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HYPOXIA-REGULATED EXPRESSION OF GLUT-1 IN GBM CELL LINES

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HYPOXIA-REGULATEDExpression OF GLUT-1 IN GBM CELl LINES

By

Marissa Kane

THESIS

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ABSTRACT

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By

Marissa Kane

Unlike normal cells, cancer cells can grow in low oxygen (hypoxic) environments. Changes in relative oxygen concentration can alter gene expression in tumors to allow for their selective growth. The result of such changes allows the tumor to adapt its cellular metabolism and promote tumor progression. Most notably, hypoxic conditions induce expression of the transcription factor hypoxia inducible factor 1 (HIF-1). HIF-1 is thought to directly affect glucose transporter 1 (GLUT1) expression levels in hypoxic conditions. This study sought to determine the relationship between HIF-1 and GLUT1 expression levels within normoxic and hypoxic environments utilizing an in-vitro GBM model. The data accumulated in this study determined that hypoxia correlates with relative expression levels of hypoxia inducible factors (HIFs) as well as GLUT1. HIF-1β and GLUT1 appear to have different expression levels in fibroblast cells than in GBM cells, solidifying that HIF-1β and GLUT1 are suitable targets for future GBM treatments.
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List of Symbols and Abbreviations

BBB: Blood Brain Barrier
CSC: Cancer Stem Cells
CT: Cycling Threshold
EP-PCR: End-Point Polymerase Chain Reaction
GBM: Glioblastoma Multiforme
GLUT1: Glucose Transporter 1
GSC: Glioma Stem Cell
HIF: Hypoxia Inducible Factor
MCT: Monocarboxylate Transporter
MGMT: O6-methylguanine–DNA methyltransferase
mRNA: Messenger Ribonucleic Nucleic Acid
qPCR: Quantitative Real-Time Polymerase Chain Reaction
TMZ: Temozolomide
VEGF: Vascular Endothelial Growth Factor
Introduction to Glioblastoma (GBM)

The current standard of care for Glioblastoma multiforme tumors (GBM) rarely cures the patient. The typical survival time for 75% of patients is 18 months after their diagnosis and less than 10% of patients survive longer than 5 years past diagnosis, despite severe surgical debulking procedures, chemotherapy, and radiation treatment regimens (Mansour, Fields, Macomson, & Rixe, 2014)(Ali, 2013).

This low survival rate is mostly due to the ability of these tumors to recur quickly and aggressively. It is thought that within the tumor there are distinct subpopulations of cells that are stem-like in their behavior (Singh et al., 2003). These stem-like cells give the tumor self-renewing properties and therefore help the tumor to grow back from only a few cells remaining after treatment (Hjelmeland et al., 2011, p. 829). GBMs are typically hard to surgically remove because the bulk of the tumor can be diffuse and often lies adjacent to important fiber tracts, blood vessels, and other structures in the brain (Park et al., 2010). Debulking surgeries reduce the size of the bulk tumor but fail to target specific subpopulations of cells within the tumor, such as the stem-like cells that can help the tumor grow back. The location of the tumor and the inherent cellular and genetic heterogeneity
within the tumor can influence how aggressive the surgeon will be when removing the tumor (Y. Wang & Jiang, 2013).

In addition to surgery, also included in the standard of care regimen are radiation and chemotherapy. Surgery targets the bulk of the GBM, but leaves behind as much of the tumor as is needed to preserve as much brain function as possible (Park et al., 2010). Radiation and chemotherapy specifically target fast-growing cells that inherently comprise the bulk of the tumor mass, but these treatments have variable effectiveness because they fail to target the characteristic hypoxic microenvironment within the tumor. The hypoxic tumor microenvironment promotes tumor aggression and stem cell formation and confers treatment resistance (Covello et al., 2006). These methods are inherently not effective at killing the slow-growing cells in the GBM and therefore are leaving the cancerous stem-like cell population mostly unharmed. However, all stem cells can't just be targeted to kill the GSCs, as there is the issue of having undesired effects by killing healthy neural stem cells in addition to the malignant stem cells which would further impair the healing process (Lawson et al., 2007).

The cellular, genetic, and epigenetic heterogeneity of GBMs makes designing targeted treatments difficult. One example of a mutation present in some GBM patients in the enzyme IDH1 (isocitrate dehydrogenase 1). IDH1 is a mitochondrial enzyme that, when mutated, has a normal loss in function and leads to the accumulation of 2-hydroxyglutarate (2-HG) (Turkalp, Karamchandani, & Das, 2014) which has been shown to inhibit the enzymatic function of several histone and DNA demethylases. This leads to uncorrected methylation on genes whose expression, when altered, can confer
tumorigenesis (Xiang & Zhi-Qiang, 2015). So indirectly, IDH1 accumulation confers tumor aggression and its expression is characteristic in secondary GBM. IDH1 acts as a prognostic marker in GBM (Parsons et al., 2008) since its expression correlates with tumor grade (Kickingereider et al., 2015). Overall, patients have genetic or epigenetic mutations in combinations that are often unique to their tumor, and often these mutations prevent treatments such as chemotherapy, radiation, or surgery from curing the patient. Consequently, there is a need for individualized treatments based on the patient’s tumor genetic profile.

In addition to genetic mutations present in GBMs, epigenetic changes are also prevalent and preclude certain chemotherapy treatments from eradicating the tumor. Epigenetic changes are evident by methylation or demethylation of promoter or enhancer regions of DNA, which can lead to gene overexpression or gene silencing. An example of this is in the gene MGMT (O6-methylguanine–DNA methyltransferase). The MGMT gene codes for a DNA repair protein that removes alkyl groups that chemotherapy drugs, such as Temozolomide (TMZ), add to tumor DNA to kill cells (Esteller et al., 2000). The alkyl groups that TMZ adds to the tumor DNA, halts cell replication followed by apoptosis in the affected cell (Hegi et al., 2004)(Ochs & Kaina, 2000)(L. Liu, Markowitz, & Gerson, 1996). The potential effects of TMZ on the tumor DNA of a person with normal MGMT will be unaffected by the chemotherapy drug because their functional MGMT protein will just fix the DNA alkylation that the TMZ caused. Therefore, when the MGMT promoter is methylated and its expression is silenced, the tumor becomes more susceptible to chemotherapy alkylating drugs, such as TMZ, and when combined with radiotherapy leads to longer overall survival for the patient (Hegi et al., 2005). Therefore, the gene MGMT is a
common prognostic factor that surgeons and oncologists use to design treatments that will be as effective as possible for the patient (Hegi et al., 2005).

In addition to the challenges presented by the cellular, genetic, and epigenetic heterogeneity of the tumor, aspects of the general biology of the brain leads to challenges, specifically the blood brain barrier. The presence of the blood brain barrier (BBB) makes it difficult to treat GBM, as it severely limits what can pass into the brain. The BBB is a neuroprotective barrier system for the capillaries of the brain and is mostly comprised of endothelial cells connected by tight junctions (Persidsky, Ramirez, Haorah, & Kanmogne, 2006) and acts to prevent toxins and pathogens from reaching the brain. Because of the challenges in permeating the BBB, drug design is difficult and existing treatments for other cancers might not be a viable option for a GBM patient (Juillerat-Jeanneret, 2008). However, nanotechnology appears to be growing more effective at treating GBM by exploiting the presence of LDL receptors on the endothelial cells (Dehouck, Dehouck, Fruchart, & Cecchelli, 1994) and are also more highly expressed in GBM (Nikanjam, Gibbs, Hunt, Budinger, & Forte, 2007).

**Hypoxia and the Tumor Microenvironment**

Abnormal physiology and abnormal location of cells contribute to the characteristic uncontrolled growth of cancer. The cancer cells proliferate with very few stop mechanisms and turn off remaining genes that, when normally translated to proteins, would act to prevent abnormal cell growth. Resulting from the constant growth is an increased
metabolism (discussed more below) and a growing distance between cancer cells and proper vasculature. Although cancer cells are abnormal in terms of their growth, metabolism, and other properties, they have need to maintain some semblance of normal cell physiology, including proper nutrient delivery and waste management. Although the cancer cells try to mimic some of the normal physiological processes such as angiogenesis (blood vessel formation), this ultimately fails and there is still variably lower oxygen in many parts of the tumor, usually in a gradient-like manner. Consequently, the typical microenvironment of bulk tumors is generally hypoxic (lower than normal oxygen), low pH, and nutrient deficient (Pouysségur, Dayan, & Mazure, 2006) since they don’t have proper vasculature.

Cancer cells have the unique ability to survive under hypoxic conditions (Hsu & Sabatini, 2008). Hypoxia has been characterized as a main reason for the poor prognosis for GBM patients (G. L. Semenza, 2002) and has been implicated in tumor cell proliferation, survival, cell migration, invasion, and treatment resistance (G. L. Semenza, 2002) (Bar, Lin, Mahairaki, Matsui, & Eberhart, 2010) (Joseph et al., 2015). Hypoxic conditions have been shown to induce changes in a myriad of genes, which lead to numerous new physiological changes that distinguish cancer from normal tissue. Resulting from the hypoxic microenvironment itself, and the genetic changes indirectly elicited by hypoxia, treatments are largely ineffective for GBMs (P. Wang et al., 2017, p. 1) (Mohyeldin, Garzon-Muvdi, & Quinones-Hinojosa, 2010) (Chiche, Ricci, & Pouysségur, 2013) (Chiche, Ricci, & Pouysségur, 2013). Some of the characteristic aggressiveness of GBMs derives from the heterogeneity of the cells within the tumor, as well as from genetic and epigenetic changes (Liberti & Locasale, 2016a).
A subpopulation of cells in GBM tumors, cancerous glioma stem cells, (GSCs) share the self-renewing properties of normal stem cells, but the malignancy and aberrant signaling of cancer cells (Singh et al., 2003) (Qiu, Fang, Luo, & Ouyang, 2015). These tumor-initiating cells or cancer stem cells (CSCs) exist within the tumor and provide it with the ability to resist treatment and to lie dormant until the cancer stem cells eventually recapitulate the tumor. CSCs might originate from non-stem cancer cells that dedifferentiate due to hypoxic conditions within the tumor microenvironment (P. Wang et al., 2017). The alleged CSCs or tumor-initiating cells have displayed the ability to recapitulate a tumor, despite only comprising a small percentage of the overall tumor mass (P. Wang et al., 2017) (Singh et al., 2003). A recent study found that the glioma stem cell (GSC) subpopulation led to tumor aggressiveness, but also that GSCs are dedifferentiated from differentiated glioma cells (P. Wang et al., 2017). This highlights the debate about the origin of GSCs, whether they derive from malignant neural stem cells or whether they are differentiated glioblastoma cells that dedifferentiated into GSCs.

Both cancer stem cells and genetic variety within the tumor are implicated in the scheme of tumor development (Liberti & Locasale, 2016b). The tumor microenvironment selects for the growth of different cell populations within GBMs, however, it seems intuitive that the opposite is true as well. The heterogeneity comprising the GBM microenvironment results in different growing conditions for cells and leads to genotypic and phenotypic variability within the tumor, which then leads to difficulties in making targeted GBM treatments (Covello et al., 2006) (Lawson et al., 2007) (Hegi et al., 2005) (Gao, Shen, Jin, Miao, & Qiu, 2016). Overall, there appears to be a relationship between the types of cells comprising these GBM subpopulations, the relative oxygen conditions and the vascularity
of the tumor microenvironment, which is discussed more in-depth in future sections. In addition to the advantages directly conferred to cancer stem cells by hypoxia, there are numerous hypoxia induced downstream changes that elicit genetic changes within GBMs that confer other advantages for the tumor (Liberti & Locasale, 2016b). These genetic changes within the tumor are the foundation for the characteristic ‘hallmark’ changes seen in cancer progression: such as angiogenesis, immune evasion, resistance to apoptosis, dormancy from cancer stem cells, inflammation, metastasis, resistance to therapies, and metabolic reprogramming (Hanahan & Weinberg, 2011)(Ravi et al., 2002), which are discussed again below. As described above, epigenetic changes within tumors also confer different factors that affect the prognosis of the patient (Hegi et al., 2005).

Due to the perpetual and uncontrolled growth of cancer cells, tumors quickly outgrow their available vascular supply. As tumors grow, proper vasculature is not created quickly enough to adequately provide for the metabolic needs of the tumor cells; most notably, oxygen delivery. Oxygen must diffuse over long distances to reach the portions of the tumor that lies farthest from the vascular supply. This mismatch of O2 consumption and delivery in certain areas of the tumor leads to a gradient of local decreased partial pressure of oxygen, which is referred to as hypoxia (Denko, 2008). Hypoxia is the relative deprivation of oxygen and a complete deprivation is called anoxia (West, 1977). Hypoxia has been shown to lead to the expression of genes that lead to some of the ‘hallmark’ changes seen in cancer, including: sustained proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis mechanisms, replicative immortality, induction of angiogenesis, and resistance to cell death (Heikkilä, Pasanen, Kivirikko, & Myllyharju, 2011)(Heikkilä, Pasanen, Kivirikko, & Myllyharju, 2011). There are also several
‘emerging hallmarks’ or major themes underlying cancer progression that are now being studied: reprogramming energy metabolism and evasion of immune destruction (Hanahan & Weinberg, 2011)(Ravi et al., 2002).

