Leiden, the Netherlands). For bisulfite sequencing, HK2 specific BSP-PCR primers targeting the promoter/1ˢᵗ intron were used generating a PCR product size of ~260bp. Sequences are as follows; forward 5'-GCAGGCTGGGCTCTGGCAAA GTG-3' and reverse 5'-GGGTGACCCGCCCCTCCAGTAAC-3'. PCR conditions are as follows using 50ng template DNA and 10 pmol of primers (94°C for 3min, 35 cycles of 94°C for 30 sec, 60°C for 30sec and 72°C for 1 min, followed by 72°C for 10 minutes and 4°C hold). PCR products were run on a 1.5% agarose gel and were gel extracted and purified using a DNA gel extraction and purification kit (Qiagen). Purified PCR product from samples was cloned into PCR TOPO 2.1 sequencing vector (Invitrogen, Inc) and transformed into chemically competent DH5 alpha cells (Invitrogen, Inc). 20-25 clones were selected and re-purified on Ampicillin agar plates (100mg/ml). Colony PCR was performed on purified clones using M13 forward and reverse primers to identify correct insert size (~450 bp). A minimum of 10 clones per cell line or normal brain sample was sequenced using the M13 reverse primer at the The Centre for Applied Genomics (TCAG, Hospital for Sick Children, Toronto). Sequencing results and data was analyzed using BiQ analyzer software v2.04 to determine percent methylation for the promoter/1ˢᵗ intron region of HK2. Universal methylated human genomic DNA was used as a positive control (Zymo Research, Orange, CA 92867 U.S.A.).
Table 2-1: Summary of normal brain adult and fetal specimens.

<table>
<thead>
<tr>
<th>Source</th>
<th>Code</th>
<th>Age</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>ADT1</td>
<td>25 years</td>
<td>M</td>
</tr>
<tr>
<td>Adult</td>
<td>ADT2</td>
<td>27 years</td>
<td>M</td>
</tr>
<tr>
<td>Adult</td>
<td>ADT3</td>
<td>33 years</td>
<td>M</td>
</tr>
<tr>
<td>Adult</td>
<td>ADT4</td>
<td>26 years</td>
<td>M</td>
</tr>
<tr>
<td>Adult</td>
<td>ADT5</td>
<td>33 years</td>
<td>M</td>
</tr>
<tr>
<td>Fetal</td>
<td>FLT1</td>
<td>26 weeks</td>
<td>F</td>
</tr>
<tr>
<td>Fetal</td>
<td>FLT2</td>
<td>26 weeks</td>
<td>M</td>
</tr>
<tr>
<td>Fetal</td>
<td>FLT3</td>
<td>22 weeks</td>
<td>M</td>
</tr>
<tr>
<td>Fetal</td>
<td>FLT4</td>
<td>16 weeks</td>
<td>M</td>
</tr>
<tr>
<td>Fetal</td>
<td>FLT5</td>
<td>18 weeks</td>
<td>M</td>
</tr>
</tbody>
</table>

2.3.7 Statistical Analyses

*In vitro* experiments were performed in triplicates. Means and standard errors were computed. Student’s t-test was performed to determine whether a significant difference exists between groups (significance level at p < 0.05).

2.4 Results

2.4.1 Bioinformatic analysis of Gene Expression Omnibus

Mining of gene expression microarray results published on NCBI’s Gene Expression Omnibus (GEO) yielded one study that looked at the gene expression profile at the oocyte, 1-, 2-, 8-cell and blastocyst stages of mouse development (data accessible at NCBI GEO database, accession GSE1749) (Zeng et al., 2004). Expression profiling of developing embryos revealed similar expression of the HK1 and HK2 isoforms in the 8-
cell stage followed by a strong induction of the HK2 isoform in the blastocyst stage (Figure 2-1). The robust increase in HK2 during blastocyst stage is in line with previous reports that total HK activity increases during compaction when the eight-cell embryo forms a blastocyst (Saito et al., 1994)

**Figure 2-1: Gene expression of HK2 and HK1 in 8-cell and blastocyst stage of mouse preimplantation embryonic development.**

Expression profiling of CF-1 x B6D2F1/J preimplantation embryos at the 8-cell and blastocyst stages (n=4/timepoint). Expression (depicted as raw intensity signal) of HK1 and HK2 were similar at the 8-cell stage (n=4). HK1 expression decreased in blastocyst embryos (n=4) while HK2 expression increased dramatically (data accessible at NCBI GEO database, accession GSE1749). Error bars represent standard deviation across 4 samples per timepoint.
Figure 2-1

[Bar chart showing raw intensity signal at 8-cell stage and blastocyst stages for Hk2 and Hk1]
2.4.2 Developmental profile of HK2 and HK1 in the mouse brain

The HKs are key enzymes in glycolytic metabolism. However, the relative contribution HK2 and HK1 expression in the developing mouse embryo including the brain is not known. We next sought to explore the temporal profile of HK2 and HK1 expression in the developing mouse embryo including the CNS, beyond the blastocyst stage. We performed qRT-PCR on RNA extracted from mouse embryos at E8.0 (whole embryo), E10.5 (developing forebrain), and brains extracted from E15.5, postnatal day 0 (P0), day 20 (preweaning) and 2 months (n=3 each timepoint). The housekeeping gene HPRT1 was employed as a reference gene and was found to be similarly expressed across the different developmental timepoints (For 100ng RNA, the mean Ct was: E8.0 = 23.3 ±0.5, E10.5 = 23.9 ±0.2, E15.5 = 23.1 ±0.7, P0 = 23.5± 0.9, P20 days = 23.1±0.3 and 2 months = 23.7±0.4).

Our results demonstrate that, relative to 2 month adult brains, HK2 expression was ~8 fold higher in the brain at E15.5, ~40 fold higher at E10.5 and ~100 fold higher in the whole embryo at E8.0 (Figure 2-2a). In contrast, HK1 expression was less than 50% of that of adult 2 month brain at E8.0 and E10.5, rose in expression at E15.5 and at birth and then decreased postnatally to adult levels. Therefore, there appears to be an inverse temporal relationship between HK1 and HK2, with HK2 strongest at early embryonic timepoints previously associated with greater reliance on aerobic glycolysis (Ramalho-Santos et al., 2009), while HK1 was strongest in the maturing brain. The upregulation of HK1 coincides with previous reports of dramatic induction of many mitochondrial ATP synthesis and OXPHOS genes late in embryonic development (Wagner et al., 2005) and with alterations in mitochondrial structure and dependence on oxygen (Fantel and Person, 2002).

Bearing in mind the importance of the enzyme lactate dehydrogenase A (LDHA) in converting pyruvate to lactate, the product of aerobic glycolysis, we next sought to examine the expression of the LDHA transcript across embryonic timepoints. We found that the LDHA transcript mirrored that of HK2 expression, such that expression was
highest at the earliest timepoints and progressively decreased to adult levels after birth (Figure 2-2b).

Figure 2-2: Developmental profile of HK2, HK1, and LDHA in the mouse embryo, including CNS.

a) QRT-PCR of HK2 and HK1 in mouse brain tissue extracted at E8.0 (pooled), E10.5, E15.5, postnatal day 1 (P0), postnatal day 20 and 2 months (n=3/timepoint). HK2 mRNA expression relative to 2month adult brain is depicted on the left y axis and HK1 mRNA on the right y axis. An inverse temporal relationship is seen between HK1 and HK2, with HK2 strongest at early embryonic timepoints (~75 fold of adult 2 month) while HK1 increases 3 fold at E15.5 and then decreases to adult levels. Samples were run in duplicates and QRT-PCR repeated in three independent experiments. b) QRT-PCR of LDHA at different developmental timepoints showing strong expression during early embryogenesis followed by reduction in expression to adult levels.
2.4.3 Expression of HK2 correlates with aerobic glycolysis in GBM cell lines

Tumour tissues including GBMs have been shown to rely on aerobic glycolysis which may promote both cell survival and proliferation (Christofk et al., 2008a; Oudard et al., 1997). The molecular basis of this metabolic switch from OXPHOS to glycolysis in GBMs remains unclear but may involve the over-expression of specific glycolytic enzymes or isoforms. We studied the expression of the HK1 and HK2 isoforms in a panel of GBM cell lines and determined how their expression relates to parameters of aerobic glycolysis including extracellular lactate and O$_2$ consumption. The following GBM cell lines were investigated: U87 (p53wt, PTEN$^-$/-), T98G (p53mt, PTEN$^-$/-), U138 (p53mt, PTEN$^-$/-), U343 (p53mt, PTEN wt) A172 (p53mt, PTEN$^-$/-) (Figure 2-3a). The U87 and T98G cell lines that had the greatest expression of HK2 was associated with high extracellular lactate (Figure 2-3b) and O$_2$ consumption levels below 2 nmol/million cells/min (Figure 2-3c). The A172 and U343 cell lines showed very little to no expression of HK2 and was associated with much lower levels of lactate (Figure 2-3b) as well as O$_2$ consumption levels above 2 nmol/million cells/min (Figure 2-3c). These results support a stronger correlation of the expression of HK2, and not HK1, with aerobic glycolysis in GBM cell lines.

2.4.4 Methylation of HK2 promoter/intron 1 in normal adult brain and GBM cell lines that do not express HK2

Considering the limited expression of HK2 in normal adult brain and A172 and U343 cell lines, and based on previous reports of potential methylation within the promoter/intron 1 of the rat HK2 gene in hepatocytes which is demethylated in hepatomas (Goel et al., 2003), we investigated the methylation status of human fetal (n=5) and normal adult brain (n=5) tissues as well as different cell lines including: non-transformed Normal Human Astrocytes (NHA), NHA immortalized with htert/E6/E7, and different GBM cell lines. Bisulfite sequencing of CpG islands within intron 1 was performed (Figure 2-4a).
Figure 2.4b depicts the heat map showing % methylation over several CpG sites in intron 1. The mean % methylation in fetal tissue (58.2+/−1.9) was significantly lower than that of normal adult tissues (79.6+/−3.4) (P=0.001). Methylation status was found to correlate with expression at the transcript level in GBM cell lines (Figure 2-4b,c). Treatment of U343 cells with 5-aza-2-deoxycytidine restored the expression of HK2 at the transcript level, supporting that HK2 may be epigenetically regulated, at least in GBM cells that do not express HK2 (P < 0.001) (Figure 2-4d). Of note, the non-transformed NHA cell line showed high methylation of HK2 promoter while NHA’s immortalized with htert/E6/E7 resulted in less methylation of HK2. This suggests that alterations in cell cycle proteins (i.e. E6/E7 impacts Rb) may alter epigenetic regulation of HK2 promoter.
**Figure 2-3: Expression of HK2 in GBM cell lines correlates with greater extracellular lactate and reduced oxygen consumption.**

a) Western blot of HK2 and HK1 in a panel of GBM cell lines with differing levels of expression. b) Extracellular lactate measurements (normalized to U87 cells). U343, U138 and A172 have significantly lower lactate levels compared to U87 cells (* p < 0.01). c) Oxygen consumption measured in nmol O₂/ million cells/min. U87 (** p < 0.001) and T98G (*p< 0.05) have significantly lower O₂ consumption compared to U343 cells.

**Figure 2-4: Epigenetic regulation of HK2 in CpG islands of intron 1.**

a) CpG island plot of first 1000bp of HK2 intron 1 reveal three CpG islands. P1 and P2 represent location of bisulfite sequencing primers used for bisulfite sequencing analysis. Bottom panel: vertical sticks represent location of individual CpG dinucleotide repeats within CpG islands. b) Heat map and percent methylation within intron 1 in 5 normal adult brain, 5 fetal brains, Normal Human Astrocytes (NHA) and different GBM cell lines. There is a significant difference in % methylation between HK2 expressors and HK2 non-expressors (P = 0.001). c) qRT-PCR of HK2 expression in different GBM cell lines and NHAs. Samples were run in duplicates and qRT-PCR repeated in three independent experiments. d) qRT-PCR of U343 GBM cell line treated with 5’aza-2-deoxycytidine (P < 0.001).
Figure 2-3

(a) Western blot analysis of HK2 and HK1 in different cell lines with B-actin as the loading control.

(b) Lactate level (relative to U87) for different cell lines: U87, T98G, U138, U343, A172.

(c) Oxygen (O2) consumption (nmol/million cells/min) for different cell lines: U87, T98G, U138, U343, A172.

- * p < 0.05 relative to U343
- ** p < 0.001 relative to U343
Figure 2-4

a) HK2 Intron 1

b) Methylation of HK2 Expressors versus HK2 non expressors, P=0.0001

Samples
NHA  70
U343  75
A172  54
Adult 1 75.6
Adult 2 76.4
Adult 3 80.8
Adult 4 83.1
Adult 5 82.3
Fetal 1 59.2
Fetal 2 59.5
Fetal 3 60.6
Fetal 4 57.3
Fetal 5 65.8
NHA (E6/E7 + hTERT) 41
U118  35
U138  40
U251  30
U87  31
T98G  26

Promoter Methylation (%)

p < 0.001
2.5 Discussion

HK is a key enzyme in glucose metabolism and the first rate-limiting step of glycolysis. The physiological roles of HK1, HK2, and HK4 have been well studied (Katzen and Schimke, 1965; Wilson, 1995). However little is known about the changes in HK isozymes expression profile during embryonic development and particularly within the brain. Furthermore, it is not clear how expression of specific HK isozymes may favour aerobic glycolysis, which has been associated with generation of greater synthetic precursor necessary for proliferation (Vander Heiden et al., 2009).

In this study, we profiled the expression of HK2 and HK1 in the brain at different embryonic and post-natal timepoints. This study’s results demonstrate that HK2 transcript is present during embryonic brain development. Greater than 8 fold expression of HK2 was present in brains at E15.5 compared to post-natal brains day 20 or 2 months (Figure 2-2a). HK2 was even more strongly expressed at earlier timepoints including E10.5 (prosencephalon) and E8.0 (total embryo). Mining of existing expression databases suggests that HK2 is also expressed more strongly than HK1 at even earlier developmental timepoints including the blastocyst stage (~E5.5) (Figure 2-1) (data accessible at NCBI GEO database, accession GSE1749) (Zeng et al., 2004). In contrast, HK1 expression was lowest at earlier timepoints but increased with brain maturation.

Aerobic glycolysis is reportedly enhanced in the blastocyst and developing embryo (Gardner and Leese, 1988; Ramalho-Santos et al., 2009). We demonstrate that earlier developmental timepoints are associated with greater expression of HK2 than HK1, in contrast to fully developed adult brains. To ascertain whether HK2 expression is associated with aerobic glycolysis, we investigated a panel of GBM cell lines with variable levels of HK2 and HK1 and dependence on aerobic glycolysis. GBMs have previously been reported to demonstrate heterogeneity in glucose metabolism with some cells relying more on OXPHOS metabolism and others on aerobic glycolysis (Griguer et al., 2005). Our results demonstrated a correlation between HK2 expression and not HK1 with greater lactate generation and decreased O2 consumption, reflecting enhanced aerobic glycolysis (Figure 2-3). The mechanisms behind HK2’s promotion of aerobic
glycolysis and generation of lactate are unclear. However, HK2 is known to interact with the outer mitochondrial membrane, particularly with activation of the PI3K/AKT pathway, and promote tumour proliferation and survival in some cancers (Pedersen et al., 2002). HK2 may form part of a growth factor mediated metabolic network, potentially involving LDHA over-expression and the PKM2 splice isoform, reportedly expressed in embryonic and tumour tissue and also associated with aerobic glycolysis (Christofk et al., 2008a; Christofk et al., 2008b; Fantin et al., 2006). Indirect support for this is the pattern of LDHA expression paralleling that of HK2 during embryonic development (Figure 2-2b).