Cancer cells, like normal cells, require nutrients and mechanisms to transport wastes. To accommodate the needs of the growing tumor, cancer cells can initiate the process of angiogenesis to create new blood vessels (Hanahan & Weinberg, 2011). However, blood vessel formation in cancer is very different from normal vessel formation. Normal physiological processes, such as in embryogenesis, wound healing, or female reproductive cycling, characteristically have new blood vessel formation. Angiogenesis is the short-lived process of forming new vessels in developing and normal tissues; the vessels are organized and lined with endothelial cells without gaps between them (Ferrara, 2010). In contrast, tumor neovasculature is aberrant and is accentuated in hypoxia (Ferrara, 2010)(Hanahan & Weinberg, 2011) and this can also lead to uneven drug distribution when drugs even make it this far into the tumor. The process of cancer-induced angiogenesis is discussed more in depth in later sections.

Introduction to the Warburg Effect

As mentioned above, angiogenesis occurs in the tumor to attempt to fulfill the vast metabolic demand of the proliferating cancer cells. However, the process does not succeed at fully oxygenating the tumor and this causes changes in the relative oxygen concentration
in the tumor microenvironment. Normal tissues anaerobically process glucose to pyruvate using glycolysis in the cytosol before further breakdown to carbon dioxide in the mitochondria, which creates 36 ATP from each glucose molecule in the aerobic process called oxidative phosphorylation (Warburg & others, 1956a). Normally, glycolysis is not the primary process used for the bulk of energy metabolism, as it is far less energy efficient than normal mitochondrial oxidative phosphorylation. Overall, glycolysis in the cytoplasm accounts for only about 10% of the ATP generation needed for a normal cell, with the other 90% being generated from pyruvate processed by oxidative phosphorylation in the mitochondria of the cell (DeBerardinis et al., 2007).

Despite the greater metabolic need of actively proliferating cancer cell subpopulations, nearly 50% of a cancer cell’s energy is produced by glycolysis (Denko, 2008). Cancer cells preferentially use glycolysis followed by lactic acid fermentation in the cytosol, despite its inefficiency at creating ATP per glucose molecule: which is reportedly ~18-fold less efficient at making ATP than is mitochondrial oxidative phosphorylation (Denko, 2008). In the 1920s, Otto Warburg characterized this phenomenon and termed it aerobic glycolysis, which is now also referred to as the Warburg Effect (Warburg & others, 1956b)(Hsu & Sabatini, 2008)(Warburg, Wein House, Burk, & Schade, 1956). The inner workings of the Warburg Effect remain elusive in cancer cell metabolism studies today, but there seems to be a consensus that this metabolic shift to glycolysis is advantageous to the tumor. In 1929, Crabtree confirmed Warburg's findings of ‘aerobic glycolysis’, but also found fermentation occurring. Warburg later proposed that dysfunctional mitochondria were to blame, but his theory was not very popular until signaling cascades were better understood, as many of the enzymes used in mitochondrial metabolic processes are
downregulated by hypoxia induced signaling changes (Denko, 2008) (Papandreou, Cairns, Fontana, Lim, & Denko, 2006). Accordingly, some experts believe that both mitochondria and aberrant aerobic glycolysis play a role in Warburg’s phenomenon. Although aerobic glycolysis in the cytosol is less efficient at generating ATP per molecule of glucose than oxidative phosphorylation in the mitochondria, lactate production derived from glucose metabolism occurs 10-100 times faster than glucose oxidation via the mitochondria (Figure 4) (Vander Heiden, 2009) (Shestov et al., 2014). This difference in the speed of glucose breakdown and subsequent rapid ATP production could account for why aerobic glycolysis is used preferentially to mitochondrial oxidative phosphorylation.

Many of the genetic and phenotypic changes described by the Warburg Effect can be attributed to the hypoxic conditions that occur within the tumor microenvironment, specifically by the family of transcription factors called hypoxia-inducible factors (HIFs) (Figures 1) (Ruan, Song, & Ouyang, 2009) (Hsu & Sabatini, 2008). Hypoxia leads to induced expression of HIFs, which have many downstream target genes: most notably, VEGF and GLUT1 (Fukumura et al., 2001). Glucose transporter 1 (GLUT1) facilitates the uptake of glucose into cells and is overexpressed in GBMs. GLUT1 overexpression allows for higher rates of aerobic glycolysis which indirectly creates an acidic tumor microenvironment (Liberti & Locasale, 2016b) due to the excess of lactate from fermentation (Figure 4). However, this pH drop is short-lived because monocarboxylate transporter (MCTs) expression increases to transport the lactate out of the cell. However, the lactate does not all leave the cell despite the addition of more MCTs; not all the lactate can be transported away from the cell due to the poor vasculature constructed via tumor induced angiogenesis (Gillies, Robey, & Gatenby, 2008) (Newell, Franchi, Pouyssegur, & Tannock, 1993).
Regardless, this decrease in pH confers advantages to the tumor, such as increasing VEGF expression (Fukumura et al., 2001), the main driver of angiogenesis: a process that facilitates more opportunities for the delivery of oxygen and other nutrients, as well as waste removal. Additionally, new vasculature creates a way for cancer cells to get transported to other areas of the body, which drives tumor invasion and metastasis (Sun et al., 2007). The preference for quick glucose metabolism, rather than more efficient metabolism, results in competition for resources between tumor cells and other cells in the vicinity such as tumor-infiltrating lymphocytes and stromal cells (Chang et al., 2015)(Ho et al., 2015). Therefore, hypoxia and the induced expression of hypoxia inducible factors play an important role in regulating aerobic glycolysis and thus GLUT1 expression in tumor cells.

**Tissue Distribution of Hypoxia Inducible Factors (HIFs)**

Hypoxia inducible factors are a family of transcription factors that respond to relative changes in oxygen or in response to oncogenic signaling mechanisms (Smith, Robbins, & Ratcliffe, 2008)(Wilkins, Abboud, Hancock, & Schofield, 2016)(Y. Liu et al., 2009a, p. -3). Three paralogs of the hypoxia-inducible transcription factor family have been identified in mammals: HIFs-1, 2, and 3 (Figure 2) (Tanaka, Wiesener, Bernhardt, Eckardt, & Warnecke, 2009). HIFs are a heterodimeric transcription factors that possess either HIF-1α or HIF-2α as the oxygen sensitive subunit and a constitutively expressed HIF beta subunit (Figure 3) (Denko, 2008). The expression of both HIF-1 and HIF-2 have been
implicated in several normal physiological processes such as erythropoiesis and angiogenesis (C.-L. Chen, Chu, Su, Huang, & Lee, 2010, p. 57). The HIF-1α subunit is ubiquitously expressed throughout the body, although two groups did not detect HIF-1α expression in normal brain tissues (Zhong et al., 1999)(Y. Liu et al., 2009a). However, it should be noted that HIF-1α expression is correlated to pathological grade of glioma (Ravi et al., 2002). HIF-1α is stabilized and upregulated in hypoxic conditions, after oncogene activation, and/or from tumor suppressor gene inactivation, which leads to downstream gene activation (Figure 5). Loss of function of the tumor suppressor gene p53 upregulates HIF-1α expression (Ravi et al., 2002). Expectedly, higher expression levels of HIF-1α and VEGF were seen in the most necrotic and least vascularized areas of the GBM (Chan et al., 1998). The correlation with HIF-1α, VEGF, and GBM pathological grade indicates that HIF-1α is likely important in regulating invasion and metastasis.

In normal tissues, HIF-2α plays a role in the process of erythropoiesis. Generally, the main actions of HIF-1α or HIF-2α are to mediate the cellular and systemic responses to hypoxic conditions in the body, but the expression of each HIF has yet to be fully elucidated (Patel & Simon, 2008). HIF-2α is expressed in certain tissues: vascular endothelial cells, neural crest cell derivatives, glial cells (Hu et al., 2006), lung type II pneumocytes (Patel & Simon, 2008), liver parenchyma, cardiomyocytes, and interstitial cells in the kidney and several other organs (Ruan et al., 2009)(Zhao, Du, Shen, Zheng, & Xu, 2015)(M. S. Wiesener et al., 1998)(M. Wiesener, 2003), neuroblastoma (Holmquist-Mengelbier et al., 2006), and glioma stem cells (Li et al., 2009). HIF-2α expression has been implicated with several types of stem cells, including embryonic stem cells (Covello et al., 2006, p. -4)(Hu et al.,
Importantly, HIF-2α makes an ideal therapeutic target because GSCs overexpress HIF-2α whereas and normal neural progenitor cells produce nearly undetectable levels (Li et al., 2009). Therefore, since HIF-2α isn’t produced in healthy progenitor cells, it seems likely that a therapy aimed at targeting HIF-2α is a viable option. By somehow blocking the expression of genes downstream of HIF-2α, a cancer specific therapy could be made that would potentially have less unwanted cell death and therefore fewer unwanted effects for patients. Contrastingly, since HIF-1α is more ubiquitously expressed, it might not be the most suitable HIF available to target only GBM and GSCs because it might cause undesired cell death in other tissues. A third HIF α isoform, HIF-3α, was discovered in 1998; HIF-3α expression is poorly characterized and the role that HIF-3α plays in hypoxia mediated functions is poorly understood. Overall, less is known about HIF-3α expression and regulation than about HIF-1α and HIF-2α (Tanaka et al., 2009).

**Induction and Regulation of HIF-1 and HIF-2**

**HIF-1**

The hypoxia inducible factor proteins HIF-1α and HIF-2α share very similar protein domains (Figure 2) and are mainly regulated via their oxygen-dependent domains (ODDs). Their shared homology is most apparent in the N-terminal basic Helix-Loop-Helix (bHLH) domain which is adjacent to the two Per-ARNT-Sim (PAS) domains: the oxygen-dependent
domains characteristic of HIF-1α and HIF-2α. Under hypoxic conditions, the PAS domains allow hetero-dimerization and DNA binding to occur; the C-terminal of HIF facilitates interactions with coactivators such as CREB binding protein and p300 (Jiang, Zheng, Leung, Roe, & Semenza, 1997)(Kallio et al., 1998)(Pugh, O’Rourke, Nagao, Gleadle, & Ratcliffe, 1997)(Pugh, O’Rourke, Nagao, Gleadle, & Ratcliffe, 1997). These coactivators facilitate transcription by interacting with sequence specific DNA binding proteins and with other RNA Polymerase II associated general transcription factors (G. Semenza, 2000).

Importantly, there are conserved proline sequences within the PAS domains of all HIF-α, which get targeted for degradation under normoxic conditions (Figure 2)(Heikkilä et al., 2011)(Heikkilä et al., 2011). The structure of HIF-3α is similar to alpha subunits of HIF1α and HIF2α, sharing 55% amino acid sequence identity and share similar domain structures (Figure 2) (Gu, Moran, Hogenesch, Wartman, & Bradfield, 1998).

Both HIF-α and β mRNAs are found in normal cells and are constitutively transcribed and translated into functional proteins. The HIF-1β subunits are constitutively active in all oxygen conditions. However, in normoxic conditions, HIF-1α proteins are rapidly degraded and have a typical half-life of five to ten minutes (Chiche et al., 2013, p. 112) (Qingdong Ke & Costa, 2006, p. 1470) (Cunningham, Candelario, & Li, 2012, p. 411)(Q. Ke & Costa, 2006). Consequently, HIF activation depends mostly on the stability of the alpha subunit (G. L. Semenza, 2002). Additionally, HIF-1α is constitutively overexpressed when the von Hippel-Lindau tumor suppressor gene is lost (Y. Liu et al., 2009a, p. - 3)(Huang, Gu, Schau, & Bunn, 1998). As mentioned earlier, there are highly conserved proline sequences in the bHLH and PAS domains of both HIF-1α and 2α proteins that are
hydroxylated by the enzyme prolyl hydroxylase (PHD). Asparagine sequences are hydroxylated in the C-terminal protein domain by enzymes called factor inhibiting HIF (FIHs). Additionally, lysine residues in the oxygen dependent degradation domain of HIFα subunits are acetylated by an acetyltransferase called arrest-defective-1 (ARD1), making it more favorable for association with the pVHL ubiquitin-elongin complex (Jeong et al., 2002). Jeong et al found that the activity of acetyltransferases is not influenced by oxygen conditions and concluded that the ARD1 facilitated acetylation of HIF-1α was irrelevant to relevant oxygen levels (Jeong et al., 2002). However, both mRNA and protein ARD1 levels were decreased in hypoxia which leads to the conclusion that there might be relatively lower levels of acetylated HIF-1α in hypoxia than in normoxia. Overall, all HIF-α is destabilized by the hydroxylation of its proline, asparagine, and lysine segments: and hydroxylation also serves as a recognition signal for ubiquitination (Denko, 2008)(Stiehl et al., 2006). The pVHL complex ubiquitinates HIF-1α subunits thereby targeting it for proteasomal degradation (Qingdong Ke & Costa, 2006, p. 1470)(Stickle et al., 2004). The pVHL complex is ubiquitously expressed in many tissues and it is mostly localized to the cytoplasm and thus facilitates HIF-α degradation to occur in either the nucleus or the cytoplasm of normoxic cells (Berra, Roux, Richard, & Pouysségur, 2001)(Berra, Roux, Richard, & Pouysségur, 2001)(Groulx & Lee, 2002).