This study also provides evidence that methylation within the intron 1 of HK2 gene may play a role in the regulation of its expression in normal brain, fetal tissue and GBMs. Epigenetic regulation of HK2 within intron 1 was previously reported in hepatocytes, with demethylation of the gene in hepatomas (Goel et al., 2003). Similarly, we show that fetal tissues and GBM cell lines expressing HK2 had low percentage methylation within intron 1 while normal brain had high methylation (Figure 2-4b,c). Treatment of the GBM cell line U343 which does not express HK2 with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine resulted in restoration of HK2 gene expression (Figure 2-4d). However, other mechanisms aside from epigenetic regulation may play a role in regulating HK2 expression in developing embryo, adult brain and GBMs. Developmental specific transcription factors may play an important role in regulating HK2 expression in fetal and GBM tissues (e.g. Hox genes, GATA, CREB). As of yet, no transcriptional repressors of the HK2 promoter have been identified. Using promoter prediction program TESS to identify putative consensus binding sequences for known transcription factors, many putative transcription factor response elements exist within intron 1 of the HK2 gene that are linked with signal transduction pathways important for gliomagenesis and metabolism (e.g. RAF, ER-alpha, CREB, among others). These may play a role in HK2 regulation in addition to transcription factors already implicated including HIF1α, c-myc and p53 (Mathupala et al., 1997b). Furthermore, HK2 mRNA stability may play a role in regulating HK2 expression. It is not clear whether HK2 mRNA degradation is regulated via AU rich elements at its 3” end. As well, no microRNAs regulating HK2 have yet been identified. However, this could be a source of future studies.
In conclusion, HK2 is expressed early on during embryonic development including brain development and may be epigenetically silenced in the mouse adult brain. In contrast, HK1 expression is lowest early on and increases with brain development and then descends down to adult levels. Both embryonic and GBM analyses support a correlation between HK2 and aerobic glycolysis. A caveat of this study is that the association between HK2 and aerobic glycolysis in embryonic development and GBM cell lines is correlational. A more comprehensive exploration of the importance of HK2 in aerobic glycolysis in GBM and potential mechanisms are pursued in Chapter 3 of this thesis.
CHAPTER 3: HEXOKINASE 2 IS A KEY MEDIATOR OF AEROBIC GLYCOLYSIS PROMOTING TUMOR GROWTH IN GLIOBLASTOMA MULTIFORME

3.1 Abstract

Proliferating tissues including embryonic and tumour tissues preferentially use aerobic glycolysis to support cell growth. This alteration in glucose metabolism is commonly referred to as the “Warburg Effect” and may confer a proliferative, invasive and survival advantage. This study provides direct evidence that the glycolytic enzyme Hexokinase 2 (HK2) is crucial for the “Warburg Effect” in human Glioblastoma Multiforme (GBM), the most common and therapeutically resistant malignant brain tumour. In contrast to normal brain and low-grade gliomas, GBMs have increased HK2 expression, particularly in the perinecrotic hypoxic regions. Increased expression of HK2 correlated with a worse overall survival of GBM patients. Stable loss of HK2 in GBM cells restored oxidative phosphorylation (OXPHOS)-mediated glucose metabolism, with increased oxygen consumption and decreased lactic acid production. This resulted in decreased proliferation and increased sensitivity to apoptotic inducers such as radiation and chemotherapy, both common adjuvant therapies of GBMs. Intracranial xenografts of GBM cells with reduced HK2 demonstrated increased survival with decreased proliferation and angiogenesis. However, in the small tumours that grew there was increased invasion along the perivascular space. In contrast, exogenous HK2 expression in GBM cells promoted proliferation, therapeutic resistance and intracranial growth. This was dependent partly on translocation of HK2 to the mitochondrial membrane, which was regulated by activated AKT, a prevalent alteration in GBMs. These observations were not found with other glycolytic enzymes such as HK1 or PKM2. Collectively, therapeutic strategies to modulate the “Warburg Effect”, such as targeting HK2 or its regulation in GBMs, may be directly beneficial or favourably alter therapeutic sensitivity.

3.2 Introduction

Cancer cells evolve a number of genetic and epigenetic changes in order to survive in unfavourable microenvironments while retaining the ability to proliferate (Vander Heiden
et al., 2009). A classic biochemical adaptation of tumour cells is a metabolic shift to aerobic glycolysis as a main source of ATP rather than oxidative phosphorylation (OXPHOS), irrespective of oxygen availability, a phenomenon referred to as the “Warburg Effect” (Warburg, 1956). This phenotype may promote a state of apoptosis resistance (Kroemer and Pouyssegur, 2008; Plas and Thompson, 2002), the generation of biosynthetic precursors for proliferation (Vander Heiden et al., 2009), as well as increased invasive ability (Stern et al., 2002).

Glioblastoma Multiforme (GBM) is the most lethal adult primary brain tumour, with an overall median survival of 12-16 months due to local recurrence and invasion (Mellinghoff et al., 2005; Stupp et al., 2005). As denoted by “multiforme”, GBMs are pathologically heterogeneous with “central” regions of necrosis surrounded by florid cellular (i.e. pseudopalisading) and hypervascularized regions under moderate levels of hypoxic stress (pO2: 2.5-5%) (Evans et al., 2004). In contrast, “peripheral” regions of GBMs consist of invading tumour cells into normal brain under relatively normoxic conditions (Evans et al., 2004). GBM cells are resistant to standard apoptotic-inducing therapies including radiation and chemotherapy and are highly invasive (Brat et al., 2004). Furthermore, GBMs demonstrate an approximate three fold increase in glycolysis relative to normal brain (Oudard et al., 1996), with variations across different GBM cell lines (Gorin et al., 2004; Griguer et al., 2005).

The molecular basis of aerobic glycolysis remains elusive and may vary across cancers. Mechanisms implicated in a switch to aerobic glycolysis include altered isoform expression profile, primary mutations and altered regulation/function of metabolic enzymes secondary to oncogenic signaling pathways or the tumour microenvironment (Vander Heiden et al., 2009). An example of primary mutations is evident in Glioblastoma Multiforme (GBM), the most common and lethal of all primary human central nervous system (CNS) tumours (Mellinghoff et al., 2005; Stupp et al., 2005). High throughput genomic screening of a large number of GBMs identified point mutation in the isocitrate dehydrogenase-1 (IDH1) gene in ~12% of all GBMs (Parsons et al., 2008), and ~80% of low-grade astrocytomas (LGA) or secondary GBMs that developed from their malignant progression (Watanabe et al., 2009; Yan et al., 2009). The effect of
this IDH1 mutation, which converts isocitrate to \(\alpha\)-ketoglutarate coupled with NADP+/NADPH, on GBM metabolism and glucose utilization and subsequent growth is not clear, but has been associated with the generation and accumulation of the metabolite 2-hydroxyglutarate in GBM tumours (Dang et al., 2009; Zhao et al., 2009). Alterations in isoform expression profile is exemplified by a reported switch in splice isoforms from the adult pyruvate kinase M1 (PKM1) to the fetal PKM2, believed to promote aerobic glycolysis and tumour growth in a lung cancer cell lines (Christofk et al., 2008a).

For several reasons, Hexokinase 2 (HK2), the first rate-limiting enzyme of glycolysis, may act as an important mediator of aerobic glycolysis particularly in GBMs and thus influence tumour proliferation and apoptosis. HK2 is expressed at low levels in normal brain, however, results from this study as well as previous published microarray data have shown its increased expression in GBMs (Dong et al., 2005; Parsons et al., 2008). HK2 expression and activity is also highly regulated by factors including Hypoxia Inducible Factor-1\(\alpha\) (HIF1\(\alpha\)) and growth factors known to promote GBM growth (Denko, 2008; Mathupala et al., 2001). GBMs have aberrant activation of growth factor receptors and/or loss of PTEN activity (Mellinghoff et al., 2005) with subsequent activation of the PI3K/AKT pathway. Upon AKT activation, HK2 has been shown in some cell systems to undergo translocation to the outer mitochondrial membrane and interact with the permeability transition pore promoting tumour cell survival (Gottlob et al., 2001; Majewski et al., 2004a; Pastorino et al., 2002). Pharmacological targeting of HK2 including its association with the permeability transition pore may significantly sensitize cancer cells to chemotherapies thereby promoting apoptotic cell death (Pastorino et al., 2005; Peng et al., 2008a).

In this study, we provide several pieces of evidence demonstrating that the glycolytic enzyme Hexokinase 2 (HK2), which is aberrantly expressed in GBMs, is an important mediator of aerobic glycolysis in GBMs, providing a proliferative and cell survival advantage. Inhibition of HK2, but not glycolytic enzymes HK1 or PKM2, restored normal OXPHOS mediated glucose metabolism with decreased extracellular lactate, increased expression of OXPHOS proteins and \(O_2\) consumption. HK2 depletion reduced \textit{in vitro} and \textit{in vivo} growth of GBMs, with increased sensitivity to apoptosis induced by
hypoxia, radiation or chemotherapy while exogenous HK2 over-expression in GBM cells promoted proliferation and intracranial growth. As described in other cancers (Gottlob et al., 2001; Majewski et al., 2004a; Pastorino et al., 2002), the growth promoting effects of HK2 in GBMs are partly dependent on growth factor induced activation of the PI3K-AKT signaling pathway.

3.3 Materials and Methods

3.3.1 Human GBM samples

Stereotactic-guided GBM samples were acquired from the T1-Gadolinium MRI enhancing “central” region and the T2 positive, T1-Gd negative “peripheral” regions. See Appendix 1 for an example of image-guided sampling of GBM “centre” and “periphery” with corresponding Magnetic Resonance Spectroscopy (MRS) spectra. Samples were verified for the presence of perinecrotic pseudopalisading tumour cells (“centre”) and infiltrating tumour cells (“periphery”). UHN REB approval was obtained.

3.3.2 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from frozen tissue samples or GBM cells using RNAeasy kit (Qiagen). cDNA was synthesized from 50ng of total RNA using the Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed on cDNA template using SYGR green (Invitrogen) and the Chromo4 Real Time PCR detector (MJ Research a Division of Bio-Rad Laboratories Ltd., MA, USA). Real-time PCR data was analyzed with the Opticon Monitor 3.1.32 analysis software. Analysis of melting curves was performed to verify specificity of PCR products. The relative quantity was measured as \( \text{efficiency}^{(\text{control})} = \frac{C_t - \text{unknown} C_t}{C_t - \text{control} C_t} \), where normal brain was the control sample. For each sample, a normalization factor (NF) was calculated as the geometric mean of the relative quantities of all reference genes (18S and HPRT1). The expression level for each sample was then obtained using the equation: \( \text{expression level} = \frac{\text{relative quantity}}{\text{NF}} \). The following primers were employed: HK1 forward 5’-GGACTGGACCGTCTGAATGTT-3’ and reverse 5’-ACAGTTCCCTTCACCCTGCTGG-3’, HK2 forward 5’-CAAAAGTGACAGTGTTGTTGGAAGTGCTGG-3’ and reverse 5’-GCCAGGTCTCTTCACTCTCGTCTC-
3’, HPRT1 forward 5’-TGACACTGGCAAAACAATGCA-3’ and reverse 5’-GGTCCTTTTCACCAGCAAGCT-3’, PGC1α forward 5’-TCAGTCCTCAGTGGGACA-3’ and reverse 5’-TGCTTCGTCGTCAAAAAACAC-3’, mTFA forward 5’-AATGGATAGGCACAGGAACC-3’ and reverse 5’-CAAGTATTATGCTGGCAGAAGTC-3’.

### 3.3.3 Immunohistochemistry

IHC was performed on formalin-fixed, paraffin-embedded 5um thick sections. Sections were de-paraffinized, hydrated, antigen retrieved in citrate buffer (pH = 6), blocked for 1 hour at RT and incubated overnight at 4°C with primary antibody. Primary antibodies and staining conditions used are listed in Table 3-1. Detection was performed using Vectastain ABC reagent and DAB chromogen (Vector Labs, Burlingame, CA). Slides were counterstained with hematoxylin and coverslipped using Permount (Fisher). TUNEL immunohistochemistry was performed with the DeadEnd™ colorimetric TUNEL System (Promega) according to manufacturer’s instructions and counterstained with hematoxylin. The apoptotic index (%) was calculated as the number of positive nuclei over total nuclei. HK2 staining was scored based on intensity of staining. The grading scale used was as follows: 0, no staining; 1, faint staining; 2 strong staining. GBM tissue microarray with prognostic information was acquired from Dr. Paul Mischel-UCLA and stained with HK2. An interrater reliability analysis using Cohen’s Kappa statistic was performed to determine consistency among the two raters.
Table 3-1: Primary antibodies used for immunohistochemical staining

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>1hr RT or 4° o/n</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>1:400</td>
<td>1hr RT</td>
<td>DAKO</td>
</tr>
<tr>
<td>MIB1</td>
<td>1:100</td>
<td>1hr RT</td>
<td>DAKO</td>
</tr>
<tr>
<td>HK2</td>
<td>1:100</td>
<td>4° o/n</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>HK1</td>
<td>1:500</td>
<td>4° o/n</td>
<td>Chemicon</td>
</tr>
<tr>
<td>HIF1α</td>
<td>1:50</td>
<td>4° o/n</td>
<td>Lab Vision</td>
</tr>
<tr>
<td>VEGF</td>
<td>1:50</td>
<td>1hr RT</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>P27-KIP1</td>
<td>1:100</td>
<td>4° o/n</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>1:50</td>
<td>4° o/n</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>
3.3.4 Cell line and siRNA treatment

The U87 human glioma cell line was obtained from the American Type Culture Collection (Manassas, VA) while U373 and immortalized NHAs were generously provided by Dr. Darrell Bigner (Duke University) and Dr. Russ Pieper (UCSF), respectively. All cell lines were maintained in DMEM containing 10% FBS and antibiotics at 37°C, 5% CO2 in a humidified chamber. Hypoxic conditions were achieved by incubating cells in a tissue culture incubator flushed with N2, 5% CO2 and O2 levels maintained constant at 2% using an oxygen controller (ProOx, Biospherix Ltd). Scramble siRNA (Qiagen) was employed as negative control. Cells were transfected with either 50nM of HK2 siRNA or 50nM of scramble siRNA or mock transfected using HiPerfect transfection reagent (Qiagen) according to the manufacturer’s protocol. The following siRNA target sequences were used:

HK2siRNA1 “5’CACGATGAAATTGAACCTGGT3’”,
HK2siRNA2 “5’CCTGGGTGAGATTGTCCGTAA3’”.

3.3.5 Cell viability assay and BrdU incorporation assay

Cell viability was measured using the sulphorhodamine B (SRB) colorimetric assay (Sigma) and trypan blue exclusion. Proliferation was measured using the BrdU incorporation assay (Roche) according to manufacturers’ protocol. Cells were seeded in 96-well plate and then transfected 24 hours later with scramble or HK2 siRNA and incubated under hypoxia or treated with Temozolomide (TMZ, 100uM), 72 hours post transfection. A standard curve with known cell number was performed. The relative luminescence units from BrdU incorporation was normalized to this standard curve in order to approximate cell number.

3.3.6 Determination of apoptosis

Cells were seeded into a 96-well plate, transfected with siRNA, and at 3 days post-transfection were administered actinomycin D (10uM), 5 Gy radiation or 24 hours of 2% hypoxia. Caspase 3, 7 activity levels were measured using the Apo-One® Homogeneous Caspase 3/7 assay (Promega) and relative fluorescence units (RFU) were computed.
Apoptosis was also assessed with fluorescence-activated cell sorting analysis (FACScan, Becton Dickinson, Heidelberg, Germany) of fixed cells stained with PI (cell cycle analysis) or live cells stained with AnnexinV-FITC/PI (BD Pharmingen). Samples were analyzed using the CellQuest Pro software.

### 3.3.7 Generation of stable cell lines

Target sequences from HK2 siRNA 1 and 2 were used to design HK2 shRNA sequences that included hairpin loop and appropriate restriction enzyme sites. The HK1 shRNA sequence employed was “5’CAC GAT GTA GTC ACC TTA CTA 3’”. The PKM2 shRNA target sequence employed was “5’ GCT GTG GCT CTA GAC ACT AAA 3’”.

ShRNA for HK2, HK1 and scramble shRNA were annealed and then cloned into RNAi Ready pSIREN-retroQ vector (Clontech, Mountain View, CA), designed to express shRNA under the human U6 promoter. U87 cells were transfected with the vector HK2shRNA and scramble shRNA and clones expressing the vector were selected using puromycin. U87HK2shRNA or U343 cells were transfected with the HK1-GFP or HK2-GFP, plasmids generously provided by Dr. Ardehali (NorthWestern). Transient transfection efficiency ranged between 60-80%.

### 3.3.8 Mitochondrial membrane permeability and immunofluorescence

Mitochondrial membrane potential was assessed with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Sigma). U87 HK2shRNA1 or 2, scramble shRNA were grown on coverslips, incubated under normoxic or hypoxic conditions for 24hrs, washed with PBS and loaded for 20 minutes with 2uM of JC-1 in HBSS at 37°C. Cells were then washed in PBS and maintained at 37°C while images were taken. Cells were excited with 540nm argon and 488nm lasers to visualize the aggregate (i.e. polarized, Texas Red/rhodamine) and monomer (i.e. depolarized, FITC) signals, respectively. Immunofluorescence of live cells stained with 50nM of Mitotracker Deep Red for 20 minutes was also performed. Images were taken on a Zeiss Axiovert 200 microscope equipped with a Hamamatsu Orca AG CCD camera and spinning disk
confocal scan head using Volocity acquisition software. Regions of interest were drawn on the image data and the software then summed the chosen pixels to generate a fluorescence ratio for each sets of images. Signal within the nucleus was used as background fluorescence. Pearson’s Correlation coefficient (R) was employed for colocalization studies. For mitochondrial mass, FACS analysis was performed on $1 \times 10^6$ cells/mL stained with nonyl acridine orange (NAO), that binds mitochondria independently of their energization state, and whose fluorescence is detectable in FL1. (Molecular Probes; 2 µM; excitation 488 nm, emission 530 nm).

### 3.3.9 Western blot analysis

See Section 2.3.4 for description of western blot procedures. Membranes were probed overnight with the following antibodies: OXPHOS (Mitoscience, 1:200), B actin (Sigma-Aldrich, 1:20,000), GAPDH (Chemicon, 1:200), PKM2, HK2, HK1, cleaved caspase 3 and caspase 3 were all from Cell Signaling (1:1000). The OXPHOS cocktail of antibodies probes for the following proteins (constituting different catalytic sites in ETC): 20 kDa subunit of Complex I (20kDa), COX II of Complex IV (22kDa), 30 kDa Ip subunit of Complex II (30 kDa), core 2 of complex III (~ 50kDa), F1α (ATP synthase) of Complex V (~ 60kDa).

### 3.3.10 Transmission Electron Microscopy

Transmission EM was performed by the EM department at the Hospital for Sick Children, Toronto. Cells were fixed in 4% glutaraldehyde prior to analysis.

### 3.3.11 Mitochondrial fractionation and cytochrome c ELISA

Ten million U87 scramble shRNA, U87 HK2shRNA1 and U87HK2shRNA2 were harvested and fractionated into cytosolic and mitochondrial fractions using a mitochondrial fractionation kit for cell culture according to manufacturer’s protocol (Pierce). ELISA for cytochrome c was performed on the cytosolic fraction according to manufacturer’s protocol (R&D) and was normalized to total protein level measured by BCA (Pierce).
3.3.12 Lactate assay and oxygen consumption

See sections 2.3.3 and 2.3.5.

3.3.13 Hexokinase assay

Hexokinase assay was performed as previously described in literature (Ahmad et al., 2002). Cells were lysed using the following buffer: 50mM potassium phosphate, 2mM dithiothreitol (DTT), 2mM EDTA, 20 mM sodium fluoride. One milliliter of buffer was used to harvest 5 million cells. After the cells were harvested, the cell homogenate was incubated on ice for 30 min followed by centrifugation at 1,000 g at 4°C for 10 min. Approximately 10 µl of freshly lysed cell supernatant was added to 1,000 µl of 100 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 10 mM ATP, 10 mM MgCl₂, 2 mM glucose, 0.1 mM NADP, and 0.1 U/ml of G6PD (Sigma-Aldrich). HK activity was determined by following the G6P-dependent conversion of NADP to NADPH spectrophotometrically at 340 nm at 37°C. One activity unit is defined as one micromole of NADPH per milliliter per minute at 37°C.

3.3.14 Subcutaneous GBM xenograft model

NODSCID mice were injected subcutaneously with 1.5 x 10⁶ U87 scr shRNA or U87HK2shRNA cells. Mice were observed for tumour formation and then sacrificed. Tumours were harvested at 4 weeks (U87 scr shRNA) or 8 weeks (U87HK2shRNA) and formalin-fixed, paraffin-embedded.

3.3.15 Intracranial GBM model

NODSCID mice were stereotactically injected with 200,000 cells of U87 control (U87 + empty vector and U87 scramble shRNA, n=10), U87HK2shRNA (n=10), U87HK2-GFP (n=10) or U87HK2-GFP+HK2shRNA-RFP (n=6). Upon development of symptoms, mice were anesthetized, perfused with ice cold saline and 4% paraformaldehyde (PFA), the brains harvested followed by flash freezing or paraffin embedding. Flash frozen specimens were sectioned at 10µm thickness using a cryostat, rinsed in PBS, counter stained with DAPI and then visualized on a confocal microscope for GFP, RFP and DAPI
expression. For quantification of extent of invasiveness, H&E slides were scanned using Zeiss Mirax scanner and analyzed using the viewer for the mean distance from the main tumour mass measured for each cluster of tumour cells.

### 3.3.16 Statistical Analyses

Real-time PCR and *in vitro* experiments were performed in triplicates. Means and standard errors were computed. Student’s t-test or Wilcoxon sign rank t-test was performed to determine whether a significant difference exists between groups (significance level at p < 0.05). Analysis of patient survival was performed using Kaplan-Meier plots generated in SPSS, using age as a covariate, and statistical significance was measured using the Cox proportional hazard model.

### 3.4 Results

#### 3.4.1 Human GBMs express HK2, correlating with poor overall survival

To establish whether HK2 is expressed in human GBM samples, we performed western blotting for HK2 on 3 normal human brain, 5 low-grade astrocytomas (LGA) and 12 GBM specimens (Figure 3-1). GBMs expressed HK2 to varying extents, while normal human brains and LGAs did not (Figure 3-1a). In contrast, normal brain, LGAs and GBMs expressed HK1. Immunohistochemical (IHC) staining of HK2 on a tissue microarray of 56 primary and secondary GBMs, demonstrated 35 (62.5%) to express HK2, while 21 (37.5%) did not. HK2 expression correlated with a poorer prognosis, even when controlling for age (Figure 3-1b) (Cox proportional hazard p<0.006) (Cohen’s κ = 0.81, p < 0.005). However, other genetic alterations and known prognosticators (e.g. loss of PTEN, Karnofsky score) were not taken into account.

Furthermore, to corroborate our prognostic results, we mined the Repository of Molecular Brain Neoplasia Data (REMBRANDT) in which human glioma samples have been collected from patients enrolled in the Glioma Molecular Diagnostic Initiative (GMDI) study. Gene expression analysis was performed using Human Genome U133 Plus 2.0
Arrays (Affymetrix) by hybridizing tumour samples collected from patients. The data from the hybridizations were used to compare the levels of expression between normal and brain tumour samples. Using two different probe sets, HK2 was shown to predict poor clinical outcome (overall survival) in glioma patients (including astrocytomas, GBMs, oligodendrogliomas, mixed tumours) when upregulated more than 3-fold (Appendix 2).

3.4.2 HK2 is strongly expressed in the perinecrotic region of human GBMs

Tumour cells within the perinecrotic “central” region grow within harsh microenvironments consisting of nutrient deprivation and hypoxia. As such, one would expect these cells to undergo greater cell death as a result of the microenvironment. To determine if this is the case, we undertook TUNEL IHC on 25 GBMs “centre” and “periphery” samples. The percentage of immunoreactive tumour cells was very low within both regions, with an average apoptotic index of less than 1% (Figure 3-2). This strongly suggests that GBM cells are resistant to apoptotic-inducing conditions.

To determine whether HK2 demonstrates differences in regional expression, we performed quantitative real-time RT-PCR (qRT-PCR) on RNA extracted from regionally dissected GBM “centre” and “periphery” samples (n=12) (Figure 3-1c). HK2 was expressed in both regions, but was higher in the “centre” compared to the “periphery” (p=0.04, Wilcoxon signed rank test) while HK1 was expressed similarly in both (p=0.20). IHC was performed for HK2 and HK1 on 25 paired GBM specimens, including the 12 samples examined by qRT-PCR, and 6 LGAs. Normal brain expressed very little HK2. Four of 6 LGAs expressed only low levels of HK2 and predominantly HK1 (Figure 3-1d). Twenty of 25 human GBM specimens expressed HK2 (80%), with 18 (90%) demonstrating increased HK2 expression in pseudopalisading cells of the “central” regions relative to infiltrating GBM cells in the “periphery”. HK1 expression was more variable across GBM samples and regions (Figure 3-1d).
Figure 3-1: HK2 is expressed in human GBMs and is associated with decreased overall survival.

a) Western blot of HK2 and HK1 in 3 normal human brains, 5 LGAs, and 12 GBM patients. HK2 was not expressed in normal brain and low grade astrocytomas but expressed to varying degrees in GBM samples. b) Tissue microarray was stained for HK2. The survival of 56 GBM patients was dichotomized by expression of HK2, with age as covariate. Cox regression p value is reported (p = 0.006). c) Real-time qRT-PCR of HK2 and HK1 in 12 paired GBM “centre” and “periphery” relative to normal human brain. Significantly stronger expression of HK2 in the “centre” compared to the “periphery” (p < 0.04, Wilcoxon signed rank test) but no significant change in HK1. d) H&E (scale = 100um) and IHC for HK1 (scale = 50um) and HK2 (scale = 50um) in representative perinecrotic “centre” of GBM, infiltrating “periphery” of GBM, LGAs, and normal brain. Strong induction of HK2 in perinecrotic regions compared to periphery while there is little expression in LGA and none in normal brain.

Figure 3-2: No difference in apoptotic index between “centre” and “periphery” of GBMs.

TUNEL immunohistochemistry showing similar % apoptotic indices in “centre” and “periphery” of representative GBM. Semi-quantitative analysis of average % apoptotic index within “centre” and “periphery” of 25 GBM samples, measured as the total number of TUNEL positive nuclei/total nuclei.
Figure 3-2

TUNEL

Centre

Periphery

Apoptotic index (%)

0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

Centre

Periphery
3.4.3 Knockdown of HK2 decreases tumour proliferation as well as cell survival, particularly under hypoxic conditions

To determine whether HK2 expression in perinecrotic pseudopalisading GBM cells confers a proliferative and/or cell survival advantage, we transfected scramble siRNA or HK2 siRNA into U87 and U373 human GBM cell lines. HK2 was almost completely knocked down 72 hours post-transfection (Figure 3-3a). Cell viability, measured by SRB assay and tryphan blue exclusion (cell count), was lower in U87 and U373 HK2siRNA transfected cells 120 hours post-transfection (p < 0.005 for HK2siRNA1 and HK2siRNA2) (Figure 3-3b). The decrease in cell viability was further enhanced by incubation under 2% hypoxia (p<0.005) (Figure 3-3c). Since HK2 expression is increased under hypoxic conditions (via its hypoxia response element), we wished to verify that hypoxia cannot overcome inhibition of HK2 by siRNA. Twenty-four hours of 2% hypoxia was not able to overcome the HK2siRNA (Figure 3-3d). Furthermore, there was no change in expression of glycolytic enzymes HK1 or downstream PKM2 with depletion of HK2.

To distinguish whether the decreased cell viability by HK2siRNA was due to decreased proliferation, increased cell death or both, we undertook BrdU incorporation and Caspase 3,7 activity assays. At 120hrs post-transfection of HK2siRNA, proliferation was significantly decreased (Figure 3-4), while the change in Caspase 3,7 activity was not pronounced under normoxic conditions (Figure 3-5a).
Figure 3-3: Inhibition of HK2 decreases cell viability particularly under hypoxia.

a) Western blot of HK2 in U87 and U373 transfected with either HK2 siRNA (50nM) or scramble (scr) siRNA (50nM) at 72 hours post-transfection. b) and c) Cell count in U87 and U373 cells transfected with mock, scramble siRNA or HK2siRNA at 120 hrs when incubated under normoxia (b) 2% hypoxia (c) (p < 0.005). d) Western blot of U87 and U373 scramble or HK2siRNA transfected incubated under 2% hypoxia for 24hrs showing HIF1α stabilization, which does not overcome HK2siRNA. There is no change in expression of HK1 or PKM2 with transient depletion of HK2.

Figure 3-4: Inhibition of HK2 decreases cell proliferation.

Decreased proliferation (BrdU incorporation) in U87 and U373 cells transfected with HK2 siRNA (50nM). In order to determine cell number, relative luminescent units was normalized to a standard curve with known number of cells.
Figure 3-3

**U87**

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2 siRNA1</th>
<th>HK2 siRNA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**U373**

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2 siRNA1</th>
<th>HK2 siRNA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**b**

Cell Viability (normoxia)

![Graph showing cell viability in normoxia](image)

*p < 0.005*

Hrs post-transfection

**c**

Cell Viability (2% hypoxia)

![Graph showing cell viability in 2% hypoxia](image)

*p < 0.005*

Hrs post-transfection

**d**

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2 siRNA1</th>
<th>HK2 siRNA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1α</td>
<td>120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK1</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKM2</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**U373**

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2 siRNA1</th>
<th>HK2 siRNA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-4

BrDU incorporation

**U87**

- U87 mock
- U87 scramble
- U87 HK2siRNA1
- U87 HK2siRNA2

* p < 0.05

**U373**

- U373 mock
- U373 scramble
- U373 HK2siRNA1
- U373 HK2siRNA2

* p < 0.05
3.4.4 Knockdown of HK2 sensitizes GBM cells to apoptosis by increasing mitochondrial membrane permeability and activating the intrinsic apoptotic pathway

Reduced cell counts of GBM cells depleted of HK2 may also reflect altered sensitivity to apoptotic inducers including chemotherapy and radiation, adjuvant treatment modalities used in therapy of human GBMs. Caspase 3/7 activity assay demonstrated increased levels in GBM cells depleted of HK2 in response to hypoxia, radiation and Actinomycin D (Figure 3-5a) (p<0.05). To complement the activated caspase 3/7 assay, AnnexinV-PI flow cytometry of U87 and U373 cells depleted of HK2 and incubated under hypoxia showed greater Annexin V positive cells, indicative of apoptotic cells (Figure 3-5b). HK2 knockdown also resulted in decreased GBM cell viability to Temozolomide (TMZ), the only current chemotherapy with demonstrated efficacy in the treatment of GBMs (Stupp et al., 2005), with more prominent sensitization to cell death in U87 cells than U373 cells (Figure 3-6). Collectively our data suggest that inhibition of HK2 significantly impairs proliferation (Figure 3-4) and sensitizes GBM cells to microenvironmental or therapeutic inducers of apoptosis (Figure 3-5).
Figure 3-5: Inhibition of HK2 sensitizes GBM cells to drug-, radiation- and hypoxia-induced apoptosis.

a) HK2 siRNA knockdown (50nM) led to significant increase in caspase 3, 7 activity in response to broad-spectrum neoplastic agent Actinomycin D, 5 Gy radiation and 24hr hypoxia (p <0.05). Caspase3,7 activity is represented as fold difference in relative fluorescence unit (RFU) relative to scramble siRNA under normoxic conditions. b) AnnexinV-PI FACS of U87 and U373 cells transfected with HK2 siRNA or scramble siRNA at 96 hours post-transfection after 24 hours of hypoxia showing enhanced apoptosis with loss of HK2.

Figure 3-6: Inhibition of HK2 sensitizes GBM cells to temozolomide.

Cell viability of U87 and U373, relative to mock transfected, was further reduced when treating with both HK2siRNA and TMZ (100uM) for 24hrs at 72 hrs post-transfection compared to HK2siRNA alone (p < 0.05).
Figure 3-5

a) Caspase 3.7 activity

<table>
<thead>
<tr>
<th></th>
<th>U87</th>
<th>U373</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normox</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Act D (10µM)</td>
<td>* *</td>
<td></td>
</tr>
<tr>
<td>Radiation (5 Gray)</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>24hr hypoxia</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Fold Difference in RFU (relative to scramble siRNA)

* p < 0.05

b) Annexin V/PI (2% Hypoxia)

<table>
<thead>
<tr>
<th></th>
<th>U87 scramble</th>
<th>U373 scramble</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL2-PI</td>
<td>3.8</td>
<td>30.3</td>
</tr>
<tr>
<td>FL1-Ann V</td>
<td>0.9</td>
<td>6.7</td>
</tr>
<tr>
<td>PI</td>
<td>95.1</td>
<td>60.9</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>U87 HK2 siRNA1</th>
<th>U373 HK2 siRNA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL2-PI</td>
<td>7.5</td>
</tr>
<tr>
<td>FL1-Ann V</td>
<td>10.1</td>
</tr>
<tr>
<td>PI</td>
<td>75.2</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
</tr>
</tbody>
</table>
In order to further study the role of HK2 in mitochondrial function and resistance to apoptosis, we created U87 GBM cell lines stably expressing two different short hairpin RNA towards HK2 (HK2shRNA1 and HK2shRNA2) as well as U87 scramble shRNA (Figure 3-7a). Since HK2 is located in the cytoplasm as well as at the outer mitochondrial membrane, we first determined whether stable loss of HK2 alters mitochondrial morphology or numbers using transmission electron microscopy (TEM) and immunofluorescence of live cells stained with a “mitotracker dye”. There was no difference in morphology or number of mitochondria between U87 scramble shRNA and U87HK2shRNA1 and U87HK2shRNA2 (Figure 3-7b,c). Furthermore, we performed FACS analysis of equal number of cells stained with Nonyl Acridine Orange (NAO) for mitochondrial mass (which stains cardiolipin) and found that this did not differ significantly either (FL1: U87scramble = 1906±337 counts, U87HK2shRNA1=1553±307 counts, U87HK2shRNA2=1675±289 counts).

HK2 has previously been reported to translocate to the mitochondria under certain growth factor signaling conditions, such as activation of AKT, and to interact with members of the permeability transition pore at the outer mitochondrial membrane (Majewski et al., 2004a; Pastorino et al., 2002). To determine the effect of stable loss of HK2 on mitochondrial membrane potential, when incubated under hypoxic conditions, live cells were stained with the ratiometric indicator JC-1 and analyzed with confocal microscopy. Greater depolarization in U87HK2shRNA1 cells was observed compared to U87 scramble shRNA under hypoxic conditions, as reflected by significant reduction in the red:green fluorescence ratio (p=0.014) (Figure 3-7d).

A critical step in the intrinsic apoptotic pathway is the release of pro-apoptotic factors, including cytochrome c, from the mitochondria. Previously HK2 was shown to regulate the release of cytochrome c via its role at the permeability transition pore (Majewski et al., 2004a). To determine whether stable loss of HK2 alters cytochrome c levels, we performed ELISA of cytosolic cytochrome c. Cytochrome c levels were found to be increased in U87HK2shRNA1 and 2 cells compared to U87 scramble shRNA (p<0.05), an effect that is more pronounced under hypoxic conditions (Figure 3-7e).
3.4.5 Stable loss of HK2 normalizes glycolytic metabolism

Since mitochondrial membrane permeability and function was altered with loss of HK2, we next wanted to determine the impact of loss of HK2 on oxidative glycolytic metabolism in GBM cells. Using western blotting, we probed for the expression of proteins of the electron transport chain (proteins of complexes I, II, III, IV and V) assisting in OXPHOS. These OXPHOS associated proteins were elevated in both U87HK2shRNA1 and U87HK2shRNA2 GBM cells, compared to U87 scramble shRNA (Figure 3-7a). There was no change in expression of downstream glycolytic enzyme PKM2 (Figure 3-7a). Extracellular lactate levels, the end-product of glycolysis, were reduced in the HK2 stably depleted cells (p<0.01) (Figure 3-7f). More direct evidence of a switch to OXPHOS metabolism was a greater than 2 fold increase in O$_2$ consumption in cells lacking HK2 compared to control cells (Figure 3-7g). Furthermore, we used qRT-PCR to measure gene expression of key transcriptions factors involved in mitochondrial function and biogenesis, including Peroxisome-Proliferator-Activated Receptor-Gamma Co-activator alpha (PGC1α) and mitochondrial transcription factor A (mTFA). Both genes were more highly expressed in GBM cells with loss of HK2 compared to scramble shRNA (p<0.05) (Figure 3-7h).