In hypoxic conditions, HIF-1α increases in stability and is therefore no longer targeted for degradation, thereby increasing functional HIF-1α protein subunits (Denko, 2008). Oxygen is a low-affinity substrate for the PHDs and so when oxygen levels decrease, PHD mediated hydroxylation decreases. Additionally, FIHs are inhibited and HIF-1α
doesn't get hydroxylated (Denko, 2008); therefore, the inactivation of PHDs and FIHs prevents HIF-1α ubiquitination mediated proteasomal degradation (Eales, Hollinshead, & Tennant, 2016). Additionally, HIF-2α has been shown to not be as responsive as HIF-1α to PDH-2 and PDH-3 induced degradation (Pasanen et al., 2010). This method of hydroxylation or acetylation-mediated regulation is the primary way HIF-1α gene expression is regulated, but there are other processes that modulate HIF-1 activity in tumors as well as for HIF-1 post-translational regulation that are not discussed in this review (Denko, 2008). In summary, HIF-1β is constitutively expressed, while HIF-1α post-translational stability is induced by hypoxic conditions (Qingdong Ke & Costa, 2006, p. 1469) (Denko, 2008).

In hypoxic conditions, stabilized HIF-1α translocates to the nucleus where it dimerizes with HIF-1β (Figure 3) and recruits cofactors such as CPB/p300 (Figure 5) (Denko, 2008) (Qingdong Ke & Costa, 2006, p. 1470) which bind to hypoxia-responsive-elements (HREs) in the enhancer or promoter regions on target DNA sequences (Chiche et al., 2013, p. 112)(Denko, 2008)(Pouysségur et al., 2006)(Pouysségur et al., 2006). In this way, HIF-1α/β initiates transcription of target genes associated with decreased mitochondrial respiration, increased glycolysis, pH regulation, erythropoiesis, apoptosis, survival, motility, basement membrane integrity, vasodilation, angiogenesis, and hematopoiesis; these HIF target genes are discussed below (Tanaka et al., 2009)(Qingdong Ke & Costa, 2006, p. 1474) (Chiche, Brahimi-Horn, & Pouysségur, 2010, p. 776)(Chiche, Brahimi-Horn, & Pouysségur, 2010, p. 776). The HIF family transcriptionally and post-translationally regulates an expansive set of downstream genes that establishes many of the characteristic
behaviors of cancer, which makes them a very relevant target to study for future cancer therapeutics.

HIF-1α is the most widely expressed of the HIFs and has been characterized in head and neck cancers, ovarian cancer, and oesopharyngeal cancer: all of which have poor prognosis and are therapy resistant (Talks et al., 2000). Another study found overexpressed levels of HIF-1α in colon, breast, gastric, lung, skin, pancreatic, prostate, and renal carcinomas; HIF-1α overexpression was also correlated with cell proliferation in this study (Talks et al., 2000) (Zhong et al., 1999). HIF-1α mRNA expression has also been implicated with wound healing (Elson, Ryan, Snow, Johnson, & Arbet, 2000) which is not surprising since stem cells grow preferentially in hypoxic environments.

**HIF-2**

Like the HIF-1α/β heterodimeric complex, HIF-2 has an oxygen regulated subunit HIF-2α and binds to a HIF-2β complex. HIF-2 expression is characterized in stem cells, endothelial cells, as well as in glioma stem cells (Covello et al., 2006). Some studies have shown that HIF-2α has a higher specificity for targeting erythropoiesis, whereas HIF-1 was said to specifically target glycolytic enzymes (Pasanen et al., 2010). Another study found that there was higher expression of HIF-2α in high-grade pediatric astrocytoma than in low-grade astrocytoma; therefore, HIF-2α might correlate with tumor aggression (Khatua et al., n.d.). These studies indicate overall differential regulation patterns amongst the members of the HIF family of transcription factors. Some tissues express higher levels of
HIF-1α than HIF-2α and vice versa (Keith & Simon, 2007) and the differential regulation also seems to depend on the oxygen concentration. For example, in neuroblastoma samples, there were strong expression levels of HIF-2α in well vascularized areas. Similarly, *in-vitro* neuroblastoma studies at 5% O₂, which simulated normal physiological oxygen conditions of an end-capillary, found stable expression of HIF-2α protein and expression of VEGF, a common downstream HIF-1α target gene, despite low HIF-1α expression levels. This was interesting because HIF-2α induced the expression of the same downstream target as HIF-1α. When cultured at 1% O₂, there was brief stabilization of HIF-1α whereas HIF-2α protein gradually accumulated and led to a more chronic hypoxia mediated response (Pasanen et al., 2010). HIF-2α knockdown reduced neuroblastoma growth in athymic mice which supports that HIF-2α expression correlates with clinical stage, and along with high VEGF expression, can predict poor prognosis for patients (Holmquist-Mengelbier et al., 2006)(Pasanen et al., 2010). More generally, this provides evidence that there is differential expression of HIF-1α and HIF-2α at different oxygen levels, even within the general classification of a hypoxic environment. These findings also promote the idea that the HIF regulation differs in acute hypoxia as opposed to chronic hypoxia (Pasanen et al., 2010). There is also evidence that HIF-2α has a larger role in the GSC population, making it an important target for continued study (Li et al., 2009).

**Induction and Regulation of HIF-3**
The regulation of HIF-3α is not as well characterized as that of HIF-1α and HIF-2α. There are various reports categorizing the expression levels of the various HIF-3α isoforms. Some groups have reported that HIF-3αv1 (variant 1) mRNA is upregulated by hypoxia, while the HIF-3αv4 was downregulated. Another reported that among other splice variants, HIF-3αv4 was actually hypoxia inducible (Pasanen et al., 2010). RNA interference experiments also found that HIF-1α and not HIF-2α was responsible for the hypoxia inducible nature of HIF-3α variants and these findings were also seen in renal carcinoma Caki cells by Tanaka et al in 2009 (Pasanen et al., 2010)(Tanaka et al., 2009).

The multiple splice variants of HIF-3α also appear to have differing functions based on the tissue they are expressed in. It is currently unknown whether HIF-3 mediated negative regulation of HIF-1 occurs ubiquitously or at all. Other studies have said that the roles of HIF-3α variants might be more versatile than just negatively regulating HIF-1α and HIF-2α (Heikkilä et al., 2011)(Heikkilä et al., 2011). More studies are needed to tease out the role of HIF-3α/β in general as well as in the context of GBM. A set of database studies predicted that there are three possibly utilized transcription initiation sites on the six splice variants and found that hypoxia upregulated expression of all three promoter sites. They also found that the promoter expression was mediated exclusively by HIF-1α (Pasanen et al., 2010). Some experts claim that rather than HIF-3α regulating HIF-1 and HIF-2, HIF-3α is oxygen dependent and is instead regulated exclusively by HIF-1α, not HIF-2α. Some studies concluded that the second splice variant of the HIF-3α isoform, HIF-3αv2, as well as HIF-3αv4, act as a negative inhibitor in HIF-1α and HIF-2α signaling (Maynard, 2005)(Heikkilä et al., 2011)(Heikkilä et al., 2011). However, one study investigated this further, but looked into whether shorter cytoplasmic HIF-3α splice
variants elicited different functions than longer nuclear HIF-3α splice variants and found varied results (Heikkilä et al., 2011)(Heikkilä et al., 2011). HIF-3α overexpression has also been shown to decrease the hypoxia regulated expression of VEGF-A and Enolase2 in human vascular cells (Augstein, Poitz, Braun-Dullaeus, Strasser, & Schmeisser, 2011). More studies are needed on HIF-3α, but based on the literature, HIF-3α either regulates HIF-1α, or is regulated by HIFs1α and 2α. Investigating this inconsistency could prove very useful to future research. If HIF-3α in fact regulates HIF-1α and HIF-2α and consequently, their target genes like VEGF-A; HIF-3α could be a great target for future studies.

**Target Genes Downstream of HIF**

As stated, as many as 100 target genes exist for HIF-1 whose activation can lead to widespread effects such as angiogenesis, vascular remodeling, erythropoiesis, pH regulation, cellular proliferation, apoptosis, vasodilation, migration/invasion, catecholamine and iron, and energy metabolism (Table 1) (Q. Ke & Costa, 2006). There is some redundancy in the target genes of HIF-1α and HIF-2α; both HIFs are hypoxia inducible and bind to HREs on many of the same target genes (Harris, 2002)(Hu et al., 2006). Most important for this study are the processes implicated with metabolic remodeling within the GBM microenvironment and are reviewed below (Denko, 2008).

**Angiogenesis**

Hypoxia mediated HIF expression mediates tumor progression, largely in part due to upregulation of genes involved with invasion, metastasis, and new blood vessel growth. As described above with the Warburg Effect, tumor cells have a growth advantage in
hypoxic, acidic and nutrient deficient areas. However, tumor cells still have metabolic requirements and need ways to transport wastes to prevent cell toxicity and death. To accommodate the need for increased nutrient and waste transport, cancer cells tend to have upregulated expression of genes involved with angiogenesis. Angiogenesis is a multistep process involved in creating new vasculature for tissues: this process is hijacked in cancer. Most notably, the endothelial-specific mitogen called vascular endothelial growth factor (VEGF), is expressed by cancer cells, which leads to recruitment of endothelial cells to hypoxic areas and subsequent endothelial cell proliferation (Mansour et al., 2014), which fails to prevent the “chronic hypoxia” characteristic within areas of the tumor (Harris, 2002)(Brown & Giaccia, 1998). Activation of VEGF and other angiogenic factors leads to the creation of more vasculature, which slightly decreases the distance that oxygen needs to diffuse into tissues while also making nutrient delivery and waste transport more possible (Q. Ke & Costa, 2006). However, the organization and structure of the new vessels are weak and hypoxic conditions can remain despite the new vasculature (Hida & Klagsbrun, 2005). It is unclear how well this tumor-induced vasculature distributes nutrients to the areas surrounding the tumor. The new blood vessels are unstable; the new vessels close randomly and then sometimes reopen (Brown & Giaccia, 1998), which provides oxygenated blood to previously hypoxic areas of the tumor in a process called ‘reoxygenation injury’ (Prabhakar, 2001). Free radicals flood the surrounding tumor microenvironment and consequently, these areas are resistant to radiation treatment because radiation targets cells using oxygen free radicals; hypoxic cells are therefore acclimated to this process already (Wouters & Brown, 1997), which provides one explanation for the ineffectiveness of radiation as a treatment for some patients.
Regardless, hypoxia has been implicated with this vascularization process and HIFs are present in various tissues and in various areas of the tumor microenvironment. Both HIF-1α and HIF-2α are highly inducible in hypoxic areas, with varying expressions based on specific oxygen levels of the tumor microenvironment and have also been shown to upregulate VEGF expression (Holmquist-Mengelbier et al., 2006) (Fukumura et al., 2001) (Forsythe et al., 1996). Additionally, cancer stem cells have been found to grow in niches near endothelium (Hira et al., 2015), which seems to correlate to HIF-2α expression since its expression has been implicated with highly vascularized areas, which typically are comprised in part of endothelium. Overall, angiogenesis is implicated with poor patient prognosis in GBM, neuroblastoma, and several other cancers (Holmquist-Mengelbier et al., 2006) (Pasanen et al., 2010).

**Metabolic Remodeling**

In low oxygen conditions, HIFs are upregulated and cause downstream changes, most notably in glucose metabolism. Although less efficient in some ways, cancer cells shift their metabolism from mitochondrial oxidative phosphorylation to the oxygen-independent process glycolysis but do so aerobically (Figure 4). Since glycolysis only generates 2 ATP molecules from each glucose molecule (Brahimi-Horn, Chiche, & Pouysségur, 2007)(Brahimi-Horn, Chiche, & Pouysségur, 2007), more glucose molecules are required to maintain cancer cell viability and subsequent aberrant growth (Q. Ke & Costa, 2006). This increased glucose uptake into the cell is achieved by up-regulating glucose transporters, GLUT1 (SLC2A1) and GLUT3 (SLC2A3) (Denko, 2008) (Q. Ke & Costa, 2006). Since the glucose transporters move glucose according to its concentration gradient, a
simple upregulation of GLUTs facilitates the increased need for intracellular glucose (Denko, 2008) (Ozbudak, Karaveli, Simsek, Erdogan, & Pestereli, 2008).

There are numerous uses for the intracellular glucose and most of them have been directly implicated with HIF-1 (Table 1). The hexokinase (HK) enzymes are responsible for phosphorylating glucose into Glucose-6-phosphate, which is a charged molecule. The phosphorylated glucose can no longer escape the cell through the plasma membrane and is instead utilized in several pathways, including glycogoprotein synthesis, metabolized via the pentose shunt to become ribose, or for glycogen synthesis (Denko, 2008). However, glucose is mostly utilized by glycolysis by further breakdown and involves the following 12 HIF-inducible glycolytic enzymes: phosphoglucone isomerase (PGI), phosphofructokinase 1 (PFK1), Aldolase, Triosephosphate isomerase (TPI), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), Phosphoglycerate Kinase (PGK), Phosphoglycerate mutase (PGM), Enolase, Pyruvate Kinase (PK), and 6-phospho-2-kinase/fructose 2,6 bisphosphatase (PFKFB1-4) (Table 1) (Denko, 2008).