To validate the above in vitro results, we performed western blot of GBM specimens with high HK2 or low HK2 expression. All the GBM specimens expressed HK1, while PKM2 expression was present in samples with high HK2 expression in the GBMs. Additionally, GBMs with high HK2 expression tended to have lower expression of OXPHOS proteins and higher LDHA expression (Figure 3-8), compared to low HK2 but HK1-expressing GBM samples.

Collectively, cell line and operative specimens suggest that increased HK2 in human GBMs is associated with high aerobic glycolysis, while inhibition of HK2 restores normal OXPHOS.
Figure 3-7: Stable loss of HK2 alters mitochondrial function, including a return to oxidative phosphorylation and increased expression of genes involved in mitochondrial biogenesis.

a) Western blot of HK2, HK1, PKM2 and OXPHOS proteins in U87 scramble (scr) shRNA, U87HK2shRNA1 and U87HK2shRNA2. b) Representative images of transmission EM of U87 scr shRNA, HK2shRNA1 and 2 (scale=500nm). c) Representative images of live cells stained with Mitotracker Deep Red (seen as purple, scale=16um). d) Altered mitochondrial membrane potential with greater depolarization (green) in U87HK2shRNA1 after 24hr hypoxia. Panels i to iii are representative images of cells under identical exposure parameters (scale = 16um). Panel i) strong J-aggregates (red) in U87 scr shRNA relative to U87HK2shRNA1. In panel ii), increased monomers (green) in HK2shRNA1 representing mitochondrial membrane depolarization iii) bright field image, iv) merged image v) Average ratio of red:green fluorescent signal intensities (p = 0.014). e) ELISA of cytosolic cytochrome-c levels (ng/mL), relative to total protein content (ug/mL) under normoxic and hypoxic conditions. Increased cytosolic cytochrome-c in U87HK2shRNA under hypoxia compared to U87 scr shRNA (p < 0.05). Western blot of cytosolic and mitochondrial fractions of U87 scr shRNA and HK2shRNA cells, under normoxic and hypoxic conditions. VDAC is a marker of mitochondrial fraction and vinculin of the cytosolic fraction. f) Extracellular lactate levels were reduced in U87HK2shRNAs relative to scr shRNA (p < 0.01). g) Greater O₂ consumption in U87HK2shRNA (nmol O₂/million cells/min). h) qRT-PCR showing increased gene expression of PGC1α and mTFA in U87HK2shRNA1,2 relative to scr shRNA (p < 0.05). Data are represented as mean ± SEM.

Figure 3-8: Expression of LDHA and OXPHOS proteins in Human GBM samples.

Samples with high expression of HK2 showed greater expression of PKM2, LDHA and decreased OXPHOS not seen in GBM samples expressing HK1 but not HK2.
Figure 3-7 (continued)

**f**

![Graph showing lactate levels](image)

Lactate (relative to scr shRNA)

- scr shRNA
- HK2shRNA1
- HK2shRNA2

**p < 0.01**

**g**

![Graph showing O2 consumption](image)

O2 consumption (mmol/million cells/min)

- scr shRNA
- HK2shRNA1
- HK2shRNA2

**p < 0.05**

**h**

![Graph showing fold difference in expression](image)

Fold Difference in expression (relative to scr shRNA)

- scr shRNA
- HK2shRNA1
- HK2shRNA2

**PGC1α**

**mTFA**

**p < 0.05**
# Figure 3-8

<table>
<thead>
<tr>
<th></th>
<th>GBMs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kDa</td>
</tr>
<tr>
<td>HK2</td>
<td>100</td>
</tr>
<tr>
<td>HK1</td>
<td>100</td>
</tr>
<tr>
<td>PKM2</td>
<td>60</td>
</tr>
<tr>
<td>LDHA</td>
<td>35</td>
</tr>
<tr>
<td>V, F1a III</td>
<td>53, 48</td>
</tr>
<tr>
<td>II</td>
<td>30</td>
</tr>
<tr>
<td>IV, I</td>
<td>22, 20</td>
</tr>
<tr>
<td>Bactin</td>
<td>45</td>
</tr>
</tbody>
</table>
3.4.6 Differential role of HK2, HK1 and PKM2 on GBM metabolism and proliferation

To determine whether HK1, which like HK2 converts glucose to glucose-6-phosphate (Wilson, 2003), and PKM2 previously implicated in aerobic glycolysis in other cancers (Christofk et al., 2008a) also regulate aerobic glycolysis in GBMs, shRNAs towards HK1 and PKM2 were generated (Figure 3-9a). Similar to loss of HK2 (Figure 3-7b,c), loss of HK1 and PKM2 did not appear to significantly alter mitochondrial number or morphology (Figure 3-9b) and resembled U87 scr shRNA (Figure 3-7c). Furthermore, overexpression of HK1-GFP in U87 cells depleted of HK2 resulting in the increase in total hexokinase activity back to control levels (Figure 3-9c), was not able to rescue extracellular lactate (Figure 3-9d), or inhibit OXPHOS restoration seen with HK2 knockdown (Figure 3-9e). This was despite total hexokinase activity levels being similar to control U87 cells expressing scr shRNA.

To determine whether alteration in HK2, HK1 or PKM2 altered glucose flux, pyruvate levels were measured. Relative to control cells, depletion of HK2 led to increase in pyruvate levels (almost double) while loss of HK1 resulted in decrease pyruvate levels (84% relative to control) (Figure 3-9f). Correspondingly, expression of pyruvate dehydrogenase E1a (PDHE1a), which catalyzes the conversion of pyruvate to acetyl-coA in the mitochondria to undergo OXPHOS, was elevated with loss of HK2 but not HK1 (Figure 3-9a). Interestingly, depletion of PKM2 resulted in elevated total HK activity (Figure 3-9c) suggesting compensatory mechanisms engaged by cells, and resulting in modest but not significant increases in pyruvate and lactate levels and a small increase in PDH expression (Figure 3-9a,d,f).

Collectively, our experimental findings from our knockdown and overexpression studies strongly support HK2 and not HK1 or PKM2, as being the main regulator of aerobic glycolysis or the Warburg effect in human GBMs.
Figure 3-9: Differential role of HK2, HK1 and PKM2 in GBM mitochondrial metabolism and survival.

a) Western blot of HK2, HK1, PKM2 in U87 scramble shRNA, U87 HK2shRNA1, U87 HK1shRNA and U87 PKM2shRNA, U87 HK2shRNA + HK1-GFP. Bands are taken from the same non-contiguous gel. b) Immunofluorescence of U87 HK1shRNA and PKM2shRNA cells stained with Mitotracker. c) Total hexokinase activity, normalized to U87 scramble shRNA, decreased in cells with knockdown of HK2 and HK1 but restored with overexpression of HK1 in cells with HK2 knockdown. d) Extracellular lactate levels were only significantly reduced in U87 HK2shRNA1 and were not found to return to scramble shRNA levels upon overexpression of HK1-GFP. e) O2 consumption was increased in U87HK2shRNA1 but not in the other cell lines. f) Pyruvate levels in the different cell lines relative to scr shRNA. Cells with loss of HK2 have increased pyruvate while cells with loss of HK1 show decreased pyruvate levels. Loss of PKM2 shows only modest increase in pyruvate levels.
3.4.7 Mechanisms of over-expression of HK2 in GBMs

Many potential mechanisms may mediate the over-expression of HK2 in GBM cells lines and human GBM tissue. Although HK2 amplification has been reported in hepatocarcinomas (Rempel et al., 1996), high throughput analyses conducted by the TCGA and other groups have not identified amplification within chromosome 2p13 in GBMs (data accessed March 2010) (Cancer Genome Atlas Research, 2008; Parsons et al., 2008). Regulatory mechanisms previously implicated in HK2 over-expression include its transcriptional regulation by key proteins including p53, HIF1α, myc among others (Mathupala et al., 2006). The importance of HK2 compared to HK1 in gliomagenesis in general is demonstrated by the increased expression of HK2 to a greater extent than HK1 in partly transformed astrocytes over-expressing oncogenes such as Ras or loss of tumour suppressors including p53 compared to the normal astrocyte counterpart (Figure 3-10).

Figure 3-10: Induction of HK2 with expression of Ras oncogene or loss of tumour suppressor p53.

Western blot of HK2, HK1 and PKM2 in Normal Human Astrocytes (NHAs) or Normal Murine Astrocytes showing greater induction of HK2 with expression of Ras in NHAs or with loss of p53 in NMAs.
<table>
<thead>
<tr>
<th></th>
<th>NHA</th>
<th>NHA-Ras</th>
<th>NMA</th>
<th>NMA-p53-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK1</td>
<td>102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKM2</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bactin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4.8 Translocation of HK2, and not HK1, to the OMM after growth factor stimulation promotes GBM proliferation

Previous studies have shown that growth factor stimulation, resulting in activation of PI3K/AKT pathway, promotes translocation of HK2 to the OMM, binding of which increases its protein stability (Bustamante and Pedersen, 1977; Gottlob et al., 2001). To test this in GBMs, U87 cells were treated with the protein synthesis inhibitor cycloheximide (CHX) with +/- EGF stimulation. Without EGF, substantially more HK2 protein was degraded following 12hrs of CHX, while HK2 protein levels were maintained for up to 20hrs after +EGF (Figure 3-11).

Figure 3-11: Increased HK2 protein stability when EGF stimulated.

U87 cells treated with the protein inhibitor cycloheximide (CHX) in the presence or absence of EGF stimulation showing enhanced HK2 protein stability under EGF stimulation. Densitometric measurements are seen below the western blots.
Figure 3-11

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>0</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HK2</td>
<td>102</td>
<td>100</td>
<td>89</td>
<td>83</td>
<td>75</td>
</tr>
<tr>
<td>Bactin</td>
<td>45</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>0</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EGF</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HK2</td>
<td>102</td>
<td>100</td>
<td>98</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td>Bactin</td>
<td>45</td>
<td>89</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To evaluate the extent of HK2 and HK1 translocation after EGF stimulation, U343 GBM cells which do not express HK2 but have some HK1 expression and are not able to grow *in vivo* were utilized along with U87 cells which express high levels of HK2 and HK1. U343 and U87 cells were transfected to express HK1-GFP or HK2-GFP and +/-EGF stimulation. U343 HK2-GFP (green) after +EGF co-localized to the mitochondria (blue) to yield light blue colour (Pearson’s correlation coefficient R=80±8.9%), in comparison to -EGF conditions (R=52±12%) (p< 0.05) (Figure 3-12). In contrast, HK1-GFP was found predominantly at mitochondria irrespective of EGF stimulation (-EGF: R=85±7%, +EGF: R=83±8%). In U87 cells, a greater proportion of HK2-GFP localized to the mitochondria under -EGF conditions (R=74±14%, +EGF = 81%±12%) compared to U343 cells (Figure 3-13a). Similar to U343 cells, HK1-GFP localization was predominantly mitochondrial in U87 cells and not altered by +/-EGF. The difference in HK2 localization under -EGF conditions may be explained by increased basal pAKT levels in U87 compared to U343 cells, although pAKT can be strongly induced in U343 cells with EGF stimulation (Figure 3-13b). In support of these findings, disruption of PI3K/AKT signaling using an EGFR tyrosine kinase inhibitor (AG1478; 24hrs at 2uM) in U87 cells decreased HK2 localization to the mitochondria (R=45±9%) (Figure 3-13a,c).

To be noted, mitochondrial fractionation of cells transfected with HK2-GFP under starved or stimulated conditions was attempted. However, technical issues arose which may be due to HK2-GFP constructs not adhering as well or being stripped off the outer membrane by detergents used in the fractionation kit. Endogenous HK2 levels at the OMM were found to be reduced in U87 cells when treated with 24 hrs of AG1478 (Figure 3-13d).

A previous study reported that AKT can phosphorylate HK2 in cardiomyocytes resulting in greater localization of HK2 to the outer mitochondrial membrane (Miyamoto et al., 2008). We wished to determine whether the same occurs in GBM cells. HK2 has a potential AKT consensus sequence ranging between amino acids 468-474. We performed immunoprecipitation of HK2 under different conditions including EGF stimulation and
probed using the phospho-Ser/Thr e AKT substrate specific antibody. No phosphorylation of HK2 was detected (Appendix 3a). In a further attempt to identify potential phosphorylation of HK2, we stimulated U87 cells with EGF (1 hour), immunoprecipitated HK2, performed proteolytic digestion followed by mass spectrometry for phospho-enriched peptides. No phospho-peptides could be identified on mass spectrometry (Appendix 3b).

Figure 3-12: GF/PI3K/AKT signaling is important for localization of HK2 but not HK1 to outer mitochondrial membrane in U343 cells.

Immunofluorescence of U343 HK2-GFP and HK1-GFP cells, under ± EGF stimulated conditions, showing co-localization of HK2-GFP and HK1-GFP with mitochondria labeled with Mitotracker Deep Red (blue). Mitochondrial co-localization is seen in light blue in merged images (scale = 16um). T2-GFP is a mutant HK2-GFP that lacks the mitochondrial localization signal. R = Pearson’s correlation coefficient.

Figure 3-13: GF/PI3K/AKT signaling is important for localization of HK2 but not HK1 to outer mitochondrial membrane in U87 cells.

a) Immunofluorescence of U87 HK2-GFP and HK1-GFP cells, under ± EGF stimulated conditions, and T2-GFP, showing co-localization of HK2-GFP and HK1-GFP with mitochondria labeled with Mitotracker Deep Red (blue). Mitochondrial localization of HK2-GFP but not HK1-GFP is disrupted with treatment of EGFR tyrosine kinase inhibitor AG1478 (2uM). Mitochondrial co-localization is seen in light blue in merged images (scale = 16um). b) Constitutive phosphoSer473-AKT in U87 cells under starved conditions but not in U343 cells. Strong induction of pAKT in U343 cells with EGF stimulation. c) Western blot showing reduced phosphoSer473-AKT levels with treatment of U87 cells with 24hrs of AG1478 (2uM). d) Fractionation of U87 cells ± inhibitor AG1478 into cytosolic (C) and mitochondrial (M) fractions. Reduced HK2 in both cytosolic and mitochondrial fraction in cells treated with AG1478. Vinculin is a marker of cytosolic fraction and VDAC of mitochondrial fraction.
Figure 3-12

<table>
<thead>
<tr>
<th>Condition</th>
<th>GFP</th>
<th>Mitotracker</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>U343 starved + HK2-GFP</td>
<td>![GFP Image]</td>
<td>![Mitotracker Image]</td>
<td>![Merged Image]</td>
</tr>
<tr>
<td>U343 + EGF + HK2-GFP</td>
<td>![GFP Image]</td>
<td>![Mitotracker Image]</td>
<td>![Merged Image]</td>
</tr>
<tr>
<td>U343 + T2-GFP</td>
<td>![GFP Image]</td>
<td>![Mitotracker Image]</td>
<td>![Merged Image]</td>
</tr>
<tr>
<td>U343 starved + HK1-GFP</td>
<td>![GFP Image]</td>
<td>![Mitotracker Image]</td>
<td>![Merged Image]</td>
</tr>
<tr>
<td>U343 + EGF + HK1-GFP</td>
<td>![GFP Image]</td>
<td>![Mitotracker Image]</td>
<td>![Merged Image]</td>
</tr>
</tbody>
</table>
Figure 3-13

a

<table>
<thead>
<tr>
<th>Condition</th>
<th>GFP</th>
<th>Mitotracker</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 starved + HK2-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87 + EGF + HK2-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87 T2-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87 + AG1478 + HK2-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87 starved + HK1-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87 + EGF + HK1-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b

<table>
<thead>
<tr>
<th></th>
<th>U87</th>
<th>U343</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starved</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EGF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HK2</td>
<td>102</td>
<td>-</td>
</tr>
<tr>
<td>Ser473-pAKT</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>AKT</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td>-</td>
</tr>
</tbody>
</table>

AG1478

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Ser473-pAKT</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>AKT</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