In glycolysis, intracellular glucose is broken down to pyruvate by several enzymes, including pyruvate dehydrogenase (PDH) (Dengler, Galbraith, & Espinosa, 2014). However, pyruvate is not utilized by the mitochondria of hypoxic cells and is mostly converted into lactate by the enzyme lactate dehydrogenase (LDHA) through fermentation. Excess lactate is one of the two known mechanisms that decreases the intracellular pH and consequently needs to be shuttled out of the cell to prevent cell toxicity (Harris, 2002). This is accomplished by releasing lactate into the extracellular space via HIF-inducible monocarboxylate transporters (MCTs) embedded in the plasma membrane (Table 1).
MCTs have been implicated in many studies and *in-vitro* knockdown has led to *in-vitro* decreased tumor cell aggressiveness with expected decrease in lactate shuttling, cell proliferation, invasion, and metastasis; there was also a distinct decrease in *in-vivo* tumor formation (Morais-Santos et al., 2015). Carbonic anhydrase (CAIX) transporters are also important in contributing to the low pH microenvironment by converting carbon dioxide and water to carbonic acid. In tumor cells with defective LDH, there is still a low extracellular pH which implies that CAIXs are a factor for the tumor’s low pH (Yamagata, Hasuda, Stamato, & Tannock, 1998). Transcription of carbonic anhydrase-9 was initiated by hypoxia in several tumor lines (Wykoff et al., 2000) and is suppressed in normoxic conditions (Loncaster et al., 2001), which implies at least an indirect relationship with HIFs, with general hypoxia, or with the pH decreases that accompany glycolytic changes induced by hypoxia. Overall, carbonic anhydrases are expressed in many tumor types and high expression correlates to poor patient prognosis (Harris, 2002) (Chia et al., 2001).

The Warburg Effect asserts that hypoxia and subsequent HIF-1 activation are correlated with lactate and pyruvate accumulation, but it has also been found that lactate and pyruvate accumulation might also lead to HIF-1α accumulation in both normoxic and hypoxic conditions (Q. Ke & Costa, 2006) (Denko, 2008) (Marin-Hernandez, Gallardo-Perez, Ralph, Rodriguez-Enriquez, & Moreno-Sanchez, 2009). As discussed, glycolytic energy metabolism might be advantageous to tumor cells due to the fast breakdown of glucose to pyruvate followed with the conversion to lactate via fermentation. Although more ATP per glucose is produced from mitochondrial oxidative phosphorylation, it is faster to perform aerobic glycolysis for energy and confers advantage over cells that are restricted to aerobic
oxidative phosphorylation in the mitochondria, which is also bypassed because of the many HIF-1 mediated mechanisms (G. L. Semenza, 2010b)(G. L. Semenza, 2010a)(Papandreou et al., 2006).

In addition to upregulating genes involved with glycolysis, such as glucose transporters, (GLUTs), HIF-1 indirectly and directly modulates mitochondrial function in hypoxic cells. HIF-1 indirectly decreases pyruvate flow to the mitochondria by activating the master kinase, pyruvate dehydrogenase kinase 1 (PDK1) which has been deemed HIF-dependent since it requires pyruvate in the Kreb’s cycle to be induced. However, PDK1 is a confirmed direct transcriptional target of HIF-1. PDK1 is a master kinase that phosphorylates the E1 segment of the enzyme phosphate dehydrogenase (PDH), thereby inactivating it. This prevents PDH from breaking pyruvate down irreversibly to Acetyl-CoA, CO₂, and NADH which are used as fuel for the Kreb’s cycle and for the electron transport chain (ETC) (Denko, 2008). Overall, HIF-activated PDK isoforms directly block the flow of pyruvate into the mitochondria (R. Thomas, 2001) and prevents oxidative phosphorylation, total O₂ consumption (Papandreou et al., 2006) by the cell, and reactive oxygen species (ROS) generation (Kim, Tchernyshyov, Semenza, & Dang, 2006). Other HIF-1 inducible mechanisms are apparent but were not reviewed in this study. Targeting aerobic glycolysis from multiple approaches might be an effective way to decrease tumor growth.

**Glucose Transporters (GLUTs)**

GLUT1 and GLUT3 belong to a family of 13 glucose transporters and primarily regulate glucose transport due to many factors: such as their wide tissue distribution and affinity for glucose (Denko, 2008)(Ozbudak et al., 2008)(Airley & Mobasher, 2007). As
previously discussed, glucose transporters (GLUTs) 1 and 3 have been involved with hypoxia and are a major component of the metabolic remodeling that is characteristic in many types of cancer including: endometrial (Ma et al., 2015), gastric (J. Liu et al., 2015), squamous cell carcinoma (Asmaa Gaber Abdou, MD, Marwa Mohammad Serag Eldien, MD, & Daliah Elsakka, MD, 2015), ovarian cancer (Labak et al., 2016), meningioma (Nes, Johannes AP, et al, 2015), and glioblastoma (GBM) (Bache et al., 2015). As expected, GLUT1 mRNA overexpression is induced by H-ras oncogenic transformation and by hypoxic conditions (C. Chen, Pore, Behrooz, Ismail-Beigi, & Maity, 2001, p. 9519). Hypoxia induced HIF-1α expression increases GLUT1 and GLUT3 expression levels (Y. Liu et al., 2009a)(Rooj, Bronisz, & Godlewski, 2016).

Past research has categorized GLUT-1 and GLUT-3 in various areas of GBM microenvironments. GLUT1 expression levels differ in different areas of the tumor: partially a product of hypoxia and of hypoxia-mediated vascularity. HIF-1α stability is regulated by the severity of the local intra-tumoral hypoxia. Not surprisingly, GLUT1 expression is correlated with the more hypoxic areas of the tumor microenvironment. As was previously discussed, HIF-1α and HIF-2α correlate with VEGF expression which is the primary protein responsible for neovasculature creation. Sites of angiogenesis have been correlated with cancer stem cell niches, as CSCs are commonly found in niches near endothelial cells, which are the main cells activated by VEGF expression. Transcription of GLUT1 and the stem cell marker Oct4 have been identified in hypoxic areas of GBM and when GLUT1 was targeted, self-renewal was inhibited in CSCs. GLUT3 was shown to regulate Oct4 in embryonic stem cells and is likely implicated in cancer stem cell self-renewal ability (Christensen, Calder, & Houghton, 2015, p. 4). These studies provide
further evidence that GLUT1 and likely GLUT3 are correlated with CSC populations within GBM and indirectly with tumor aggression, treatment resistance, tumor recurrence, and invasion, to name a few (Labak et al., 2016). Many of the studies investigating the role of GLUT1 in GSCs haven’t determined whether elevated GLUT1 expression can directly confer tumor initiating abilities and stem cell self-renewal potential. Recent studies have found that GSCs have increased glycolytic metabolism compared to non-stem cancer cells (Hanahan & Weinberg, 2011)(Zhou et al., 2011), which supports the data concluding that GLUT1 upregulation is more pronounced in GSC populations. It is unknown whether GLUT1 directly regulates stemness in GSCs, but this is an area for future investigation (Shibuya et al., 2015). Targeting glycolysis as a cancer therapeutic has limitations due to it being utilized in many tissues, both healthy and cancerous throughout the body (Hanahan & Weinberg, 2011)(DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008). The selective overexpression of GLUT1 in GSC populations makes GLUT1 a strong contender for future targeted treatments (Flavahan et al., 2013). There are few therapies developed for GLUT3 expression in CSCs, but its expression in GBM, suggests that it remains a good option for future targeted GBM therapies (Flavahan et al., 2013).

**Summary**

The data presented in this study sought to investigate the relationship between GLUT1 and HIF-1α/β in GBM and fibroblast cell lines. A time course assay was completed to determine if GLUT1 relative mRNA expression levels changed in hypoxic conditions, the amount of hypoxia exposure was required to induce these changes, and if the GLUT1 expression levels correlate to relative mRNA expression of HIF-1α/β. This data can be
utilized for future studies to identify a direct relationship between HIF-1α/β heterodimers and GLUT1 in GBM.
Figure 1: Spatial relationship between a blood vessel, hypoxia, and tumor micro-environment (S. Thomas et al., 2013).


The figure above depicts the correlation HIF-1 expression and relative oxygen concentration in the context of hypoxic tumor microenvironment in tumors and their proximity to blood vessels. In normoxia, HIF-1α subunits are rapidly degraded, but the mechanisms that degrade them in normoxia are less effective in hypoxic conditions resulting in stabilized HIF-1α and subsequent translocation in the nucleus where it binds to HIF-1β which together comprise an active HIF-1 heterodimeric protein.
The three HIF paralogs: HIF-1α, HIF-2α, and HIF-3α and the HIF-3α splice variant called inhibitory PAS domain protein (IPAS) which might to negatively regulate HIF-1 mediated transcription, but this is controversial. All the HIF alpha subunits hetero-dimerize with HIF-β/ARNT subunits, possibly except for the IPAs HIF-3α splice variant, which might only bind to a specific HIF-3β subunit. All the HIFs possess highly similar bHLH and PAS domains which mediates the characteristic HIF-α/β hetero-dimerization and DNA binding. All the HIF-α subunits contain oxygen dependent domains (ODDDs) and N-terminal transactivation domains (NADs). The oxygen dependent domains (ODDDs) contain the proline sequences that are hydroxylated by PHDs in normoxic conditions, as discussed in Figure 6 and Chapter 1. The hydroxylation of these sequences and others targets HIF-1α for proteasomal degradation which confers HIF-α instability in normoxic conditions. HIFs 1 and 2 contain oxygen dependent C-terminal transactivation domains (CADs). Both CADs and NADs facilitate the recruitment of coactivators and transcription intermediates to the HIF-α/β complex which facilitates association with transcriptional activation of HIF-downstream genes.
After HIF-α stabilization, it translocates to the nucleus and binds to the constitutively active HIF-1β subunit, forming a heterodimeric complex. The heterodimerization of the complex is essential to binding to HRE binding. The basic residues near the N-terminus of each protein facilitates binding to the nucleotides of the hypoxia response element (HRE) in the promoter region of DNA sequences of target genes (Lisy & Peet, 2008). The heterodimeric HIF-α/β also recruits cofactors and RNA polymerase II and initiates transcription genes that mediate cell metabolism, angiogenesis, erythropoiesis, and many other properties that are normal and also that can promote cancerous phenotypes.

**Figure 3: HIF dimerization and DNA binding.**

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The figure above depicts the major differences between normal cellular metabolic processes and abnormal cancer metabolism. When in the presence of oxygen, differentiated and non-proliferating tissues metabolize a portion of their glucose into pyruvate anaerobically in the cytoplasm by glycolysis, which produces 2 ATP. Then in environments with oxygen present, oxidative phosphorylation completely oxidizes most of the pyruvate made in glycolysis in the mitochondria. Oxygen is required for oxidative phosphorylation to occur, as oxygen is the final electron acceptor for oxidizing glucose. When oxygen is reduced, the pyruvate generated by glycolysis can be used to generate lactate by anaerobic fermentation as opposed to mitochondrial oxidative phosphorylation, which is only done aerobically. Generation of lactate cycles NADH into NAD+ and propagates glycolysis repeatedly. Warburg found that cancer cells and normal proliferating cells convert most of their glucose to lactate despite whether oxygen was present, aerobic glycolysis. Despite the mitochondria being used less for oxidative phosphorylation, it is fully functional at producing ATP.
FIGURE 5: MECHANISMS OF HYPOXIA-INDUCIBLE FACTOR 1(HIF-1α) STABILIZATION

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HIF-1α is unstable in well-oxygenated tissues due to many mechanisms that degrade it within five to ten minutes of its translation (Chiche et al., 2013, p. 112) (Qingdong Ke & Costa, 2006, p. 1470) (Cunningham et al., 2012, p. 411) (Q. Ke & Costa, 2006). However, HIF-1α stabilization can be a product of a hypoxic environment or oncogene activation associated activation of genes such as Ras, RAF, MAPK, phosphoinosotide-3 kinase (PI3K), PTEN, or Akt pathways. In Figure 6a, the HIF-1α domain structure is shown. In normoxia, the proline (P) sequences 402 and 546 and the asparagine (N) 803 sequences are hydroxylated thereby targeting the HIF-1α protein for degradation. In Figure 6b, the classical pathway of HIF-1α stabilization is depicted. In normoxia, the very conserved bHLH and PAS domains of HIF-1α have proline sequences (P) that are hydroxylated by the enzyme prolyl hydroxylase (PHD); the asparagine sequences (N) are hydroxylated by enzymes called factor inhibiting HIF (FIHs). HIF-1α hydroxylation of the various proline, asparaginide, and lysine (discussed in Chapter 1) serves as a recognition signal for ubiquitination (Denko, 2008) (Stiehl et al., 2006). The hydroxylated proline sequences are recognized by the ubiquitin ligase pVHL that is complexed to elongins B and C, which makes the VHL-elongin complex shown above (Jeong et al., 2002) (Stickle et al., 2004). The VHL complex binds to the hydroxylated proline sequences and ubiquitinates it and thus
targets it for proteasomal degradation (Qingdong Ke & Costa, 2006, p. 1470) (Stickle et al., 2004). However, in hypoxic conditions, the PHD and FIH enzymes do not function as effectively and HIF-1α protein remains stable because it is no longer targeted for degradation (Denko, 2008). Additionally, Kreb’s cycle intermediates such as succinate or fumarate or mitochondrial reactive oxygen species (ROS) can also inhibit the function of PHDs which would also lead to increased HIF-1α stabilization and subsequent heterodimerization with the constitutively active HIF-1β subunit. This heterodimeric complex then translocates to the nucleus of the cell before binding to the hypoxia response element (HRE) in the promoter region of target DNA sequences.
Expression of hypoxia inducible factors (HIFs) has been shown to mediate much of the characteristic metabolic remodeling that occurs in cancer cells. Specifically, glucose entry is upregulated by upregulating expression of GLUT1 and GLUT3 transporters. Additionally, glycolytic enzymes are upregulated to promote glycolysis and fermentation as opposed to the normal aerobic oxidative phosphorylation that occurs after brief glycolysis. Also, monocarboxylate transporters are upregulated to shuttle out the excess lactate that gets produced by fermentation, to promote continued cell viability.

**Table 1: HIF-1 targets that regulate glucose metabolism (Denko, 2008).**

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Objectives and Hypotheses

Many studies have discussed the roles of HIF-1 and GLUT1 in cancer metabolism. However, it is unknown whether there is a direct relationship between them in a Glioblastoma (GBM) in-vitro model. In other systems, the heterodimeric HIF-1α/β complex binds directly to the Hypoxia Response Element (HRE) on the GLUT1 promoter to drive expression of GLUT1 (Amann et al., 2009). In this study, changes in HIF-1 and GLUT1 relative mRNA expression were measured in response to changes in relative oxygen concentrations utilizing reverse transcription followed by End-Point Polymerase Chain Reaction (EP-PCR) and Quantitative Polymerase Chain Reaction (qPCR) in a GBM in-vitro model. EP-PCR was conducted to test whether the genes of interest were expressed at all. Then qPCR was conducted to quantify the amount of mRNA transcripts that were transcribed over time in a hypoxic environment.

**Hypothesis 1:** GLUT1 and HIF-1 expression in GBM cell lines is directly correlated with in-vitro hypoxic conditions.

**Overall Research Questions:**

- Does the expression of HIF-1 and GLUT1 transcripts change in hypoxic conditions? How long are hypoxic conditions required to induce these changes?
Does HIF-1 gene expression directly correlate with GLUT1 and relative oxygen concentrations?

**Aim 1:** Determine the presence or absence of HIF-1α, HIF-1β, and GLUT1 in U87MG GBM cells and in MSU1.1 cells in both *in-vitro* normoxic and hypoxic environments using EP-PCR and gel electrophoresis.

- **Aim 1a:** Generate and test the designed PCR primers and confirm the presence or absence in expression of HIF-1α, HIF-1β, and GLUT1 in both U87MG GBM cells and MSU1.1 cells.
- **Aim 1b:** Characterize the presence or absence of expression of HIF-1α, HIF-1β, and GLUT1 in both normoxic (20% O₂) and hypoxic (5% O₂) environments using EP-PCR and gel electrophoresis.

**Aim 2:** Quantify the relative expression levels of HIF-1α, HIF-1β, and GLUT1 in GBM using qPCR.

- **Aim 2a:** Quantify relative gene expression levels of HIF-1α, HIF-1β, and GLUT1 in both U87MG glioblastoma cells and MSU1.1 fibroblast cells while grown *in-vitro* in a normoxic control (20% O₂) environment.
- **Aim 2b:** Quantify relative gene expression levels of HIF-1α, HIF-1β, and GLUT1 in both U87MG glioblastoma cells and MSU1.1 fibroblast cells while grown *in-vitro* in a hypoxic environment (5% O₂).

**Aim 3:** Determine whether there is a positive correlation between relative expression of HIF-1α, HIF-1β, and GLUT1 in U87MG and MSU1.1 cells and whether it correlates with *in-vitro* hypoxic conditions over time (0, 3, 6, 12, or 24 hours).
Methods

PCR Primer Design

Splice variants of the genes of interest were evaluated using the UCSC Genome Database. The NCBI Gene Bank provided further information about splice variants and the mRNA sequences. The NCBI primer design tool was used to pick primers that amplified the exon-exon junctions, to amplify mRNA sequences of the genes of interest. Primers were picked based on their predicted PCR amplicon size. The primers with predicted amplicons between 80-400 bp were chosen, as they would be more successful in both End-Point and qPCR. The IDT Oligo Analyzer tool (www.idtdna.com) was used to ascertain the likelihood of the primers forming primer dimers and other secondary structures, as they impede successful PCR. Further information on the designed PCR primers is shown in Table 2.

Cell Culture
Human Glioblastoma cell lines U87MGMG and LN229 cells as well as the human foreskin fibroblast line MSU1.1 were utilized in this study. Both U87MG and LN229 cell lines are commonly used as in-vitro models for glioblastoma research studies. MSU1.1 is a fibroblast cell line and was used as a control cell line due to their non-cancerous phenotype. Although the efficacy of using MSU1.1 cells as a control is a limiting factor in the study, it was deemed the most appropriate option available to us. The use of MSU1.1 cells in this study was also highly controlled; passage numbers were kept as low as possible and cells were not grown past 65% confluency to minimize the chance of changes to the cell that acquire different characteristics like a cancer cell or cancer cell line.

All cell lines were removed from cryostasis in liquid nitrogen and revived back into culture. Cell cultures were expanded in T-75 flasks and then frozen back in 1.5 mL aliquots of freeze media at a ratio of 10% DMSO and 90% Media. Frozen aliquots were placed in cryostasis for the ease of culturing cells as needed for experimental assays. This method helped to control for gene expression changes that would potentially occur because of differing cell passage numbers.

All cultured cell lines were expanded in standard cell conditions of 37°C, 5% CO₂, 20% O₂ at 100% humidity. U87MG and MSU1.1 cells were cultured in EMEM + 10% Fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA). LN229 cells were cultured using MEM + 10% FBS. U87MG and LN229 cultures were expanded in T-75 cell culture flasks until they were 70-80% confluent. LN229 cells were discontinued from the project due to time and budget constraints. MSU1.1 cultures were only grown to approximately 60% confluence to minimize any chance of phenotypic changes induced by over-growth. For the hypoxia time course assay, cells were plated and were grown in a normoxic (20% O₂) environment.
overnight (12-15 hours), were washed with 1X PBS (Lonza, Cat# 17-516F/12), to remove any cellular wastes that had accrued overnight followed by the application of fresh cell media. The cells were then placed in the hypoxic chamber (5% O₂) for various amounts of time.

**Hypoxia Time Course Assay: Cell Culture Design**

In this experiment, hypoxic conditions of 5% oxygen were used as the treatment prior to gene expression analysis. In preparation for each assay, cells were revived from cryostasis, split once in a 1:4 ratio and were then plated for the assay immediately after the last cell culture split. Cells were split in this manner and were not counted due to time constraints. Experimental cells were plated in 10 cm treated cell culture plates at 30-50% confluency and the and the cells adhered to the plate overnight (approximately 12-15 hours) in the presence of 20% O₂ chamber.

The following morning, the experimental cell plates were removed from the incubator. The cell culture media was aspirated, and cells were washed with 2-3 mL of sterile 1% PBS. The cells were then fed 10 mL of fresh media, respective to each cell line. The experimental cell cultures were then transferred to the hypoxic (5% O₂) chamber for specified times of 3 hours, 6 hours, 12 hours, and 24 hours. The control cells were only grown in normoxic conditions, were either lysed after spending 0 hours in hypoxia or after 24 hours of growth in hypoxia. In the data analysis below, the control cells are referred to as 0 hours spent in a hypoxic chamber. At each time point, the cell culture dishes were
removed individually from the hypoxic chamber and were lysed directly in their cell culture plates using the RLT lysis buffer from the Qiagen RNeasy kit (Cat# 74104). This was repeated for each of the three biological replicates for each hypoxia exposure time point in the assay. Overall, the experiment was done once, but included three biological replicates for each hypoxia time point or control sample.

**Hypoxia Time Course Assay: Direct Cell Lysis**

Direct cell lysis in 10 cm cell culture plates, as opposed to T-75 flasks, to allow for quicker cell lysis. The direct cell lysis was done to reduce any potentially reversible changes in the hypoxia induced gene expression from occurring. In total, three control (normoxia exposed) plates were lysed and three cell experimental culture plates grown in hypoxia were lysed after each time point in the assay: 3 hours, 6 hours, 12 hours, or 24 hours. This design was repeated for each cell line: MSU1.1 (control), LN229 (glioblastoma), and U87MG (glioblastoma). However, LN229 was quickly removed from the project due to time and budget constraints.

**RNA Isolation and First Strand cDNA Synthesis**

RNA was isolated and homogenized using a QiaShredder (Qiagen, Cat # 79654). RNA was isolated from cell lysates using a RNeasy mini kit (Qiagen). The full RNA isolation
procedure is included in Appendix C. The isolated RNA was quantified using a ThermoScientific Nanodrop 2000c. The RNA was then converted into single strand cDNA using either Promega AMV Reverse Transcriptase (Promega, Cat # M5108) or ThermoFisher Maxima Reverse Transcriptase (Cat # EP0741) and incubated in a Bio-Rad T100 Thermal Cycler. Further details on cDNA synthesis are included in Appendix A. All the qPCR data collected was with cDNA synthesized using the ThermoFisher reverse transcriptase and the Promega RT was utilized only in the End-Point PCR.

Relative mRNA expression levels were analyzed in place of the proteins in question. This method confirmed that the mRNA coding for the hypoxia inducible factor family of transcription factors was being transcribed. The presence or absence of mRNA of the alpha and beta subunits of HIFs 1-3 were evaluated with End Point PCR (EP-PCR). HIF-1α and HIF-1β were evaluated further using Quantitative Real Time PCR (qPCR) to note the relative expression levels. Since the transcription and translation of glucose transporters, specifically GLUT1, is elevated in certain cancers, the transcription of GLUT1 was also evaluated in this study with EP-PCR and qPCR.

**End-Point Polymerase Chain Reaction**

End-Point PCR (EP-PCR) was conducted to determine whether the designed PCR primers were functional and then to determine presence or absence of HIF-1α, HIF-1β, and GLUT1 mRNA transcripts in both normoxic and hypoxic environments. Additional genes of interest were evaluated in the End-Point PCR study: HIF-2α, HIF-2β, HIF-3α, HIF-3β, and
GLUT3. End-Point PCR was conducted on samples from U87MG-MG, LN229, and MSU1.1 cells.

The reaction was performed using a Bio-Rad T100 Thermal Cycler. The program initiated at $95^\circ C$ for 3 minutes. Then the reaction performed 35 cycles of: $95^\circ C$ for 30 seconds for denaturing, decreased to $55^\circ C$ for 30 seconds for primer annealing, and was raised to $72^\circ C$ for 1 minute for DNA elongation. After the 35 cycles, the reaction was terminated with an incubation at $72^\circ C$ for 5 minutes before incubating at $12^\circ C$. The PCR product was either subjected to gel electrophoresis or was stored at -20°C freezer.

To determine the presence of DNA products produced from the End-Point PCR reaction, the products were electrophoresed in a 1.0% agarose gel in Tris/Borate/EDTA (TBE) buffer. When the mixture was cool to the touch, 1 µL of 10 µg/µL ethidium bromide was added to the solution and then was mixed. The mixture was further cooled in the fume hood and was then poured into a standard gel box setup. A total of 5 µL of Blue/Orange 6X Dye (Promega, Cat# G1881) was added to each 25 uL PCR product, yielding a total 30 µL total volume per lane. A 1kb and/or a 50 bp ladder were included on each gel. The gel electrophoresis was conducted using the following parameters; ~120 V, for 60-75 minutes in a running buffer solution comprised of 0.5x TBE buffer. Gels were imaged using a NMU Gel Doc Imaging System (BioRad, Hercules, CA).

Real Time (Quantitative) Polymerase Chain Reaction
Real time PCR (qPCR) was conducted to determine the amount of time that was needed to induce relative expression changes to occur in HIF-1α, HIF-1β and GLUT1 mRNA expression levels after exposure to hypoxic conditions (5% O2).

The real-time PCR (qPCR) reaction was performed with the GoTaq qPCR Master Mix (Promega, Cat# A6001). The master mix consisted of a propriety SYBR green dsDNA intercalating dye, primers, dH2O, and a low level of carboxy-X-rhodamine (CXR) reference dye. The reaction size recommended by the manufacturer was 50µL, but the reaction size was scaled down to 20µL to minimize project cost. The small volume necessitated extra care to be taken in pipetting evenly throughout all the individual PCR reactions. The 20 µL reaction had the following components:

- 10 µL Master Mix
- 0.2 µL CXR reference dye
- 0.8 µL Forward Primer
- 0.8 µL Reverse Primer
- 7.4 µL Nuclease-free water

Total: 19.2 µL Master Mix

A total of 19.2uL of master mix without primers was added to each of the three negative control wells in the 48 well sample PCR plate. 0.8uL of PCR grade nucleotide free water (Promega, Cat#A6001) was added to each of the control wells. 0.8 µL of cDNA was added to the experimental plates. 20uL total volume was added to each well in the 48 sample PCR plate. The reaction was performed using an Applied Biosciences StepOnePlus thermocycler and was programmed for 1 cycle at 95°C for 10 minutes and then 40 cycles of
15 seconds at 95°C to denature the cDNA, followed by a decrease in temperature to 55°C for 1 minute for annealing, and then increased for elongation to 73°C for 30 seconds.

In qPCR, fluorescent signal is accumulated and denotes a positive signal in the PCR reaction. The number of cycles needed for the accumulated fluorescence to pass a threshold value is referred to as the cycling threshold (CT) value. CT values are inversely proportional to the amount of nucleic acid. Therefore, housekeeping genes or highly expressed genes will have a low CT value. The comparative Ct method was used to analyze the data which was exported to Excel. The comparative Ct method was used to analyze gene expression levels, as it seemed appropriate due to having a large sample size: differently treated samples, two cell lines, and three genes of interest (Wong & Medrano, 2005). This method involves normalizing the gene of interest to a housekeeping gene: GAPDH in this case to calculate a delta Ct (dCt) (Brugè, Venditti, Tiano, Littarru, & Damiani, 2011). The delta Ct values of the hypoxia treated cells were compared to the delta Ct values of the normoxia exposed (control) cells to get a delta delta Ct (ddCT) value (Livak & Schmittgen, 2001). Relative gene fold change was calculated by $2^{\Delta\Delta Ct}$. Relative fold change shows whether there is any change in relative gene expression. A fold change above a value of one denotes a gene up-regulation; a negative value for relative gene fold change indicates that the gene was down-regulated, thereby reducing the number of gene transcripts made (Wightman, 2016).
Statistical Analysis

Many methods were initially used to test the significance of the qPCR data including factorial analysis using SPSS software and GraphPad-PRISM software for two-way ANOVA with repeated measures and student t-tests. Two-way ANOVA was utilized to create graphs depicting mRNA expression fold change of GLUT1, HIF-1 α, and HIF-1β in U87MG cells and MSU1.1 cells at each of the time points in the hypoxia time course assay (Figures 11-13).