AG1478

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>VDAC</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Vinculin</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>
We next determined the impact of over-expressing HK2 on the growth of U343 cells, which do not express HK2 endogenously. Proliferation of U343 HK2-GFP cells, measured via BrdU incorporation and normalized to a standard curve, was increased compared to control (GFP) and U343 HK1-GFP expressing cells after 3 days of +EGF (Figure 3-14a). To characterize the contribution of glucose phosphorylation and mitochondrial binding of HK2 to its effects on proliferation, U343 cells were transfected with truncated HK2 (T2), mutant HK2 (M2) and a combined truncated and mutant HK2 (TM2) cDNA, all previously characterized in HEK293 cells (Sun et al., 2008). The T2-HK2 construct lacks the N-terminal mitochondrial-binding domain, hence can phosphorylate glucose but cannot bind mitochondria. In contrast, the catalytically inactive M2-HK2 construct retains mitochondrial-binding ability, but cannot phosphorylate glucose. Cells transfected with HK2-GFP proliferated more by day 3 compared to T2-GFP (p < 0.05) and control GFP (p < 0.003). As such, both glucose phosphorylation and mitochondrial binding contributed to enhanced proliferation in cells transfected with HK2 constructs, although glucose phosphorylation to a greater extent (as seen by greater reduction in proliferation with M2-GFP construct compared to T2-GFP construct) (Figure 3-14a). In addition to enhanced proliferation, HK2-GFP overexpression increased extracellular lactate levels (~11%, Figure 3-14b). This was not observed with HK1-GFP overexpression, even though total hexokinase activity was similar between U343 transfected HK2-GFP and HK1-GFP cells, both of which were greater than control U343 cells (p < 0.05) (Figure 3-14c).
Figure 3-14: Both glucose phosphorylation and mitochondrial localization contribute to HK2’s growth promoting effects in GBMs over and above HK1.

a) BrdU incorporation assay showing greater proliferation by 3 days in U343 cells over-expressing HK2-GFP compared to control (GFP only) transfected u343 cells (p < 0.003). HK2-GFP cells had greater proliferation than T2-GFP transfected cells (p < 0.05). No significant difference between HK1-GFP, M2-GFP, TM2-GFP and control transfected cells. Cell number was derived by comparing RFUs to a standard curve with known number of cells. b) Extracellular lactate levels increased by 11% in cells over-expressing HK2-GFP but not HK1-GFP relative to U343 control cells (p < 0.05). c) Total hexokinase activity in U343 transfected cells with HK2-GFP and HK1-GFP relative to control U343 cells (p < 0.05).
Figure 3-14

(a) Cell number over days 1, 2, and 3 for different conditions:
- U343 control
- U343 HK2-GFP
- U343 M2-GFP
- U343 T2-GFP
- U343 TM2-GFP
- U343 HK1-GFP

Significance:
- p < 0.05
- p < 0.003 relative to control

(b) Lactate level (relative to control):
- U343 control
- U343 HK2-GFP
- U343 HK1-GFP

Significance:
- * p < 0.05 relative to control

(c) HK activity (relative to U343 control):
- U343 control
- U343 HK2-GFP
- U343 HK1-GFP

Significance:
- * p < 0.05 relative to control
3.4.9 Subcutaneous growth of GBM cells is impaired when HK2 deficient

To determine the effect of stable loss of HK2 on *in vivo* tumour growth within the subcutaneous microenvironment, we injected 1.5 million U87 scramble shRNA (n=7) or U87 HK2shRNA1 cells (n=8) subcutaneously into NOD-SCID mice and followed their growth. By 4 weeks, the U87 scramble shRNA group had substantial tumours while no visible tumour was seen in mice injected with U87HK2shRNA1 (*Figure 3-15a*). By 8 weeks, 3 of 8 mice injected with HK2shRNA1-expressing U87 cells were tumour free. HK2shRNA1 tumours had a much smaller mean tumour volume at 8 weeks compared to scramble shRNA tumours harvested at 4 weeks (p<0.001) (*Figure 3-15a*). The scr shRNA tumours showed large regions of necrosis (N) and HIF1\(\alpha\) positivity with decreased apoptosis as measured by cleaved caspase 3 compared to U87HK2shRNA1 xenografts (1% vs 4%; p < 0.05) (*Figure 3-15b*). Furthermore, the U87HK2shRNA1 xenografts that grew demonstrated upregulation of the cell cycle inhibitors p27\(^{KIP1}\) (23% vs. 7%; p< 0.05) (*Figure 3-15b*) compared to scr shRNA xenografts.

*Figure 3-15: Stable loss of HK2 decreases in vivo GBM tumour growth in a subcutaneous xenograft model.*

a) Mean tumour volume of subcutaneous NOD-SCID xenografts at time of necropsy for U87 scr shRNA (n = 7) at 4 weeks and U87HK2shRNA1 (n = 8) at 8 weeks (p < 0.001).
b) Histopathology of subcutaneous U87 scr shRNA and U87HK2shRNA1 tumours including H&E staining (scale = 200um), HK2 (scale = 100um), HIF1\(\alpha\) (scale = 100um), cleaved caspase 3 (scale = 100um) and p27\(^{KIP1}\) (scale = 50um). Necrosis (N) is observed in U87 scr shRNA tumours but not HK2shRNA1.
**Figure 3-15**

(a) Subcutaneous Xenograft

![Box plot showing tumor volume comparison between scr shRNA (4 weeks) and HK2shRNA1 (8 weeks). The p-value is < 0.001.](image)

(b) Immunohistochemistry images comparing scr shRNA and HK2shRNA1

- H&E
- HK2
- HIF1α
- cleaved caspase 3
- p27kip1
3.4.10 HK2 regulates survival and invasion in orthotopic xenograft models of GBM

Since loss of HK2 had a profound impact on growth in our ectopic GBM model, we sought to investigate the response in a more physiologically relevant model. We injected intracranially 200,000 cells of U87 control (either U87 scramble shRNA or U87-empty vector; n=10), U87HK2shRNA tagged with RFP (n=10), U87 overexpressing HK2-GFP (n=10) or a mixed U87 HK2shRNA-RFP+U87HK2-GFP group (n=6). Mice injected with U87HK2shRNA survived significantly longer than those injected with U87 control, U87HK2-GFP or mixed cells (p<0.01) (Figure 3-16a). HK2 overexpressing mice or the mixed group did not live as long as the U87 control group (p<0.01). No difference in survival or pathology was observed between the U87HK2-GFP and mixed group; however, only the U87HK2-GFP cells were observed in mixed tumours, reflecting a clear growth advantage for cells overexpressing HK2 (Figure 3-16a,b,d).

The majority of tumours with loss of HK2 did not form a large tumour bulk seen for U87HK2-GFP and U87 control groups (Figure 3-16b). However, U87HK2shRNA-RFP tumour cells were found invading within perivascular Virchow-Robin spaces and tracking along leptomeninges in both hemispheres, a phenomenon not seen in U87 control tumours. Extent of invasiveness was quantified by measuring the mean distance (um) of invading tumour clusters away from the tumour mass and was increased in both U87HK2-GFP and U87HK2shRNA-RFP tumours compared to U87 control (p<0.01) (Figure 3-16c). However, the invasion by the over-expressing HK2 tumours was more local into the adjacent parenchyma and not the long distance perivascular invasion present in the U87HK2shRNA-RFP tumours.

The tumour vasculature (vWF positivity) was reduced in U87HK2shRNA-RFP. HK2shRNA-RFP tagged tumour cells appeared to cluster along existing mouse brain vessels (Figure 3-16d,e), although this was not formally assessed. In comparison, U87HK2-GFP tumours showed numerous irregularly shaped and distorted vessels, especially at the leading edge (Figure 3-16d,e) to be contrasted with the smaller vessels found throughout the U87 control tumours. The U87HK2-GFP and U87 controls, but not
the tumours with decreased HK2, showed sporadic expression of HIF1α and expression of VEGF (Figure 3-16e). U87HK2shRNA-RFP tumours had a decreased proliferative index and stained strongly for nuclear p27^Kip1, suggesting cell cycle arrest (Figure 3-16e,f). In comparison, the U87HK2-GFP tumours had a higher proliferative index (MIB1), even compared to U87 controls (Figure 3-16e,f). Cleaved caspase 3 levels were elevated in tumours with reduced HK2 (12±3.5%) compared to control (3±1.5%) and HK2-GFP (2±1.9%) (p < 0.05) (Figure 3-16e). In summary, decreased HK2 restricted growth of human GBMs within the intracranial environment with decreased angiogenesis and yet enhanced perivascular invasion.
Figure 3-16: HK2 regulates GBM survival and invasion in an orthotopic xenograft model.

a) Survival curve of mice injected intracranially with 200,000 cells of U87HK2shRNA, U87 control, U87 overexpressing HK2-GFP and U87 mixed group (HK2-GFP/HK2shRNA-RFP). B) H&E stain of U87 HK2-GFP, U87 control and U87HK2shRNA tumours, showing presence of large tumour bulk in HK2-GFP and control tumours but not in tumours lacking HK2 (scale = 2000um). c) Extent of invasiveness was significantly greater in U87HK2-GFP and U87HK2shRNA tumours relative to U87 control (p < 0.01), determined by measuring the mean distance (um) of invading tumour clusters away from the main tumour mass. d) Top-Immunofluorescence of U87HK2-GFP, U87 mixed and U87HK2shRNA-RFP injected tumours (scale = 28um). In the U87 mixed tumours, the GFP labeled U87HK2-GFP cells are dominant, demonstrating a growth advantage by the HK2-overexpressing cells; Bottom- Staining of the vasculature (vWF) showing the presence of abnormal and distorted vessels in U87HK2-GFP and U87 mixed tumours, while U87HK2shRNA tumours did not show numerous vessels and appeared to aggregate around larger vessels (scale = 39um). e) Histopathology of U87HK2-GFP, U87 control and U87HK2shRNA intracranial tumours including vWF (scale = 100um), VEGF (scale = 100um), HIF1α (scale = 50um), MIB1 (scale = 100um), cleaved caspase-3 (scale = 100um), p27KIP1 (scale = 100um), and HK2 (scale = 100um), f) Semi-quantification of percentage of cells staining for MIB1 and p27KIP1 over total number of cells in U87HK2-GFP, U87 control and U87HK2shRNA tumours.
Figure 3-16

**Intracranial xenograft**

(a) Survival curve showing the survival rate over time (days).

(b) Images of U87 HK2-GFP, U87 control, and U87 HK2shRNA groups.

(c) Bar graph showing distance (μm) with statistical significance (*p < 0.01 relative to control). ** indicates p < 0.01.

(d) Images showing vascularity for U87 HK2-GFP, U87 mixed, and U87 HK2shRNA-RFP.

(e) Immunohistochemistry images for vWF, VEGF, HIF1α, MIB1, p27kip1, Cleaved caspase 3, and HK2.

(f) Graph showing the percentage of positive nuclei relative to total nuclei with statistical significance (*p < 0.05, **p < 0.01 relative to control).
3.5 Discussion

Proliferating cells employ aerobic glycolysis to assist in their growth (Vander Heiden et al., 2009). Warburg originally hypothesized that cancer cells preferentially shift to glycolysis due to mitochondrial mutations (Warburg, 1956). Recent data supports that mitochondrial dysfunction in cancers is reversible with active suppression of OXPHOS, resulting in enhanced proliferation, apoptosis resistance and invasion (Bonnet et al., 2007; Gatenby and Gillies, 2004; Plas and Thompson, 2002). This study provides evidence that the enzyme HK2, expressed in a large percentage of GBMs but little in normal brain or LGAs (Figure 3-1), is a major regulator of the Warburg Effect in GBMs.

Despite a hypoxic microenvironment in the perinecrotic regions, we and others have demonstrated limited apoptosis in GBMs in vitro and in vivo with average indices below 1-2% (Figure 3-2)(Heesters et al., 1999; Kuriyama et al., 2002). These perinecrotic regions were associated with high levels of HK2 vs. the periphery. GBMs expressing HK2 had a worse prognosis (Figure 3-1b), corroborating a previously published report (Dong et al., 2005). HK2 impacts GBM growth by providing 1) a cell survival advantage via its effects on the intrinsic apoptotic pathway (Figure 3-5, 3-7) and 2) a proliferative advantage associated with enhanced aerobic glycolysis (Figure 3-4, 3-14).

Mechanistically, our in vitro experiments demonstrate a role for HK2 in survival by depolarizing mitochondrial membrane potential, thereby altering mitochondrial function and regulating intrinsic apoptosis, seen particularly under hypoxic conditions (Figure 3-7). This is consistent with previous reports of a role for HK2 at the outer mitochondrial membrane interacting with the permeability transition pore (Majewski et al., 2004a; Pastorino et al., 2002). Inhibiting HK2 in GBM cells in vitro resulted in increased sensitivity to apoptosis, seen by increased release of cytochrome c and activation of downstream caspases, when subjected to apoptotic stimuli such as chemotherapy and radiotherapy (Figure 3-5). These results have important therapeutic implications supporting the strategy of targeting HK2’s interaction with the mitochondria with ensuing normalization of mitochondrial membrane potential and sensitization to chemotherapeutic agents.
Our experimental results also demonstrate that HK2, and not HK1 or PKM2, alters mitochondrial function by impacting OXPHOS in addition to its effects on intrinsic apoptotic regulation. We demonstrate that stable HK2 downregulation in GBM cells, but not HK1 or PKM2, inhibited aerobic glycolysis as seen by a return in the expression of OXPHOS proteins, increased O$_2$ consumption and reduced lactate (Figure 3-7, 3-9). One possible mechanism through which depletion of HK2 can promote a return to OXPHOS is by increased expression of the transcription factors PGC1$\alpha$ and mTFA, important for the expression of metabolic-associated genes including OXPHOS genes (Figure 3-7g). PGC1$\alpha$ has previously been shown to act as a dominant regulator of mitochondrial function, respiration and biogenesis (Rohas et al., 2007). Considering recent evidence of the role of the inactive dimeric form of PKM2 in promoting aerobic glycolysis (Christofk et al., 2008a), it is surprising that decreased total PKM2 did not significantly alter lactate levels in our GBM cells. However, total HK activity was increased with loss of PKM2 suggesting reduced feedback inhibition. The addition of HK1 to cells lacking HK2, resulting in rescue of total hexokinase activity, did not rescue the phenotype of aerobic glycolysis in GBM cells.

Upon growth factor stimulation and activation of AKT, HK2 protein has been shown to undergo translocation to the outer mitochondrial membrane and mediate survival and anti-apoptotic effects via its interaction with VDAC in HeLa cells, MEF or Rat1a fibroblasts either in a Bax and/or MPTP dependent or independent fashion (Majewski et al., 2004a; Pastorino et al., 2002). Mitochondrial binding of HK2 is believed to yield several other advantages including reduced sensitivity to feedback inhibition by its product glucose-6-phosphate, greater protein stability and enhanced glycolytic flux and overall ATP levels by preferentially utilizing mitochondrial ATP (Bustamante and Pedersen, 1977). The results of this study support the importance of the GF/PI3K/AKT signaling in maintaining HK2 localization to the mitochondria but not that of HK1 (Figure 3-12, 3-13). Interruption of this pathway or lack may result in reduced localization of HK2 to the mitochondria and potentially faster protein degradation (Figure 3-11). However, we were not able to prove that AKT directly phosphorylates HK2. Furthermore, overexpression of HK2 but not HK1 in U343 GBM cells promoted aerobic glycolysis and cell proliferation, particularly under growth factor stimulation.
associated with greater mitochondrial localization of HK2 (Figure 3-14). The exact mechanisms of how HK2 inhibits OXPHOS and promotes proliferation and lactate formation remain unclear however both glucose phosphorylation and mitochondrial localization of HK2 were found to contribute, although glucose phosphorylation to a much greater extent (Figure 3-14). U343 GBM cells do not grow in vivo and thus it would be of interest to determine whether stable over-expression of HK2 can promote in vivo tumourigenesis.

Consistent with both our knockdown and over-expression studies demonstrating a crucial role for HK2 in aerobic glycolysis, expression profiling of developing embryos revealed greater expression of HK2 at the earlier embryonic stages including the blastocyst stage (data accessible at NCBI GEO database (Zeng et al., 2004), accession GSE1749), a switch deemed necessary to accommodate for the large increase in proliferation and reliance on aerobic glycolysis. Mice homozygous for HK2 deficiency die at approximately 7.5 days post-fertilization, indicating that HK2 is necessary for mouse embryogenesis after implantation and before organogenesis (Heikkinen et al., 1999). Taken all together, these results strongly favour that the isoform HK2 is a major regulator of the Warburg Effect in GBMs.

Since HK2 expression is limited in the normal adult brain, targeting HK2 may be a way of selectively killing GBM cells with limited toxicity. Consistent with this therapeutic potential, growth of GBM xenografts subcutaneously was reduced drastically when stably depleted of HK2 (Figure 3-15), with increased apoptosis and expression of cell cycle inhibitors p27Kip1. Similar to the subcutaneous tumours, HK2 reduction inhibited intracranial GBM growth, with significant prolongation of survival (Figure 3-16). Mixing experiments with HK2 depleted and HK2-overexpressing GBM cells clearly demonstrated a proliferative advantage of the latter (Figure 3-16a,d).