However, more in-depth analysis was needed to determine which variables had correlative relationships. The large number of dependent variables in the study: three genes of interest, two cell lines, and five treatment time points, using a factorial cell culture setup, necessitated the use of a multivariate analysis of variance, MANOVA (Stevens, 2009)(Warne, 2014). The data was log transformed and then was analyzed with MANOVA using SPSS software. One outlier was present in the data and was removed from the data set. However, the removal of the outlier did not significantly change the results of the MANOVA analysis. MANOVA is an extension from the univariate analysis of variance (ANOVA). An ANOVA can evaluate the statistical differences among a single dependent variable. For example, an ANOVA could determine whether there were changes in the relative expression of GLUT1 as a product of the time spent in a hypoxic environment. The MANOVA can evaluate the effects of several dependent variables and compiling them to create a single continuous variable. This study analyzed the effects of cell line (U87MG and MSU1.1) as well as the combined effects of both hypoxia treatment and cell line. The independent variable in the study was the time that cells spent in either normoxic (control) or hypoxic (experimental) environments prior to being lysed for subsequent RNA isolation.
The many multiple dependent variables in the study necessitated the use of a MANOVA to understand whether any statistical significance was due to variance within any of the dependent variables or whether it was due to the time spent in a hypoxic environment (independent variable). Pairwise comparison analyses were conducted to find correlations between the expression levels of the genes of interest in the cell lines that were tested while also testing whether different amounts of time in a hypoxic environment also had an effect (Tables 8-12). These pairwise comparisons were depicted visually in Figures 14-16.

**Limitations of Study**

Limitations were present in the usage of MSU 1.1 cells as a control line, as they are a cell line made by transfecting diploid fibroblasts with the viral v-Myc gene (Hurlin, Maher, & McCormick, 1989). Making a cell line inherently gives the cells, cancerous or not, different genetic and phenotypic profiles that limit the replicability of the study as well as the applicability when applying this study to *in-vivo* GBM studies. However, the growth of MSU1.1 cells was carefully controlled in this study: cells were not grown past their recommended confluency and were examined carefully for any phenotypic changes prior to hypoxic exposure and subsequent lysis. Since normal human control cells were not readily available, the MSU 1.1 fibroblast cell line was used. Although the sample size for PCR was relatively small, there were sufficient replicates done for the data sets to pass the needed statistical tests for variance and normality. Likely due to human error there was
some variability in the delta CT values. However, this variability was accounted for in the statistical analysis and proved to be insignificant.

**Table 2: PCR Primer Sequences**

The following sequences were designed for this study and were utilized in EP-PCR and qPCR studies. As discussed on page 29, PCR primers were designed to have predicted amplicon sizes between 80-400 base pairs, to increase qPCR success, as small amplicons sizes are required to have successful qPCR. All primers were utilized in EP-PCR and qPCR studies and tested for expression of the following target genes in U87MG and MSU1.1 cells: HIF-1α, HIF-1β sets 1 and 2, HIF-2α, HIF-2β, HIF-3α, HIF-3β, GLUT1, and GLUT3.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer Sequences (5'-3')</th>
<th>Reverse Primer Sequences (5'-3')</th>
<th>Use of Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>ACCTATGACCTGCTTGGTGTC</td>
<td>GGCTGTGTGACAGTGAAAGA</td>
<td>End Point and qPCR</td>
</tr>
<tr>
<td>HIF-1 Set 1</td>
<td>CCCACACAAAGGAGCA</td>
<td>AGAAAAGCTGAGCGGTAGTG</td>
<td>End Point and qPCR</td>
</tr>
<tr>
<td>HIF-1 Set 2</td>
<td>CTTTCTGCCCAGTGCTA</td>
<td>ATGGAGTCTGAAAGCTGCC</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>TACAATCTCAGCGCATGTC</td>
<td>GAGGCTGTCAGACCCGAA</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>HIF-2</td>
<td>CGGCAGCTAAAACCAGAGTA</td>
<td>ACTTTCAACGAACCTGAGG</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>HIF-3α Set 1</td>
<td>ATAAAGTCAGGAGGGAGGAG</td>
<td>TAGCAGGTCCTCAGTGGTTC</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>HIF-3α Set 2</td>
<td>CAGTAGGCAGTGGAGAGA</td>
<td>GGCTCAATTCAAGTCA</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>HIF-3α Set 3</td>
<td>GACACTGAGCGAGGAC</td>
<td>CATTCAGGTTCAGGAGG</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>HIF-3</td>
<td>AGAAGGTGCCCACAGAGA</td>
<td>GGAGGCTACGTCAGTGT</td>
<td>End Point PCR</td>
</tr>
<tr>
<td>GLUT1</td>
<td>GTGACAAAGACACCCAGAGG</td>
<td>CCTGAGCCGCTTAAGTCC</td>
<td>End Point and qPCR</td>
</tr>
<tr>
<td>GLUT3</td>
<td>GGAAAGGCGAGAGGAAGAGA</td>
<td>ACAGTCATGAGCGGAAACAA</td>
<td>End Point PCR</td>
</tr>
</tbody>
</table>
Results

In previous work, the heterodimeric HIF-1 complex activity was shown to positively correlate with a hypoxic tumor microenvironment (G. L. Semenza, 2002). After hypoxia induced stabilization of HIF-1, the HIF transcription factor translocated to the nucleus and bound to the hypoxia response element on target DNA: one such target is GLUT1 (G. L. Semenza, 2010b). This study sought to establish the relative mRNA expression levels of HIF-1α, HIF-1β, and GLUT1 in U87MG GBM and MSU1.1 fibroblast cells in hypoxic and normoxic conditions. Additionally, this study sought to determine whether there is a direct correlation between HIF-1 and GLUT1. As a first step, we investigated whether HIF-1α/β heterodimeric complexes were being transcribed into mRNA. This study served as a precursor step to future studies investigating the HIF-1 complex at the protein level and its interaction with GLUT1.

PCR primers for HIF-1α, HIF-1β, HIF-2α, HIF-2β, HIF-3α, HIF-3β, GLUT1, and GLUT3 were designed (Table 2) and tested using End-Point PCR and subsequent gel electrophoresis for U87MG cells. HIF-1α and two sets of HIF-1β products from U87MG cell lysates were detected in both normoxic and hypoxic conditions with the correct band length for their expected PCR product sizes: 98 bp (HIF-1α), 55 bp (HIF-1β-Primer Set 1), and 80 bp (HIF-1β-Primer Set 2) (Figure 6). Similarly, in Figure 7, the PCR products for HIF-2α (183 bp) and HIF-2β (234 bp) are shown at the correct product size. HIF-3α amplification was not conclusively established in U87MG cells in this study. There were multiple bands in lanes 8 and 9 rather than having a single band of 70 bp using one of two
primer sets designed for HIF-3α (Figure 7). The second primer set designed for HIF-3α should have had a product of 72 bp in lanes 2 and 3 of the gel shown in Figure 8. Both attempts to detect a PCR product for HIF-3α using either primer set were unsuccessful. However, there was successful amplification of HIF-3β (177 bp) and GLUT1 (231 bp) in U87MG cells (Figure 8). GLUT3 expression was also found in U87MG PCR reactions from both U87MG cells in a normoxic and hypoxic environment (Figure 9). In Figure 10, the presence of HIF-1α, HIF-2α, HIF-2β, HIF-3α, HIF-3β, and GLUT1 were tested in MSU1.1 cells (Figure 10) and the presence of all but HIF-3α were confirmed.

Overall, the expression of genes HIF-1α, HIF-2α, HIF-2β, HIF-3β, GLUT1, and GLUT3 were observed in U87MG cells in normoxic and hypoxic conditions. GLUT3 expression was not tested in MSU1.1 cells using EP-PCR. Most of the primers were tested in MSU1.1 cells grown in hypoxia for 48 hours, except for HIF-1β, due to issues designing an effective PCR primer (Figure 10). However, HIF-1β expression was measured using qPCR. Agarose gels were also run using normoxic control cDNA from MSU1.1 cells (data not shown).

There were also recurrent issues with designing a HIF-3α primer. Preliminary EP-PCR experiments could not confirm HIF-3α expression in either U87MG cells or in MSU1.1 cells. Inappropriately sized bands were present in all EP-PCR amplification agarose gels conducted with U87MG cell mRNA (Figures 7 and 8) and in MSU1.1 cell mRNA samples (Figure 10). Consequently, the study of HIF-3α was discontinued and subsequent qPCR experiments were not performed. New PCR primers were needed to continue the study of HIF-3α but a new set of primers was not designed due to time and budget constraints.

The End-Point PCR was followed up with qPCR to measure the relative mRNA expression levels of HIF-1α, HIF-1β, and GLUT1 in U87MG and MSU1.1 after hypoxia.
exposure for 0, 3, 6, 12, or 24 hours. The resulting CT values for each gene for each run of qPCR that was conducted. These values were compiled and the technical replicate values for each biological replicate were averaged (data not shown). As discussed previously, the comparative CT method was utilized to analyze the results of each qPCR. This method is described below using GLUT1 as the example gene of interest.

**Equation 1:** Calculate the $\Delta CT$ of hypoxia treated relative genes (Experimental Treatment)

$$\Delta CT \text{ (hypoxia treated)} = CT \text{ (GLUT1)} - CT \text{ (GAPDH)}$$

**Equation 2:** Calculate the $\Delta CT$ of normoxia treated qPCR runs (Control treatment)

$$\Delta CT \text{ (Normoxia Treated)} = CT \text{ (GLUT1)} - CT \text{ (GAPDH)}$$

**Equation 3:** Calculate the difference between $\Delta CT$ Hypoxia (Experimental Treatment) and $\Delta CT$ Normoxia (Control Treatment).

$$\Delta \Delta CT \text{ GLUT1} = \Delta CT \text{ (Hypoxia)} - \Delta CT \text{ (Normoxia)}$$

Using Equation 1, the delta CT values were calculated for each gene of interest after exposure to hypoxia at the various time points in the hypoxia time point assay. These delta CT values were referred to as ‘$\Delta CT$ (hypoxia treated)’. Then in Equation 2, the delta CT values of the normoxia exposed gene of interest, GLUT1, were measured and then normalized to the CT of the normoxia exposed housekeeping gene GAPDH. In Equation 3, the $\Delta \Delta CT$ of GLUT1 was calculated by normalizing the effects of hypoxia to the effects shown in normoxia. The $\Delta CT$ values for each gene of interest measured at each time point in the hypoxia time point assay in both U87MG and MSU1.1 cells are depicted in Table 4. The $\Delta \Delta CT$ values were converted into relative fold change by using Equation 4 below.

**Equation 4:** Fold Change of Gene of Interest (GOI) = $2^{\Delta \Delta CT \text{ of Gene Of Interest}}$
The relative fold change data was log transformed and one outlier was removed from the data, which did not significantly change the overall results (data not shown). The data was then analyzed for significance using Multivariate Analysis of Variance (MANOVA) using SPSS software. MANOVA was conducted to test whether each of the independent variables: cell line, relative oxygen concentration, and time spent in variable relative oxygen conditions had effects on the dependent variables: the expression of the genes HIF-1α, HIF-1β, and GLUT1. In this study, several multivariate test statistics were used: Wilks’ Lambda, Pillai’s Trace, Hotelling’s Trace, and Roy’s Largest Root, shown in Table 4. Of interest was the Wilks’ Lambda statistic from the F test, which represents the percentage of variance within the dependent variables, which are the genes of interest: HIF-1α, HIF-1β, and GLUT1. The desired result entails that the p-value denoting significance fall in the range of zero to one, ideally as close to zero as is possible.

The Wilk’s Lambda test indicated that there was a statistically significant effect (p=0.000) between the independent variable, cell line, and the expression level of all the dependent variables when analyzed together as a group: HIF-1α, HIF-1β, and GLUT1 (Table 4). Likewise, the other independent variable, hypoxia treatment, had a statistically significant effect (p=0.000) on the expression levels of the entire group of genes of interest: HIF-1α, HIF-1β, and GLUT1 (Table 4). It was hypothesized that both HIF-1α, HIF-1β, and GLUT1 expression levels would be directly proportional to in-vitro hypoxic conditions. Therefore, our null hypothesis was rejected in regard to the expressions of HIF-1α and GLUT1 and confirmed for HIF-1β expression. It was expected that both HIF-1α and HIF-1β expression would be proportional to relative oxygen concentrations in GBM cells as HIF-1 expression has been found in several types of cancer (C. Chen et al., 2001, p. 1) (G. L.
Semenza, 2007). Likewise, it was expected that GLUT1 expression would also increase in hypoxic conditions, as it is thought to be directly downstream from HIF-1, as well as HIF-2 and possibly HIF-3 in an indirect manner (Richardson, Knowles, Tyler, Mobasher, & Hoyland, 2008). GLUT1 upregulation has been identified in several types of cancer (Amann et al., 2009, p. 1) (Krzeslak et al., 2012).