In contrast to the poorly vascularized subcutaneous microenvironment, HK2-depleted tumour cells in the brain, a highly vascular microenvironment, were much more invasive and tracked along perivascular spaces deep in both hemispheres (Figure 3-16b-d). The increased invasive nature is highly reminiscent of the longer-term effects of VEGF.
inhibition and anti-angiogenic therapies on GBM growth in intracranial models and human clinical trials (Blouw et al., 2003; Paez-Ribes et al., 2009). A possible molecular explanation of the increased invasion by GBMs may involve HIF1α, which was decreased relative to control tumours or tumours overexpressing HK2. Reduction of HIF1α would inhibit its transcriptional targets such as VEGF, and provide a plausible explanation of the phenotypic similarity between HK2 knockdown, VEGF or HIF1α knockdown in *in vivo* GBM models (Blouw et al., 2003; Paez-Ribes et al., 2009). Tumours that overexpressed HK2 also showed greater infiltration than control tumours (*Figure 3-16c*), although this was a much more local invasion, potentially involving different mechanisms than the long distance perivascular invasion seen with HK2 inhibition. In summary, our orthotopic xenograft results indicate that singular permanent reduction of aerobic glycolysis by inhibiting HK2 reduces tumour growth and increases survival; however, one must be cognizant that the growth pattern of a localized highly proliferative and vascular GBM may be changed to one that is slow-growing yet infiltrating. Whether the increased sensitivity to radiation and chemotherapy conferred by reduction of HK2, as per our *in vitro* results offsets this longer-term increased invasion requires further *in vivo* evaluation. Likely, additional current and future biological therapies in conjunction with HK2 targeting will be required.

The exact mechanism though which HK2 may be exerting anti-apoptotic effects are not clear and have been reported to be both dependent and independent on the permeability transition pore. In this study, although we saw greater release of cytochrome c and activation of caspases when cells were depleted of HK2, we did not address the exact mechanism though which HK2 may be interacting with the permeability transition pore or the relative importance of other putative anti-apoptotic mechanisms including: a) inhibition of MPTP and cytochrome c release via interaction at VDAC, b) inhibition of OXPHOS thereby decreasing sensitization of cells to Bax-induced apoptosis c) inhibition of OXPHOS thereby reducing generation of ROS and oxidative stress d) increase in NAPDH formation via the PPP resulting in increased anti-oxidant functions. Furthermore, the mechanisms by which HK2 may be promoting proliferation are unclear. HK2 expression in GBMs may promote proliferation and inhibit apoptosis by impacting activation of cell cycle inhibitors including p53 and p27 and potentially DNA repair.
enzymes important in resistance to temozolomide (MGMT, APNG). The next chapter is a preliminary attempt to elucidate the mechanisms implicated in reduced proliferation and sensitivity to apoptosis with loss of glycolytic enzymes HK2, HK1 and PKM2.
4.1 Abstract

Cells have central metabolic sensing pathways that regulate nutrient uptake, proliferation and cell death. The EGFR/PI3K/AKT signaling pathway is activated in many cancers including Glioblastoma Multiforme (GBMs), mediating cell growth and proliferation. AMPK is a metabolic checkpoint downstream of the LKB1 tumour suppressor that integrates growth factor receptor signaling with cellular energy status. The AMPK and PI3K/AKT pathways converge and regulate mTORC1 function and cell cycle progression. This study aimed at investigating the impact of loss of glycolytic enzymes HK1, HK2 and downstream PKM2 in GBM cells on PI3K/AKT and AMPK signaling, cell proliferation, cell death and in vivo growth. Our results support that targeting glycolytic enzymes impacts in vitro and in vivo proliferation; however, glycolytic enzymes may differentially influence the PI3K/AKT/mTOR and AMPK signaling, thereby potentially influencing the mode of cell death.

4.2 Introduction

Glioblastoma Multiforme (GBM) is the most common malignant adult primary brain tumour that remains highly resistant to cell death inducing therapies including radiation and chemotherapy. One potential therapeutic approach is to sensitize or prime tumours cells to apoptosis. GBMs are highly glycolytic tumours, generating lactate and over-expressing many enzymes of glycolysis, particularly HK2 (see Chapter 3) (Oudard et al., 1996; Oudard et al., 1997). Targeting metabolic enzymes may offer one approach to sensitizing tumour cells to radiation and/or chemotherapy in GBMs. Disruption of the binding of the glycolytic enzyme HK2 to VDAC at the outer mitochondrial membrane has been shown previously to potentiate chemotherapy-induced cytotoxicity in certain cancer cells, including GBMs (Chapter 3) (Pastorino et al., 2005). The combination of 2-deoxyglucose, an inhibitor of HK and glucose uptake, along with chemotherapies including cisplatin has been effective in promoting apoptosis in preclinical studies of
different cancers (Ahmad et al., 2008; Simons et al., 2007). Dichloroacetate (DCA) has recently been proposed as an anti-cancer agent by shifting metabolism from glycolysis to glucose oxidation via the inhibition of mitochondrial pyruvate dehydrogenase kinase (PDK). DCA was shown to decrease mitochondrial membrane potential, increase ROS and eventual lead to the induction of apoptosis (Bonnet et al., 2007). The combination of DCA with radiation and temozolomide is currently in Phase 1 clinical trials in Alberta in patients with newly diagnosed GBMs.

Cells have important metabolic sensing pathways that regulate nutrient uptake, proliferation and cell death. The EGFR/PI3K/AKT/mTOR signaling pathway is activated in many cancers including GBMs (Mellinghoff et al., 2005). To mediate cell growth and proliferation, mTOR acts through the canonical PI3K pathway via 2 distinct complexes, mTORC1 and mTORC2. mTORC1 uniquely integrates growth factor signaling through S6K1 promoting protein translation and cell growth (Engelman, 2009). However, strategies aimed at targeting mTOR (e.g. rapamycin) are limited in their effectiveness, partially due to a feedback loop to the mTORC2 complex (Cloughesy et al., 2008). AMPK is a metabolic checkpoint downstream of the LKB1 tumour suppressor that integrates growth factor receptor signaling with cellular energy status. AMPK inhibits mTORC1 by activating the tuberous sclerosis complex 2 and by direct inhibitory phosphorylation of the mTOR binding partner raptor to limit cellular proliferation (Shackelford and Shaw, 2009). Furthermore, AMPK activation results in suppression of anabolic pathways of fatty acid and cholesterol synthesis and promotes catabolism of glucose and fatty acids (Carling et al., 1994). Therefore, activation of AMPK is currently being investigated as potential therapeutic approach including in GBMs due to its inhibitory effects on mTOR signaling and also lipogenesis important for proliferation (Guo et al., 2009). AMPK activation has also been shown to directly result in metabolic induced arrest at the G1/S transition by phosphorylation of p53 at Ser15, which can occur without significantly affecting translation (Jones et al., 2005). The PI3K/AKT/mTOR pathway has also been implicated in apoptosis and type II programmed cell death also known as autophagy. Autophagy is the process of lysosomal degradation of cytosolic contents and can act as cell survival or cell death mechanism depending on the cellular
context. Studies have shown that inhibition of mTOR (e.g. with rapamycin) may promote autophagic cell death (Mathew et al., 2007).

It is unknown how targeting different glycolytic enzymes impacts cellular metabolic sensing including PI3K/AKT/mTOR and AMPK, the type of cell death and *in vivo* tumour growth. Using GBM cells, we wished to compare the effects on cell proliferation, survival, sensitivity to different chemotherapeutics and *in vivo* growth when altering the glycolytic enzymes HK1, HK2 and PKM2. Our results suggest that targeting each of these enzymes may affect proliferation; however, glycolytic enzymes may differentially impact PI3K/AKT/mTOR and AMPK signaling which may thus impact whether the cell preferentially undergoes autophagy or apoptosis as a mode of cell death.

### 4.3 Materials and Methods

#### 4.3.1 GBM cell lines and BrdU incorporation

U87 cells were seeded into 96 well plates, treated with siRNA targeting HK2, HK1 or PKM2 and followed over 5 days. See section 3.3.4 for siRNA treatment procedures. The following siRNAs target sequences were used: HK2siRNA1: 5’CACGATGAAATTGAACCTGGT3”, HK1siRNA: 5’CACGATGTAGTCACCTTACTA 3”, PKM2siRNA: “5’GCTGTGGCTCTAGACACTAAA 3”. BrdU incorporation assay was performed according to manufacturer’s protocol from Day 1 post transfection to Day 5 (Roche). Stable cell lines were generated by expressing short hairpin RNA towards HK2, HK1 and PKM2 in U87 GBM cells. See Section 3.3.7 for details on the establishment of these cell lines. For drug studies, cells were treated for 48hrs with the following drugs and concentrations: AICAR (0.5mM), doxorubicin (2uM), actinomycin D (10uM), rapamycin (100ng/mL).
4.3.2 Cell cycle Propidium Iodide (PI) analysis

GBM cells were trypsinized, counted and washed in PBS followed by fixation in 80% ethanol. Cells were incubated with PI and RNAseA for 30 minutes. Cell cycle analysis was performed using FACS analysis in MoFlo software.

4.3.3 Western blot analysis

See Section 2.3.4 for details of western blot procedures. Membranes were probed overnight with the following antibodies: AMPK, Thre172-pAMPK, AKT, Ser473-pAKT, p70-S6K, Thre389-phospho-p70-S6K, cleaved caspase-3, caspase-3, cleaved PARP from Cell Signaling (1:1000), Light chain 3 (LC3; 1:1000, MBL), B actin (1:10000, Sigma), p53 (1:200, Santa Cruz).

4.3.4 Immunofluorescence for LC3-GFP

Cells were grown on coverslips in 6 well plates, transfected with LC3-GFP plasmid using Fugene transfection reagent, and then treated 48 hrs later with AICAR or rapamycin. After 48 hrs of drug treatment, cells were then washed in PBS, fixed in 4% PFA and stained with DAPI. Images were taken on a Zeiss Axiovert 200 equipped with a Hamamatsu Orca AG CCD camera and spinning disk confocal scan head using Velocity acquisition software. Cells were analysed for the presence of accumulation of LC3-GFP into autophagosomes (i.e. punctae).

4.3.5 Intracranial GBM model and Immunohistochemistry

See Section 3.3.15 for details on procedures. In addition to U87 control and U87HK2shRNA groups, 6 mice were injected with 200 000 cells of U87 HK1shRNA and 6 with U87 PKM2shRNA. Two hours prior to sacrifice, mice were injected intraperitoneally with 300 mg/kg of 5-bromodeoxyuridine (BrdU; Sigma) which is a thymidine analog that incorporates into the DNA of dividing cells during S phase and can be detected immunohistochemically. Mouse brains were harvested, formalin-fixed and paraffin embedded. See section 2.3.1 for details on immunohistochemistry. Table 3.1 provides details of antibodies employed. In addition, tissue sections were incubated
overnight with the following antibodies: PKM2 (1:100, Cell Signaling), BrdU (1:250, BD Bioscience).

4.4 Results

4.4.1 Transient and stable loss of glycolytic enzymes HK1, HK2 and PKM2 decreases cell proliferation under normal growth conditions

Glycolytic metabolism and particularly aerobic glycolysis has been hypothesized to assist in the generation of biosynthetic precursors required for proliferation. We wished to determine the effect of targeting different glycolytic enzymes on GBM cell proliferation. We first determined the impact of transient loss of glycolytic enzymes HK1, HK2 and PKM2 on GBM cell proliferation over 5 days under normal growth conditions in U87 GBM cell line that expresses high levels of all three proteins (Figure 4-1). U87 cells were transfected with 50nm of HK1, HK2 or PKM2 siRNA. Loss of each enzyme was found to decrease proliferation by day 5 (p < 0.05).

To further explore the effects of loss of different glycolytic enzymes on proliferation and cell death, we established U87 cells with stable expression of shRNA for HK2, HK1 and PKM2 (See Chapter 3). We performed cell cycle analysis and only small differences in cell cycle profile compared to U87 scramble controls under normal growth conditions (Figure 4-2). Cells depleted of HK2 had a slightly greater G1/S block while HK1 and PKM2 had greater G1 peaks. Although not performed here, these differences in cell cycle profiles may be enhanced under hypoxic or death inducing conditions.
Figure 4-1: Decreased cell proliferation in U87 cells with transient loss of HK2, HK1 and PKM2 glycolytic enzymes.

BrdU incorporation over 5 days in U87 cells transfected with HK2 siRNA, HK1 siRNA and PKM2siRNA under normal growth conditions.

Figure 4-2: Cell cycle analysis of U87 cells with stable loss of HK2, HK1 and PKM2.

Greater G1/S arrest in U87HK2shRNA and G1 arrest in U87HK1shRNA and U87 PKM2shRNA compared to U87 scramble control cells.
Figure 4-1

The graph shows the cell number over days for different groups:
- U87 scr siRNA
- U87 HK1 siRNA
- U87 HK2 siRNA
- U87 PKM2 siRNA

Cell number increases over days, with significance indicated by an asterisk (*) where the p-value is less than 0.05.
Figure 4-2

- **U87 scramble shRNA**
  - FL2-A: 53.8, 13.8, 25.5
  - # Cells: 0.47

- **U87HK2shRNA1**
  - FL2-A: 54.7, 6.46, 32.3
  - # Cells: 0.31

- **U87HK1shRNA**
  - FL2-A: 59.4, 8.26, 26.3
  - # Cells: 1.71

- **U87PKM2shRNA**
  - FL2-A: 58.2, 8.96, 28.6
  - # Cells: 0.44
4.4.2 Impact on PI3K/AKT/mTOR and AMPK signaling with stable loss of HK2 and HK1

To understand the mechanisms resulting in decreased proliferation with loss of glycolytic enzymes, we next sought to determine the impact of loss of HK2 and HK1 on the activity of signaling pathways and proteins that sense the metabolic state of the cell including AMPK, AKT and p70-S6K as reflecting mTOR function. Loss of HK2 (HK2shRNA1, HK2shRNA2), and HK1 to a lesser extent, resulted in increased expression of phosphoThr172-AMPK, especially under 24 hrs of 2% hypoxia (Figure 4-3). However, total AMPK levels were higher in cells depleted of HK2 as well. PhosphoSer473-AKT levels were maintained in U87HK2shRNA but slightly lower in U87HK1shRNA. Correspondingly, phosphoThr389- p70-S6K and p70-S6K levels, a protein downstream of mTOR and reflecting mTOR function, were maintained and even higher in U87HK2shRNA but decreased in U87HK1shRNA under hypoxic conditions. These results suggest that the cell’s metabolic sensing pathways are differentially altered with loss of HK2 compared to HK1 glycolytic enzyme.

Figure 4-3: Differential effects on PI3K/AKT/mTOR and AMPK signaling with depletion of HK2 and HK1.

Western blot showing altered signaling in U87HK2shRNA compared to U87HK1shRNA with respect to expression of metabolic signaling proteins including phospho-AMPK, phospho-AKT and phospho-p70-S6K, particularly under hypoxic conditions. Densitometric values, normalized with B actin and relative to U87 scr shRNA, are represented below western blots.
### Figure 4-3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2, HK1</td>
<td>HK2shRNA</td>
<td>HK2shRNA</td>
</tr>
<tr>
<td>HK2, HK1</td>
<td>scr</td>
<td>HK1 shRNA</td>
</tr>
<tr>
<td>HK2, HK1</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>Thr172-pAMPK</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>AMPK</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Ser473-pAKT</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>AKT</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Thr389-phospho-p70-S6k</td>
<td>85, 70</td>
<td>100, 113, 117, 99, 103, 111, 111, 90</td>
</tr>
<tr>
<td>p70-S6K</td>
<td>85, 70</td>
<td>100, 113, 117, 99, 103, 111, 111, 90</td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>
4.4.3 Enhanced autophagosome accumulation with loss of HK1 and PKM2 but not HK2

Considering the important role of mTOR in autophagic cell death and the observed decrease in mTOR signaling with loss of HK1 but not HK2 under hypoxic conditions, we next sought to determine the impact of loss of HK2, HK1 and PKM2 on autophagy when induced with different chemotherapeutic agents that inhibit mTOR signaling. We treated GBM cell lines with agents that directly inhibit mTOR (rapamycin) or activate AMPK and thereby inhibit mTOR indirectly (AICAR). We found that loss of HK1 appeared to sensitize U87 GBM cells to rapamycin, which was associated with increased levels of the cleaved LC3-II fragment on western blot, a marker of autophagy (Figure 4-4a). This was not seen with stable loss of HK2. With treatment with AICAR, LC3-II levels were similarly increased in scr shRNA and HK1shRNA but low in HK2shRNA. These results were corroborated by immunofluorescence of treated cells transfected with LC3-GFP. There was greater formation of punctate structures in U87HK1shRNA and U87PKM2shRNA compared to U87HK2shRNA, suggesting the accumulation of autophagosome (Figure 4-4b). Taken together our results suggest that the metabolic status of GBM cells, which can be altered by altering glycolytic enzymes, plays an important role in the nature of their response to different chemotherapeutic agents.

Figure 4-4: Increased sensitivity to autophagosome accumulation with loss of HK1 and PKM2 but not HK2.

a) Treatment of cells with rapamycin or the AMPK activator, AICAR, leads to increased LC3-II in U87 scramble and U87HK1shRNA but not in U87HK2shRNA. b) Immunofluorescence of cells transfected with LC3-GFP and treated with rapamycin or AICAR shows greater formation of punctate autophagic vacuoles in U87HK1shRNA while U87HK2shRNA shows a diffuse pattern of staining.
Figure 4-4

(a) Western blot analysis with Rapamycin and AICAR treatments. LC3 and B-actin bands are shown.

(b) Immunofluorescence images showing U87 scr, HK2shRNA, HK1shRNA, and PKM2shRNA under Norm, Rapamycin, and AICAR conditions.
4.4.4 Differential sensitivity to apoptotic inducing chemotherapeutic agents with loss of HK1, HK2 and PKM2

We wished to look at the effect of treating these cell lines with different chemotherapeutic agents inhibiting DNA synthesis (doxorubicin, actinomycin D) or inducing energy crisis by activating AMPK (AICAR). Cells with stable loss of HK2 but not HK1 and PKM2 were particularly susceptible to doxorubicin and actinomycin D with greater formation of cleaved caspase 3 (Figure 4-5a). Previously we found that cells lacking HK1 or PKM2 and treated with AICAR were predisposed to accumulate autophagosomes, suggesting enhanced autophagy. However, for GBM cells lacking HK2 and treated with AICAR, these cells appear predisposed to undergo apoptosis seen by increased levels of cleaved caspase 3 and cleaved PARP (Figure 4-5b). Considering AMPK’s important role in directly linking sensing of nutrient concentration and bioenergetics to cell-cycle progression, we next looked at the effect of activation of AMPK on the cell cycle inhibitor p53. Previous reports have shown that activation of AMPK can result in a glucose-dependent checkpoint at the G1/S boundary mediated by p53 (Jones et al., 2005). Upon treatment with AICAR, GBM cells depleted of HK2 showed higher p53 induction, not seen with loss of HK1 or PKM2 (Figure 4-5b).

Figure 4-5: Sensitivity to apoptosis in GBM cells with stable loss of HK2 but not HK1 or PKM2.

a) Treatment of cells with actinomycin D (10uM) or doxorubicin (2uM) led to increased cleaved caspase 3 in U87HK2shRNA but not U87 scramble, U87HK1shRNA or U87 PKM2shRNA. B) Treatment with AICAR (0.5mM) resulted in increased cleaved caspase 3, cleaved PARP and p53 in cells depleted of HK2 but not HK1 or PKM2.
Figure 4-5

a

Doxorubicin

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2</th>
<th>HK1</th>
<th>PKM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>cleaved caspase 3</td>
<td>19</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caspase 3</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Actinomycin D

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2</th>
<th>HK1</th>
<th>PKM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>cleaved caspase 3</td>
<td>19</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caspase 3</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b

AICAR

<table>
<thead>
<tr>
<th></th>
<th>kDa</th>
<th>scr</th>
<th>HK2</th>
<th>HK1</th>
<th>PKM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>cleaved PARP</td>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cleaved caspase 3</td>
<td>19</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B actin</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.4.5 Impact of loss of HK2, HK1 and PKM2 on *in vivo* intracranial growth

Since stable loss of HK1, HK2 and PKM2 appeared to alter *in vitro* cell growth via different mechanisms, we sought to determine the impact of loss of these glycolytic enzymes on *in vivo* intracranial growth. We injected intracranially 200,000 cells of U87 control, U87 HK1shRNA, U87 HK2shRNA and U87 PKM2shRNA. Mice injected with HK2, HK1 or PKM2shRNA tumours survived significantly longer than those injected with U87 control tumour (*Figure 4-6a*).

We previously reported the effects of loss of HK2 on *in vivo* growth (Chapter 3). These tumours did not form large tumours but were found to be more invasive into perivascular Virchow-Robin spaces and along leptomeninges in both hemispheres. Although tumours lacking HK1 and PKM2 were slower growing than control, this phenotype of increased invasiveness was not present (*Figure 4.6b*). Tumours appeared relatively large and circumscribed similar to control tumours. However, the proliferative index (BrdU incorporation) was reduced in all three groups of tumours compared to U87 control, although the most significant change was seen in tumours depleted of HK2 (control = 26±4%, HK2shRNA = 8.5±3%, HK1shRNA = 19±3%, PKM2shRNA = 14.5±3%) (*Figure 4.6c*). Increased cleaved caspase 3 immunopositivity was seen only in the tumours lacking HK2 (12±3.5%) compared to U87 control (3.5±1.5%), HK1 (0.8±0.5%) and PKM2 (0.9±0.5%), suggesting that only with loss of HK2 is their enhanced *in vivo* apoptosis (*Figure 4.6c*).
Figure 4-6: Increased survival and reduced proliferation with depletion of HK2, HK1 and PKM2 in GBM orthotopic xenograft model.

a) Survival of mice injected intracranially with 200,000 cells of U87 controls, U87HK2shRNA, U87 HK1shRNA and U87 PKM2shRNA. b) Histopathology of U87 control, U87HK2shRNA1, U87 HK1shRNA and U87PKM2shRNA tumours including H&E staining (scale = 200um), BrdU (scale = 100um), and cleaved caspase 3 (scale = 100um). c) Semiquantification of BrdU and cleaved caspase 3 staining for each group.
Figure 4-6

(a) Survival (days) comparison among control, HK2shRNA, HK1shRNA, and PKM2shRNA groups. Error bars indicate standard error of the mean.

(b) Histological images of U87 cells stained with H&E, BrdU, and Cleaved Caspase-3. Images are labeled U87 control, U87HK2shRNA, U87HK1shRNA, and U87PKM2shRNA.

(c) Bar graph showing the percentage of positive cells for BrdU and Cleaved Caspase-3 in Control, HK2shRNA, HK1shRNA, and PKM2shRNA groups. Error bars indicate standard error of the mean.

* p < 0.05
** p < 0.01
4.5 Discussion

A fundamental requirement of all cells including tumour cells is that nutrient availability be coupled to growth factor signals driving proliferation only when nutrients are sufficiently abundant to guarantee proper cell division. The molecular mechanisms interconnecting the signaling pathways controlling metabolism and cell growth are only now being identified, making this an active area of investigation in cancer research in search of potential therapeutic targets. Targeting of specific glycolytic or metabolic enzymes may predispose tumour cells to cell death. However, whether targeting specific glycolytic enzymes may differentially impact growth factor and metabolic signaling and sensitivity to cell death in GBM cells is unclear.

Previously, we have reported that GBM cells specifically upregulate the glycolytic enzyme HK2 resulting in enhanced aerobic glycolysis and resistance to apoptosis (Chapter 3). Depletion of HK2 promoted a return to OXPHOS not seen with depletion of the HK1 isoform or the downstream glycolytic enzyme PKM2. In this study, we attempted to characterize the effect of loss of these three glycolytic enzymes HK1, HK2 and PKM2 on GBM growth factor cell signaling, proliferation, sensitivity and type of cell death and in vivo growth. Our results support that cell proliferation is decreased with loss of each of these enzymes (Figure 4-1, 4-2); however, the mechanisms resulting in reduced proliferation were found to differ.

AMPK is a central metabolic switch found in all eukaryotes that governs glucose and lipid metabolism in response to alterations in intracellular energy levels. During nutrient deprivation or hypoxia, AMP levels accumulate and activate AMPK (Shackelford and Shaw, 2009). Loss of HK2, and HK1 to a lesser extent, appeared to activate AMPK in GBM cells, enhanced under hypoxic conditions. However loss of HK1 resulted in reduced pAKT/mTOR signaling compared to HK2 loss in GBM cells. Loss of HK1 appeared to have effects analogous to those reported for 2-deoxyglucose or glucose withdrawal where AMPK is activated and results in decreased mTOR signaling and enhanced autophagy (DiPaola et al., 2008). Associated with the loss of HK1 and decreased mTOR signaling was increased LC3-II and an accumulation of LC3-GFP.
autophagosomes, potentially reflecting enhanced autophagy in response to the mTOR inhibitor rapamycin and AMPK activator AICAR (Figure 4-4). The impact of depletion of PKM2 on chemotherapeutic response appeared analogous to that of HK1 (Figure 4-4). Autophagy refers to the process whereby organelles and cytoplasm are engulfed and targeted to lysosomes for proteolysis. Autophagy is believed to act as a protective mechanism and sustain cell survival under conditions of nutrient stress but may result in eventual cell death. To directly ascertain whether GBM cells are undergoing autophagy, autophagic flux should be measured to complement autophagosome accumulation. Further support that depletion of HK1 and PKM2 results in autophagy would be to inhibit beclin 1, an essential regulator of autophagy, to determine whether autophagy is abrogated.

On the other hand, stable loss of HK2 increased pAMPK and total AMPK levels without impacting mTOR signaling (Jones et al., 2005). Our results show that persistent AMPK activation with depletion of HK2 in addition to AICAR treatment resulted in stabilization of p53 and sensitization to apoptosis reflected by increased cleaved PARP and cleaved caspase 3 (Figure 4-5). Enhanced apoptosis was also seen with inhibitors of DNA synthesis including actinomycin D and doxorubicin (Figure 4-5).

There is some evidence to suggest that whether a cell undergoes autophagy or apoptosis will depend on the cells’ ability to undergo apoptosis (Degenhardt et al., 2006). Under metabolic stress, tumours cells may undergo apoptosis unless those pathways are inactivated thereby pushing toward autophagy. Loss of HK2 may promote a return or sensitization in apoptotic pathways not seen with HK1 or PKM2 depletion, thus resulting in apoptosis under inducing conditions rather than autophagy. In addition, the induction of persistent G1 arrest while maintaining the ability to undergo translation and cell growth is the hallmark of cellular senescence. AMPK activation may therefore result in metabolic induced arrest, with eventual cellular senescence (Jones et al., 2005). Thus, in addition to enhanced apoptosis, cells depleted of HK2 may be predisposed to senesce. B-galactose staining as a marker for senescence should be performed to ascertain this.
Depletion of HK2, HK1 and PKM2 resulted in enhanced survival in orthotopic mouse xenograft models (Figure 4-6). However, the pattern of tumour growth differed between tumour groups. Tumours depleted of HK2 had reduced proliferation and enhanced apoptosis. They did not form a large tumour bulk seen in control tumours, were unable to form new vasculature and were much more invasive. Tumours lacking HK1 and PKM2 had reduced proliferation but were more similar to control tumours with large tumour bulk, highly vascular and limited invasiveness. Overall, our in vivo xenograft results support that altering different glycolytic enzymes and the ensuing impact on growth factor and nutrient sensing signaling can result in phenotypically different tumours.

The exact mechanisms resulting in altered AMPK and AKT signaling with loss of HK2 compared to HK1 or PKM2 are not clear. The extent of energy crisis (ATP loss) that occurs with loss of each enzyme may well dictate the cell’s response. Although the results of this study lack mechanistic insight, it reinforces the concept that targeting glycolytic enzymes may sensitize to cell death. However, the type of cell death may vary with specific enzymes of glycolysis or depending on the impact on growth factor and AMPK signaling and may not be as straightforward as a decrease in overall glucose flux. A major limitation of this study is that it is conducted in only one GBM cell line in which p53 is wild-type in nature. It would be important to determine the role of loss of glycolytic enzymes, especially HK2, and the impact of p53 status on proliferation and sensitivity to apoptosis. HK2’s effects may be minimized or altered in a p53 mutant background, as reflected by the decrease in sensitivity to temozolomide in the U373 cell lines (See Chapter 3, Figure 3-6).

Our results with respect to HK2 depletion in GBM cells reinforce the need to further understand the mechanisms that underlie its effects on proliferation and sensitization to apoptosis, which may include: a) impact of glucose phosphorylation on energy sensing pathways and cell cycle inhibitors, b) alteration of MPTP and cytochrome c release via interaction at VDAC, c) alteration of OXPHOS which can sensitize cells to Bax-induced apoptosis d) alteration of OXPHOS and generation of ROS and oxidative stress and thus impact on cell cycle inhibitors (e.g. p53) d) impact on NAPDH formation via the PPP resulting in increased anti-oxidant functions. Future experiments should be aimed at
deciphering the relative importance of these different mechanisms. This may be achieved by employing mutant proteins of HK2 either lacking its kinase function and/or truncated HK2 which does not localize to the mitochondria, within background of p53 wildtype or p53 mutant. A better understanding of these mechanisms is crucial in establishing the best therapeutic targeting strategies if aimed specifically at HK2.

In conclusion, our results suggest that altering different glycolytic enzymes may differentially impact AMPK and PI3K/AKT/mTOR signaling, reducing proliferation and predisposing cells to either autophagy or apoptosis. Therapeutic targeting of glycolytic enzymes or metabolism in general should thus take these findings into account.
CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Summary and Discussion of thesis findings

This thesis attempts to answer several biological questions relating to the expression, regulation and function of the glycolytic enzyme HK2 in embryonic development and GBMs, a very aggressive primary brain tumor that remains refractory to all conventional treatments. In summarizing the findings of this thesis, we will refer back to the original biological questions set forth in the introduction.

5.1.1 Expression of HK2 in embryonic tissue, normal brain, low-grade astrocytomas and GBMs

Expression profiling of HK2 and HK1 at different mouse developmental timepoints revealed an inverse temporal relationship between HK2 and HK1 in the embryo and developing brain with HK2 expressed strongly during early embryonic development and negligently in adult mouse brain, while HK1 was strongest in the maturing brain, and then declined to adult levels. The pattern of HK2 expression across embryonic development mirrored that of expression of LDHA, the enzyme catalyzing the conversion of pyruvate to lactate.

Similarly, human normal brain and low-grade astrocytomas expressed little HK2 and predominantly HK1. Screening of GBMs at the western and immunohistochemical level revealed greater than 50% expressed HK2. These results are validated by high throughput screens of GBMs conducted by TCGA, Rembrandt, and Parsons et al. (2008) showing a high percentage of GBMs (~60-80%) expressing HK2 but not HK1. Furthermore, expression of HK2 is found to predict poor overall survival of GBM patients in this study. HK2’s prognostic ability is further confirmed in the Rembrandt database and results published by Dong et al., (2005).
5.1.2 Regulation of HK2 in GBMs

HK2 is a highly regulated form of HK. In this thesis, we demonstrate that HK2 is regulated at the epigenetic, transcriptional and protein level in GBMs. Epigenically, HK2 intron 1 was highly methylated in normal brain tissues. Within fetal and GBM cell lines, a high percentage of the CpG islands within intron 1 were demethylated and correlated with HK2 expression. Furthermore, HK2 is transcriptionally regulated by a variety of proteins including HIF1α, strongly induced in perinecrotic centres of GBMs. HK2 is also shown to be induced downstream of activation of important oncogenes in gliomagenesis including Ras and loss of p53. At the protein level, HK2 is stabilized upon binding to the OMM, particularly under growth factor stimulation and/or constitutive activation of the PI3K/AKT pathway.

5.1.3 The role of HK2 in proliferation and apoptosis in GBMs

HK2 has been associated with increased proliferation and/or a cell survival advantage in variety of cancers. This thesis demonstrates that HK2 provides GBM cells with a proliferative advantage as well as a cell survival advantage, particularly under harsher microenvironments including hypoxia. Furthermore, HK2 expression protected against clinically relevant apoptotic inducers including chemotherapy (e.g. actinomycin D, temozolomide) and radiation. These effects were demonstrated in GBM cell lines using both over-expression and knockdown studies. HK2 was demonstrated to translocate to the OMM under conditions of growth factor stimulation or constitutive PI3K/AKT signaling, mediating in part both proliferative and survival effects. Valuable for GBMs is that HK2 is not expressed strongly in normal brain, in contrast to HK1, making it an attractive therapeutic target with limited toxicity.

5.1.4 The role of HK2 in promoting aerobic glycolysis

The glycolytic phenotype may emerge as a consequence of one or multiple reversible and/or irreversible adaptations that confer a growth advantage. Results from this thesis demonstrate that the increased lactate and decreased pyruvate and OXPHOS function in GBMs is reversible and can be restored with stable depletion of HK2. This was
associated with increased expression of transcription factors important for mitochondrial biogenesis including PGC1α and mTFA. Altogether, targeting of HK2 has multiple effects on GBM cells including 1) inhibition of the glycolytic phenotype, 2) sensitization to apoptosis potentially via different mechanisms, 3) decreased proliferation.

5.1.5 Relationship between aerobic glycolysis and angiogenesis in GBMs

Results from our \textit{in vivo} studies demonstrate a link between aerobic glycolysis and angiogenesis in GBM tumours. Depletion of HK2 in GBM cells resulted in an inability to generate new vessels and co-option of existing vasculature, resulting in long distance invasion. Over-expression of HK2 in GBM cells produced more numerous irregularly shaped and distorted vessels, especially at the leading edge. This phenotype may denote an essential role of tumour aerobic glycolysis in mediating tumor-stromal interactions, including endothelial cells. Whether the effect of inhibiting aerobic glycolysis on angiogenesis is mediated via HIF1α and/or VEGF dependent pathways, or potentially other pathways including AMPK, remains to be investigated.

5.1.6 Impact of glycolytic proteins on growth factor signaling and metabolic sensing pathways

Although we did not show an effect of depletion of HK1 or PKM2 on return of OXPHOS metabolism as observed with HK2, depletion of these enzymes did alter \textit{in vivo} tumor growth and proliferation. Altering specific glycolytic enzymes may result in different downstream effects on metabolic sensing pathways including AMPK and PI3K/AKT/mTOR, potentially impacting on proliferation and its response to different chemotherapeutic drugs. Preliminary evidence suggests that targeting PKM2 and HK1 may predispose more to autophagy type of cell death while HK2 loss favoured sensitivity to apoptosis in response to chemotherapeutic drugs such as doxorubicin or AICAR. HK1 would not be a feasible therapeutic target in GBMs due its widespread expression in the normal CNS, resulting in toxicity when targeted. The PKM2 gene on the other hand is a splice isoform where PKM1 is expressed in adult brain and not PKM2, which is
expressed in tumors including GBMs. Specific targeting of HK2, potentially in combination with PKM2, may provide an effective therapeutic strategy in GBMs.