The combined effects of the two independent variables (cell line and hypoxia treatment) had significant interactional effects (p=0.004) on the expression of HIF-1α, HIF-1β, and GLUT1 as well (Table 4). Overall, the multivariate analyses supported our hypothesis in the context of all the genes of interest when they are lumped together as one dependent variable. Additionally, the significance values found with the Wilk's Lambda analysis were also seen with the other three multivariate tests mentioned above and are shown in Table 4. However, the multivariate analyses were not capable of determining whether the independent variables, cell line and hypoxia exposure, led to expression changes in each individual gene.

This general significance found using the MANOVA overall test necessitated further study of the effects that each independent variable had on the entire group of dependent variables, the genes of interest (HIF-1α, HIF-1β, and GLUT1) (Table 5). To conduct this analysis, two-way ANOVAs were utilized and can be visualized in Figures 14-16. The results from these ANOVAs concluded that the independent variable, cell line, had a significant univariate effect on the dependent variables of HIF-1α expression (p = .001) and GLUT1 expression (p = .000). Cell line had a significant control over the expression of these two genes individually. Therefore, the affected expression levels of HIF-1α and GLUT1 cannot be correlated to one another from the data provided in Table 5. This ANOVA also
identified that the *in-vitro* hypoxia exposure had a single univariate effect on the expression of the following genes: HIF-1β (*p* = .018), and GLUT1 (*p* = .000). When the effects of both independent variables (cell line and hypoxic treatment) were combined, there was only a significant effect seen in GLUT1 expression (*p* = .002), shown in Table 5. Overall, this univariate ANOVA was useful in determining the effects that each independent variable had on each dependent variable (gene expression levels) individually but could not correlate the effects seen in the dependent variables together as was possible in the MANOVA depicted in Table 4.

In this study, a two-way MANOVA (Table 4) and multiple univariate ANOVAs (Table 5) were utilized to analyze the gene expression data from the qPCR gene expression studies. To better understand the data collected, a MANOVA was used. The two-way MANOVA revealed that there were statistically significant effects on gene expression levels from the following independent variables: cell line, hypoxia exposure treatment, and interaction effects from the combined effects of cell line and hypoxia exposure treatment (Table 4). Subsequent two-way ANOVAs further analyzed the effect that each individual independent variable, cell line or hypoxia exposure, had on each gene expression level (HIF-1α, HIF-1β, and GLUT1) (Table 5). Put differently, the above studies analyzed the general effect that each independent variable had on the entire group of dependent variables with the MANOVA (Table 4) followed by the specific effect that each independent variable had on each dependent variable individually using multiple univariate ANOVAs (Table 5).

These studies were followed with analysis using a pairwise comparison model from the univariate ANOVAs that followed the MANOVA (Tables 6-8). These analyses
characterized the effects that all of the various time points spent using hypoxia environment exposure treatment (0, 3, 6, 12, and 24 hours) had on the expression of each gene of interest individually, as opposed to looking at the effects of general hypoxia without specifying significance to any hypoxia time point. Overall, the pairwise comparison analysis compared the expression levels of each gene after the cell lines spent various amounts of time (0, 3, 6, 12, or 24 hours) in a hypoxic environment. Between the time zero of the assay and 3 hours spent in hypoxia, HIF-1α expression showed a statistically significant change, but no other significant HIF-1α expression changes were evident between any of the other hypoxia time points or to the time zero in the assay (Table 6). Since HIF-1α expression has been deemed acutely hypoxia dependent, it was surprising to see that the only significant change in expression occurred in the first 3 hours spent in hypoxia. Initially, it was expected that HIF-1α expression would be upregulated for the first 6 hours of the study. However, the literature seems to support that HIF-1α expression peaks transiently in the first few hours of hypoxia before being degraded (Holmquist-Mengelbier et al., 2006).

However, several other studies have found that HIF-1α stabilization and expression tends to occur after short periods (acute) of exposure to hypoxia rather than when exposed to hypoxia more chronically (12+ hours) (Holmquist-Mengelbier et al., 2006). This study was not designed to determine the amount of time necessary to stabilize HIF-1α, but rather to determine that it was expressed and whether its expression and stabilization correlated with HIF-1β, other HIFs, and GLUT1. Since HIF-1 expression appears to be more related to acute exposure to hypoxia as opposed to chronic exposure (Holmquist-Mengelbier et al., 2006), it was not surprising that HIF-1 expression was not expressed at significant levels between all of the other time points spent in a hypoxic environment in this study (Table 6).
As described above, pairwise comparison analyses of HIF-1β expression levels between each of the time points in the hypoxia exposure assay. Between time zero of the assay and six hours in hypoxia (p = .005), the expression of HIF-1β changed in a statistically significant manner. Other significant HIF-1β expression changes occurred between time zero and 12 hours in hypoxia (p = .002) as well as between 12 hours and 24 hours (p = .037) in a hypoxic environment (Table 7). Since HIF-1β is the binding partner for HIF-1α, although it is allegedly constitutively expressed, it would be assumed that the expression of the two binding partners would coordinate with one another. HIF-1 downstream signaling could depend on the amount of HIF-1β present. It was expected that HIF-1β expression would be stable this study and that HIF-1α would be variable and it was thought to display its hypoxia responsive downstream signaling within the first three hours of HIF-1α being stabilized, as HIF-1α has been said to mediate an acute response in hypoxia whereas HIF-2α supposedly mediates downstream signaling in a more chronic response in a hypoxic environment. Some reports claim that HIF-1β binds to alpha subunits of other HIF isoforms, most notably HIF-2α.

The pairwise comparison then measured the expression changes of GLUT1 (Table 8 and Figure 16), which was more widely affected by time spent in a hypoxic environment than HIF-1α or HIF-1β. Statistically significant changes in GLUT1 expression were evident between the following hypoxia time points: 0-3 hours in hypoxia (p = .000), 0-6 hours in hypoxia (p = .000), 0-12 hours in hypoxia (p = .001), 0-24 hours in hypoxia (p = .000).

There have been reports that both HIF-1 and HIF-2 expression modulate glucose metabolism and have been shown to upregulate genes involved with metabolism, notably GLUT1 and GLUT3. As reported above in this study, HIF-1α and HIF-2α expression changes
occur after differing lengths of time spent in a hypoxic environment, which could mean that GLUT1 expression is being constantly upregulated due regardless of which HIF is upregulated at the time, since both HIFs 1 and 2 have been implicated with GLUT-1 upregulation.

Next, pairwise comparisons were conducted to analyze the effects of cell line on the expression of each gene. Shown in Table 9, HIF-1α expression was significantly different in U87MG versus MSU1.1 cells, which was expected, but the MSU1.1 line has not been well characterized to this point making this finding potentially novel. Most notably is that HIF-1α expression can be upregulated by oncogenic signaling, which is typical in cancerous cells but not in healthy cells, which in this study are represented by the MSU1.1 cell line. Additionally, aberrant signaling can mimic hypoxic conditions by initiating the expression of HIF downstream genes, but while bypassing HIF. This is likely true with the expression of vascular endothelial growth factor, VEGF. Aberrant signaling of many genes can initiate hypoxic conditions and the subsequent expression of HIF-1α, which can account for why there are significantly different expression levels based on the cell line they are expressed in (U87MG cancer cells versus MSU1.1 fibroblast cells). The HIF-1β expression changes were not significantly resulting from the cell line it was expressed in. Not surprisingly, the expression of GLUT1 was significantly tied to the cell line. It is well documented that GLUT1 expression is typical in normal tissue and is overexpressed in cancerous tissues. Knowing this, it is not surprising that GLUT1 expression is significantly elevated in the cancer cell line U87MG as opposed to the ‘normal’ MSU1.1 cells used in this study. However, after conducting EP-PCR, this study only encompassed two cell lines (MSU1.1
fibroblasts and U87MG cancer cells) for the qPCR aspects. Therefore, subsequent studies are needed to confirm and expand on this study.

The estimated marginal means were calculated using all of the variables in the study: cell line, hypoxia time points, shown in the context of changes in expression of the genes of interest in this study (Table 10). Additionally, the means calculated in Table 10 comprise the data points for Figures 14-16. In Figure 14, HIF-1α expression was significantly correlated (p = .001) to the cell line in which it was expressed (U87MG versus MSU1.1). Surprisingly, as discussed above with Table 5, there were no significant HIF-1α expression changes evident after hypoxia exposure, except in the first three hours in a hypoxic culture environment (p = .215). Additionally, the combined effects of cell line and hypoxic environment treatment did not elicit any statistically significant changes in HIF-1α expression (p = .558) (Table 5). The estimated marginal means of the effects of cell line, hypoxic exposure, and the combined effects on HIF-1β expression were analyzed in Table 10 and then visualized in Figure 15. HIF-1β was not significantly affected by the IV cell line (p = .911) but was surprisingly correlated with time spent in a hypoxic environment (p = .018). The combined effects of both cell line and hypoxic exposure did not lead to significant changes in HIF-1β expression (p = .332) (Table 10 and Figure 15). Overall, hypoxic exposure was the only variable that affected HIF-1β expression in this study, which was surprising and will be discussed more in depth in the following chapter. The estimated marginal means of GLUT1 expression were also depicted in Table 10 and visualized in Figure 16. GLUT1 expression correlated to the cell line it was expressed in (p = .000) and was also significantly affected by the hypoxic treatment (p = .000) and when combined
effects of cell line and hypoxia treatment (p = .002). The significance of GLUT1 expression changes in reference to hypoxia and cell line will be discussed in the following section.
**Figure 6:** Expression of the genes HIF-1α and HIF-1β was confirmed in both hypoxia treated U87MG cells as well as in U87MG cells that only grew in a normoxic environment.

From left to right: 50 bp DNA ladder, HIF-1α (98 bp) after hypoxic exposure (treatment), HIF-1α (98 bp) expression after no hypoxic exposure (control), blank lane, HIF-1β (55 bp) expression after hypoxic environment exposure (treatment), HIF-1β (55 bp) expression after no hypoxic environment exposure (control), blank lane, HIF-1β (2nd primer set-80 bp) expression after hypoxic environment exposure (treatment), HIF-1β (2nd primer set-80 bp) expression in normoxia- after no hypoxic environment exposure (control), blank well.
Figure 7: Expression of the genes HIF-2α and HIF-2β was confirmed in both hypoxia treated U87MG cells as well as in U87MG cells that only grew in a normoxic environment. HIF-3α was neither confirmed to be expressed in hypoxia treated U87MG cells nor in control normoxia treated U87MG cells.

From left to right: 50 bp DNA ladder, HIF-2α (183 bp) after hypoxic exposure (treatment), HIF-2α (183 bp) expression after no hypoxic exposure (control), blank lane, HIF-2β (234 bp) expression after hypoxic environment exposure (treatment), HIF-2β (234 bp) expression after no hypoxic environment exposure (control), blank lane, HIF-3α (1st primer set) (72 bp) expression after hypoxic environment exposure (treatment), HIF-3α (1st primer set) (72 bp) expression after no hypoxic environment exposure (control), blank well.
**Figure 8:** Expression of the genes HIF-3β and GLUT1 was confirmed in both hypoxia treated U87MG cells as well as in U87MG cells that only grew in a normoxic environment. The second primer set designed for HIF-3α neither confirmed HIF-3α expression in hypoxia treated U87MG cells nor in control normoxia treated U87MG cells.

From left to right: 50 bp DNA ladder, HIF-3α (2nd primer set) (70 bp) after hypoxic exposure (treatment), HIF-3α (2nd primer set) (70 bp) expression after no hypoxic exposure (control), blank lane, HIF-3β (177 bp) expression after hypoxic environment exposure (treatment), HIF-3β (177 bp) expression after no hypoxic environment exposure (control), blank lane, GLUT1 (231 bp) expression after hypoxic environment exposure (treatment), GLUT1 (231 bp) expression after no hypoxic environment exposure (control), blank well.
**Figure 9:** Expression of GLUT3 was confirmed in both hypoxia treated U87MG Cells as well as in control (Normoxia) U87MG Cells.

From left to right: 50 bp DNA ladder, GLUT3 (75 bp) after hypoxic exposure (treatment), GLUT3 (75 bp) in U87MG cells only exposed to normal oxygen conditions (control).
FIGURE 10: EXPRESSION OF HIF-1α, HIF-1β, HIF-2α, HIF-2β, HIF-3β, AND GLUT1 IN MSU1.1 FIBROBLAST CELLS AFTER 48 HOURS OF HYPOXIA EXPOSURE.

From left to right: 50 bp DNA ladder, control lane with no template MSU1.1 cDNA, HIF-1α (98 bp), HIF-2α (183 bp), HIF-2β (234 bp), HIF-3β (177 bp), and GLUT1 (231 bp).
Table 3: Summary of End-Point PCR Study

The following table depicts the results of the End-Point PCR and subsequent gel electrophoresis of the genes in U87MG and MSU 1.1 cells: HIF-1α, HIF-1β (1st and 2nd Primer Pair Sets), HIF-2α, HIF-2β, HIF-3α (1st and 2nd Primer Pair Sets), HIF-3β, GLUT1, and GLUT3.