5.2 Future Directions

5.2.1 Impact of inhibition of HK2 in vivo tumour regression

Our *in vivo* subcutaneous and intracranial results support that depletion of HK2 can profoundly impact tumour growth. However, this data was based on U87 cell lines stably expressing shRNA towards HK2. It would be clinically relevant to determine the impact of inhibiting HK2 on an established tumour, using either injection of primary cell lines or using transgenic mouse models of differing genetic background (EGFRvIII/PTEN-/-, RasB8 mice), followed by 1) Adenoviral or Lentiviral injection of HK2shRNA in grown tumors, or 2) employing inducible HK2shRNA systems (eg. Tetracycline, Estrogen Receptor/Tamoxifen). Currently, there are no specific inhibitors targeting solely HK2 without also impacting HK1.

5.2.2 Impact of inhibition of HK2 in combination with classical and novel chemotherapies in *in vivo* animal models

Results from Chapter 3 demonstrate that inhibiting HK2 in GBM cells can significantly impact sensitivity to radiation and different chemotherapies including temozolomide. Further *in vivo* evaluation combining specific HK2 inhibition (e.g. Adenoviral or Lentiviral HK2shRNA or inducible HK2shRNA system) along with radiation and/or temozolomide would be very informative. Furthermore, this would help determine whether increased sensitivity to radiation and chemotherapy conferred by inhibition of HK2 offsets the longer-term enhanced invasiveness seen in our *in vivo* results.

5.2.3 Mechanisms of HK2 at the OMM

Knockdown of HK2 in GBM cells was found to sensitize these cells to apoptosis in responses to radiation, chemotherapy and hypoxia. This was associated with depolarization of the mitochondrial membrane, increased release of cytochrome c and activation of downstream caspases. Furthermore, this thesis demonstrated that HK2
Localization to the mitochondria may be regulated by GF/PI3K/AKT signaling, and to a greater extent than HK1. U87 cells with greater constitutive AKT expression resulted in greater localization of HK2 to the OMM, previously reported in other cancer systems (Gottlob et al., 2001) compared to U343 cells, which require EGF stimulation to enhance HK2 mitochondrial localization. Inhibition of EGFR signaling decreases percent HK2 at OMM. However, a major unanswered question of this thesis includes the exact mechanism through which HK2 exerts its anti-apoptotic effects, particularly in contrast to HK1, both of which can localize to the OMM. Putative anti-apoptotic mechanisms, occurring independently or in combination, include:

1) Inhibition of MPTP and cytochrome c release via interaction at VDAC and/or GSK3.

2) Inhibition of OXPHOS, resulting in resistance to Bax-induced apoptosis.

3) Inhibition of OXPHOS reducing ROS generation and oxidative stress.

4) Increase in NAPDH formation via the PPP resulting in increased anti-oxidant functions.

The mechanisms resulting in increased OXPHOS function with loss of HK2 and not HK1 also remain unclear but may be linked with the sensitization to apoptosis. Although we show increased expression of transcription factors PGC1α and mTFA, the mechanisms behind this elevation are unclear. PGC1α has been reported regulated by a variety of cell signaling pathways including p38 MAPK, calcineurin and CaMK, among others (Lin et al., 2005; Rohas et al., 2007). Another hypothesis is that HK2 and HK1 differentially impact the enzyme PDH regulating entry of pyruvate into mitochondria. Future studies should attempt to elucidate mechanisms specific to HK2 in promoting resistance to apoptosis and inhibition of OXPHOS and enhanced aerobic glycolysis.

We were not able to demonstrate direct phosphorylation of HK2 by AKT by means of immunoprecipitation, pSer/Thr AKT specific substrate antibody or mass spectrometry. However it remains a possibility that AKT may directly impact HK2 function via phosphorylation, as was reported in cardiomyocytes (Miyamoto et al., 2008).
An initial set of experiments to begin addressing these mechanistic questions may be to determine the relative contribution of glucose phosphorylation and mitochondrial localization to cell survival and OXPHOS function. To address this, GBM cell lines can be transfected with full length HK2-GFP, mutant kinase HK2-GFP, truncated HK2-GFP (lacking mitochondrial localization) and truncated-mutant constructs provided by Dr. Ardehali (NorthWestern). Using live cell imaging after induction of apoptosis with radiation or chemotherapy +/- growth factor stimulation, the following can be analysed: 1) HK2 localization 2) mitochondrial membrane permeability (e.g. JC1) 3) ROS generation (e.g. mitoSOX), 4) NAPDH generation 5) cytochrome c release, 6) caspase 3/7 induction 7) extracellular lactate and O2 consumption. The relative importance of MPTP, VDAC and ANT for HK2’s survival effects can determined by: 1) use of specific activators of MPTP and determine whether over-expression of HK2 protects against these, 2) use of VDAC or ANT depleted or null GBM cell lines and verify if HK2 remains protective. Furthermore, to confirm the importance of PI3K/AKT/mTOR on HK2’s protective effects, GBM transfected cells can be treated with inhibitors to EGFR tyrosine kinase (AG1478), PI3K (LY-294002) or mTOR (rapamycin).

### 5.2.4 Mechanisms through which HK2 promotes proliferation and resistance to radiation and temozolomide

The mechanisms through which HK2 may be promoting proliferation are also unclear. HK2 expression in GBMs may promote proliferation and inhibit apoptosis by impacting activation of cell cycle inhibitors including p53 and p27 and potentially DNA repair enzymes important in resistance to temozolomide (MGMT, APNG). This may be dependent or independent of HK2’s effects at the mitochondria. Preliminary evidence of GBM cells depleted of HK2 and treated with temozolomide showed greater sensitization in U87 cells compared to U373 cells. To determine whether HK2’s resistance effects are partially p53-dependent, isogenic p53 mutant and p53 wildtype GBM cell lines depleted of HK2 can be treated with temozolomide followed by measuring of p53 phosphorylation (pSer15), cell viability and proliferation and caspase 3/7 activation.
More recent evidence suggests a potential role of HK2 in the nucleus (Neary and Pastorino). In yeast, HK2 acts as a transcription repressor under glucose plentiful conditions (Pelaez et al., 2009). The possibility that HK2 possesses non-traditional functions in the nucleus may be another mechanism through which HK2 impacts on cell proliferation and remains an interesting avenue of investigation.

5.2.5 Mechanisms underlying the association of aerobic glycolysis and angiogenesis

Our intracranial *in vivo* data from Chapter 3 revealed that permanent loss of HK2 and resulting impact on aerobic glycolysis led to an inability to form new vasculature and enhanced migration of tumour cells along existing vasculature. The mechanisms promoting this enhanced invasion can only be hypothesized at this point. Looking at the angiogenic literature, recent evidence points to a similar enhanced invasive phenotype with anti-angiogenic treatment (e.g. avastin), or with HIF1α and/or VEGF deletion (Blouw et al., 2003; Paez-Ribes et al., 2009). It is possible that by inhibiting aerobic glycolysis, tumors are unable to grow beyond the point where HIF1α is stabilized. Conversely, inhibition of HK2 may directly thwart HIF1α stabilization under normoxic conditions. Consequently, VEGF levels remain low and vessel cooption rather than angiogenesis is favoured. Whether or not the enhanced perivascular invasiveness is HIF1α and/or VEGF-dependent or independent is another avenue to be explored. A recent yet to be published clinical trial conducted at MD Anderson looked at the effect of metformin (AMPK agonist) on inhibiting GBM growth in patients. Similar to inhibition of VEGF, activation of AMPK may promote an enhanced invasive phenotype. Therefore, activation of AMPK may be another mechanism through which depletion of HK2 favours invasiveness.

A corollary to this question is the relationship and timing between glycolytic and angiogenic switches and whether these are inter-dependent. Acidosis may precede angiogenesis and aerobic glycolysis/lactate may stimulate HIFα expression independent of hypoxia or stimulate angiogenesis independent of HIFα (Fukumura et al., 2001; Lu et al., 2002; Shi et al., 2001).
5.2.6 Altered metabolism as a network: a systems biology approach

Rather than employing reductionist strategies focusing only on one protein, it would be more beneficial to utilize a systems biology approach, such as examining the patterns of altered metabolism and expression of enzymes and how they co-vary depending on the microenvironment. Examination of embryonic tissue at different developmental timepoints identified the covariation of HK2 and LDHA transcripts. Metabolomics would assist in the quantification of metabolites in cells or tissues, which would be particularly informative in pathologically heterogeneous human GBM tissues. The metabolomic profile could then be correlated with the transcript or proteomic profile of these tissues within different regions. Understanding how these metabolic networks co-vary and importance for proliferation will allow identification of key therapeutic targets.

There may be a variety of mechanisms resulting in similarly altered metabolic profile. For example, high-throughput sequencing of GBMs has reported a 12% mutation rate of the metabolic enzyme IDH1 (Yan et al., 2009). IDH1 mutations result in the new ability of the enzyme to catalyze the NADPH-dependent reduction of αKG to 2HG (Dang et al., 2009), although the consequences of this accumulation on metabolism are unclear. This thesis focused on the role of HK2 in promoting aerobic glycolysis and inhibiting OXPHOS, potentially in a different subset of tumours than IDH1 mutated tumors since HK2 is not expressed strongly in low-grade astrocytomas. A metabolomics approach may identify other potential metabolites accumulating within tumors or co-occurring compared to normal brain. These metabolites may also be found within the serum as reported for 2HG (Dang et al., 2009) and thus be useful diagnostically.

5.2.7 Expression profile and functional relevance of HK2 in GBM brain tumour initiation cells (BTIC)

Embryonic stem cells have been reported to have altered metabolism with greater reliance on aerobic glycolysis and decreased mitochondrial function (Ramalho-Santos et al., 2009). In order for embryonic stem cells to differentiate, a switch from glycolysis to
OXPHOS may be required (Chung et al., 2007). Similarly, a role for mitochondria in differentiation of adult stem cells has also been described (Carriere et al., 2004; Chen et al., 2008). In GBMs, BTICs are postulated to be cell of origin of GBMs and harbor different growth and therapeutic characteristics distinct from the bulk of the GBM (Piccirillo et al., 2009). The metabolic profile of BTICs including the expression of HK2 and reliance on aerobic glycolysis to maintain stemness has yet to be explored. Future studies may wish to characterize the expression of HK2 and other glycolytic and metabolic enzymes in anchorage independent neurospheres and adherent GBM BTICs.

5.2.8 Epigenetic mechanisms regulating HK2 expression in GBMs

Results from Chapter 2 of this thesis support the epigenetic regulation of HK2 via methylation within the intron 1. HK2 gene methylation may play a role in the regulation of its expression in normal brain, fetal tissue and GBM cell lines, such that extent of methylation is inversely correlated with HK2 expression. To strengthen these findings, the methylation status of HK2 within human GBM samples should be determined by bisulfite treatment and sequencing and methyl specific PCR. Extent of methylation can be correlated to gene expression in matching tissue sections. Furthermore, the mechanisms that result in demethylation of the HK2 promoter in GBMs are also unknown, another avenue merit ing investigation. Direct evidence of silencing of HK2 in NHAs and the adult brain remains to be acquired. Since NHAs do not express much HK2, treating these cells with DNA methyltransferase inhibitor (e.g. 5-aza-2-deoxycytidine) and HDAC inhibitor (trichostatin A) may give an indication of its epigenetic regulation (to complement findings in the U343 GBM cell line). Another possible experiment is to clone the HK2 promoter/intron 1 in front of a luciferase reporter gene and then transfect this construct into HK2 non-expressing cell (NHA, U343 cells) and then determine if the promoter gets methylated over time.

5.2.9 microRNA regulation of HK2

The HK2 gene is regulated transcriptionally by a variety of important factors including HIF1α, myc, among others (Mathupala et al., 2006). There is emerging evidence that
deregulation of microRNAs (miRNAs) plays an important role in the development of human cancers including GBMs. Several miRNAs have been reported to be down-regulated in GBMs including miR-128-1, miR-7, miR-181a/b/c, which when over-expressed can suppress cell growth and induce apoptosis (Pang et al., 2009). Correspondingly, HK2 has predicted miRNAs target sites for miR-181b/181c and miR-451. It remains to be investigated if the HK2 transcript is regulated by these miRNAs.

5.2.10 Retrograde mitochondrial signaling in GBMs

There is an intricate pathway of signaling that occurs from the mitochondria back to the nucleus which can impact the metabolism of the cell, also known as “retrograde mitochondrial signaling”, initially identified in S. cerevisiae (Butow and Avadhani, 2004). Our results show that permanent knockdown of HK2 in GBM cell lines led to an increase in key transcriptions factors involved in mitochondria biogenesis including PGC1α. The mechanisms behind this are not known. Mitochondrial dysfunction has been found to result in stress signaling responses analogous to the retrograde mitochondrial signaling pathway described in mitochondria depleted yeast cells (Parikh et al., 1987). Disruption of mitochondrial membrane potential, either due to mitochondrial genetic stress (partial depletion of mtDNA) or treatment with mitochondrial ionophores, was found to be associated with increased glucose uptake, glycolysis, resistance to apoptosis and invasiveness (Guha et al., 2007). This effect was associated with increased calcineurin signaling resulting in enhanced autophosphorylation of the IGF1 receptor (Guha et al., 2007). Similar mechanisms may be activated in GBMs upon HK2 over-expression and hyperpolarization of mitochondria.

5.3 Conclusion

Metabolism is a central process implicated directly or indirectly in almost all cellular functions including apoptosis, proliferation, differentiation, and invasion. The regulation of proliferating cell metabolism is now an exciting area of investigation. This thesis aimed to increase our knowledge of altered metabolism in GBMs by demonstrating a crucial role of the glycolytic enzyme HK2 in promoting aerobic glycolysis, proliferation and resistance to apoptosis. Further understanding of the network of metabolic alterations
in GBMs will have a major impact on our current knowledge of GBM biology, potentially providing new serum and tissue biomarkers and attractive therapeutic targets.
References


kinase is homologous to yeast and plant protein kinases involved in the regulation of carbon metabolism. J Biol Chem 269, 11442-11448.


translational, and mutational events that lead to a critical role for type II hexokinase. J Bioenerg Biomembr 29, 339-343.


dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. J Clin Oncol 27, 4150-4154.


Appendix 1: Image-guided sampling of representative GBM “centre” and “periphery”.

Representative images showing biopsy tissue taken from T1-Gadolinium positive “Centre” and T1-Gadolinium negative/T2 positive “Periphery” along with the surgical trajectory view, corresponding MR spectra, and pathological stains including H&E, MIB1 (proliferation) and factor vIII (vasculature).
Appendix 1

Conventional MRI

Surgical trajectory view

MR spectroscopy

H&E

MIB1

Factor VIII
Appendix 2: REMBRANDT database: Kaplan-Meier survival plot for Glioma samples with differential HK2 gene expression.

Glioma samples (n = 204) including astrocytomas, GBMs, oligodendrogliomas and mixed tumours were dichotomized based on the expression of HK2 (upregulated: 3 fold upregulation vs intermediate). Upregulation of HK2 (n = 103) was found to significantly predict poor overall survival in glioma patients (Log-rank p value = 0.033). (National Cancer Institute. 2005. REMBRANDT home page. http://rembrandt.nci.nih.gov, Accessed data October 2009).
Appendix 2

Kaplan-Meier Survival Plot for Samples with Differential HK2 Gene Expression

Number of samples in group:

Upregulated: 103
Intermediate: 101

Log-rank p-value
(for significance of difference of survival between group of samples)
Up-Regulated vs. Intermediate: 0.0333123579
Appendix 3: Immunoprecipitation of HK2 and LC-MS mass spectrometry in U87 GBM cells EGF stimulated.

a) U87 GBM cells were incubated under hypoxia, zero glucose, starved or EGF stimulation for 30 minutes and an hour +/- Caliculyn A. Cells were incubated overnight with HK2 antibody followed by immunoprecipitation. Immunoblots were probed with HK2 and PSer/Thre AKT substrate antibody. b) EGF stimulated U87 cells were IPd for HK2, protein digested, enriched for peptides using TiO2 columns followed by LC-MS at the UHN Mass spectrometry facility. No phosphorylated HK2 was detected at sites including at the AKT kinase substrate motif.
Appendix 3

a

<table>
<thead>
<tr>
<th>IP: HK2</th>
<th>Nom</th>
<th>Hypoxia</th>
<th>No glucose</th>
<th>Starved</th>
<th>EGF (30min)</th>
<th>EGF (1hr)</th>
<th>EGFl + CaA</th>
<th>HK2shRNA (+)</th>
<th>whole cell lystate</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK2</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSer/Thr</td>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT substrate</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b

g(19390703) (100%), 100,479.9 Da

*Chain A, Crystal Structure Of Human Hexokinase II, g(19390704)*

Chain B, Crystal Structure Of Human Hexokinase II

36 unique peptides, 43 unique spectra, 53 total spectra, 446,902 amino acids (49% coverage)

93-102: AMPK kinase substrate motif
468-474: AKT kinase substrate motif
588-591: Abl/Src/Insulin receptor kinase substrate motif