<table>
<thead>
<tr>
<th>Genes Tested</th>
<th>U87 Glioblastoma Cells</th>
<th>MSU1.1 Fibroblast Cells (Control Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Present</td>
<td>Present</td>
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<tr>
<td>HIF-1b</td>
<td>Present</td>
<td>Present</td>
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<tr>
<td>HIF-2α</td>
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<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>HIF-3α- 1st Primer Set</td>
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<td>Inconclusive</td>
</tr>
<tr>
<td>HIF-3α- 2nd Primer Set</td>
<td>Inconclusive</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>HIF-3β</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>GLUT3</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>
Relative expression level of HIF-1α had a statistically significant peak in the first 3 hours of hypoxic exposure treatment. The qRT-PCR results appear to show a trend in U87MG cells: HIF-1α levels increase over time in hypoxia and have a significant peak after 12 hours of hypoxia exposure. However, the trend showing a gradual increase over time, with a peak at 12 hours that is not statistically significant. The expression levels in MSU1.1 cells are generally higher than in U87MG cells, but there is no trend in relation to time spent in hypoxia.
Relative Expression of HIF-1β After Exposure to Hypoxic Environment

**Figure 12: Relative Expression of HIF-1β in U87MG Cells and MSU1.1 Cells After Hypoxia Treatment.**

Two-way ANOVA was utilized for analysis of the relative fold change observed in both U87MG and MSU1.1 cells during a hypoxia time course assay. qRT-PCR was conducted on cDNA from RNA isolated from U87MG cells and MSU1.1 cells. In U87MG cells, HIF-1β levels increase over time in hypoxia and peak at 12 hours, but the trend is not statistically significant. No distinct trend is apparent in MSU1.1 cells. There was a distinctly higher baseline expression of HIF-1β seen in MSU1.1 cells than in the other genes investigated in this study: HIF-1a in Figure 11 and GLUT1 in Figure 13.
**Figure 13: Relative Expression of GLUT1 in U87MG and MSU1.1 Cells After Hypoxia Treatment.**

qRT-PCR results appear to show a trend in U87MG cells: GLUT1 levels increased over time spent in hypoxia and peaked at 24 hours. Using MANOVA pairwise comparisons, the mRNA fold change levels of in U87MG and MSU1.1, that were measured at each hypoxia time point (3, 6, 12, and 24 hours), were normalized to the baseline GLUT1 mRNA expression from U87MG and MSU1.1 cells that were never exposed to hypoxic conditions.
**TABLE 4: ΔCT VALUES USED FOR MANOVA MULTIVARIATE ANALYSIS**

The following chart depicts the ΔCT values determined by the StepOnePlus Thermocycler software. The following values were utilized for MANOVA analysis. The ΔCT was utilized over the ΔΔCT to keep the normoxia ΔCt values to use as a control factor in the MANOVA analysis. The MANOVA results are shown in Table 4.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Δ CT HIF1</th>
<th>Δ CT HIF1</th>
<th>Δ CT GLUT1</th>
</tr>
</thead>
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<td>Normoxia</td>
<td>0.390237808</td>
<td>8.862180233</td>
<td>6.975073179</td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>4.681705793</td>
<td>11.3155982</td>
<td>11.833498</td>
<td></td>
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<tr>
<td>Normoxia</td>
<td>4.256052971</td>
<td>10.21491146</td>
<td>12.66498693</td>
<td></td>
</tr>
<tr>
<td>3 Hour</td>
<td>0.959792773</td>
<td>10.76535384</td>
<td>8.108195623</td>
<td></td>
</tr>
<tr>
<td>3 Hour</td>
<td>3.697454453</td>
<td>9.111228943</td>
<td>8.134410222</td>
<td></td>
</tr>
</tbody>
</table>

| U87       | 3.657238324  | 9.22144858  | 10.88333511|
| 6 Hour    | -1.579455058 | 7.83086268  | 8.561536789 |
| 6 Hour    | 4.533939342  | 7.603870074 | 7.842486064 |

| 6 Hour    | 4.254323324  | 8.735117594 | 11.44408994|
| 12 Hour   | 0.86288929   | 7.628475507 | 7.218052864|
| 12 Hour   | 0.994344076  | 6.518761953 | 6.66847229 |
| 12 Hour   | 2.983535767  | 7.028027217 | 11.17832947|
| 24 Hour   | 0.192746798  | 8.47631565  | 0.073177338|
| 24 Hour   | 5.204382261  | 10.5066309  | 6.624696732 |
| 24 Hour   | 4.931388855  | 8.970122019 | 9.319644292 |

| Normoxia  | 3.142354965  | 8.140199025 | 5.464523315 |
| Normoxia  | 5.871913433  | 10.85678816 | 11.15261587 |
| Normoxia  | 4.286186854  | 10.8575236  | 10.87225914 |
| 3 Hour    | 2.596813202  | 8.067699432 | 4.847998301 |
| 3 Hour    | 2.570558548  | 7.713668823 | 9.600447973 |
| 3 Hour    | 3.153512319  | 9.428974152 | 7.063512166 |
| 6 Hour    | 2.392367045  | 7.469454447 | 5.085081418 |
| 6 Hour    | 3.588159243  | 9.148351351 | 8.939088821 |
| 6 Hour    | 4.331070582  | 8.967363993 | 9.863989512 |

| MSU1.1    | 3.747689565  | 8.277808507 | 4.958525976 |
| 3 Hour    | 3.419293086  | 8.271207809 | 9.395822525 |
| 12 Hour   | 1.733569463  | 8.98453482  | 10.72193146 |
| 24 Hour   | 3.216023127  | 8.594952901 | 4.716258367 |
| 24 Hour   | 3.787295659  | 8.864278793 | 9.2711188 |
| 24 Hour   | 5.77850914   | 10.09346962 | 8.524061203 |
Table 5: MANOVA tests of between-subjects effects

The MANOVA was utilized to analyze the general effect that each independent variable had on the group of dependent variables: HIF-1α, HIF-1β, and GLUT1. However, the MANOVA analyzed the effects that these independent variables (cell line and hypoxia treatment) had on the dependent variables clumped as a group of genes. Significant effects were using all four of the multivariate statistical analyses: Pillai’s Trace, Wilks’s Lambda, Hotelling’s Trace, and Roy’s Largest Root. P-values were all less than 0.05, which indicates a strongly significant effect resulting from the independent variables labeled in the first column of the table.

*Statistically significant values are highlighted in the figure below.

<table>
<thead>
<tr>
<th>MANOVA Test of Between Subjects Effects</th>
</tr>
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<tbody>
<tr>
<td>Effect</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Intercept</td>
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<tr>
<td>Pillai’s Trace</td>
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<tr>
<td>Wilks’ Lambda</td>
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<tr>
<td>Hotelling’s Trace</td>
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<tr>
<td>Roy’s Largest Root</td>
</tr>
<tr>
<td>CellLine</td>
</tr>
<tr>
<td>Pillai’s Trace</td>
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<td>Hotelling’s Trace</td>
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<tr>
<td>Roy’s Largest Root</td>
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<tr>
<td>Treatment</td>
</tr>
<tr>
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<tr>
<td>Wilks’ Lambda</td>
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<tr>
<td>Hotelling’s Trace</td>
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<tr>
<td>Roy’s Largest Root</td>
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<tr>
<td>Cell Line * Treatment</td>
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<tr>
<td>Pillai’s Trace</td>
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<td>Wilks’ Lambda</td>
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<tr>
<td>Hotelling’s Trace</td>
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<tr>
<td>Roy’s Largest Root</td>
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</tbody>
</table>

a. Design: Intercept + Cell Line + Treatment + Cell Line * Treatment
b. Exact statistic
c. The statistic is an upper bound on F that yields a lower bound on the significance level.
d. Computed using alpha = .05
Table 6: Test of Between-Subjects Effects Shown for Each Gene of Interest

Univariate ANOVAs were performed after the MANOVA to determine whether the independent variables (cell line, in-vitro hypoxic exposure, and the combined effects from the two variables) influenced the expression of the each of the dependent variables (HIF-1α, HIF-1β, and GLUT1), when viewed autonomously from the other genes. The univariate tests concluded that cell line had a significant effect on the individual expression levels of HIF-1α (p = .001) and GLUT1 (p = .000), but not on HIF-1β. In-vitro hypoxia exposure had a significant effect on the individual expression of HIF-1β (p = .018) and GLUT1 (p = .000) and surprisingly not on HIF-1α (p = .215), which is surprising since it is considered mainstream that HIF-1α is successfully activated by a hypoxic environment. Then the effects of both independent variables were combined to determine their mutual effect on each gene’s expression level; when combined these two factors had a significant effect on GLUT1 expression (p = .002).

*Statistically significant values are bolded in the figure below.

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<th>Source</th>
<th>Type I Sum of Squares</th>
<th>df</th>
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<tr>
<td>HIF1α Log</td>
<td>3.480a</td>
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<td>0.367</td>
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<td>0.037</td>
<td>23.565</td>
<td>0.803</td>
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<td>HIF1β Log</td>
<td>1.734b</td>
<td>9</td>
<td>0.193</td>
<td>2.272</td>
<td>0.063</td>
<td>20.452</td>
<td>0.733</td>
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<td>GLUT1 Log</td>
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<td>9</td>
<td>8.433</td>
<td>13.708</td>
<td>0.000</td>
<td>123.375</td>
<td>1.000</td>
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<td>Intercept</td>
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<td>82.285</td>
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<td>GLUT1 Log</td>
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<td>0.237</td>
<td>1.633</td>
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<td>5.885</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HIF1α Log</td>
<td>6.285</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF1β Log</td>
<td>3.346</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT1 Log</td>
<td>87.587</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. R Squared = .554 (Adjusted R Squared = .342)
b. R Squared = .518 (Adjusted R Squared = .290)
c. R Squared = .867 (Adjusted R Squared = .803)
d. Computed using alpha = .05
Table 7: Pairwise Comparisons of Various Time Points in Hypoxic Environment and Effects Seen in the Expression of HIF-1α Conducted by Multiple Univariate ANOVA.

The following table depicts the results of multiple univariate ANOVAs that were conducted to determine the effects that each hypoxia exposure time point had on the expression of HIF-1α. This chart does not include any effects from cell line, only the effects of hypoxia exposure treatment. Between 0-3 hours of hypoxia, there was a significant change in the relative expression of HIF-1α.

*Statistically significant values are highlighted and bolded in the figure below.

<table>
<thead>
<tr>
<th>Pairwise Comparisons</th>
<th>Mean Difference [I-J]</th>
<th>Std. Error</th>
<th>Sig. b</th>
<th>95% Confidence Interval for Differenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>03 Hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06 Hour</td>
<td>-0.193</td>
<td>0.222</td>
<td>0.395</td>
<td>-0.658 to 0.271</td>
</tr>
<tr>
<td>12 Hour</td>
<td>-0.056</td>
<td>0.235</td>
<td>0.814</td>
<td>-0.549 to 0.436</td>
</tr>
<tr>
<td>24 Hour</td>
<td>-0.139</td>
<td>0.222</td>
<td>0.539</td>
<td>-0.603 to 0.326</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-0.509*</td>
<td>0.222</td>
<td>0.033*</td>
<td>-0.973 to -0.044</td>
</tr>
<tr>
<td>06 Hour</td>
<td>0.193</td>
<td>0.222</td>
<td>0.395</td>
<td>-0.271 to 0.658</td>
</tr>
<tr>
<td>12 Hour</td>
<td>0.137</td>
<td>0.235</td>
<td>0.567</td>
<td>-0.355 to 0.630</td>
</tr>
<tr>
<td>24 Hour</td>
<td>0.055</td>
<td>0.222</td>
<td>0.808</td>
<td>-0.410 to 0.519</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-0.316</td>
<td>0.222</td>
<td>0.171</td>
<td>-0.780 to 0.149</td>
</tr>
<tr>
<td>12 Hour</td>
<td>0.056</td>
<td>0.235</td>
<td>0.814</td>
<td>-0.436 to 0.549</td>
</tr>
<tr>
<td>06 Hour</td>
<td>-0.137</td>
<td>0.235</td>
<td>0.567</td>
<td>-0.630 to 0.355</td>
</tr>
<tr>
<td>24 Hour</td>
<td>-0.083</td>
<td>0.235</td>
<td>0.730</td>
<td>-0.575 to 0.410</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-0.453</td>
<td>0.235</td>
<td>0.069</td>
<td>-0.945 to 0.040</td>
</tr>
<tr>
<td>24 Hour</td>
<td>0.139</td>
<td>0.222</td>
<td>0.539</td>
<td>-0.326 to 0.603</td>
</tr>
<tr>
<td>06 Hour</td>
<td>-0.055</td>
<td>0.222</td>
<td>0.808</td>
<td>-0.519 to 0.410</td>
</tr>
<tr>
<td>12 Hour</td>
<td>0.083</td>
<td>0.235</td>
<td>0.730</td>
<td>-0.410 to 0.575</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-0.370</td>
<td>0.222</td>
<td>0.112</td>
<td>-0.835 to 0.094</td>
</tr>
<tr>
<td>Normoxia</td>
<td>0.509*</td>
<td>0.222</td>
<td>0.033*</td>
<td>0.044 to 0.973</td>
</tr>
<tr>
<td>06 Hour</td>
<td>0.316</td>
<td>0.222</td>
<td>0.171</td>
<td>-0.149 to 0.780</td>
</tr>
<tr>
<td>12 Hour</td>
<td>0.453</td>
<td>0.235</td>
<td>0.069</td>
<td>-0.040 to 0.945</td>
</tr>
<tr>
<td>24 Hour</td>
<td>0.370</td>
<td>0.222</td>
<td>0.112</td>
<td>-0.094 to 0.835</td>
</tr>
</tbody>
</table>
**TABLE 8: PAIRWISE COMPARISONS OF THE EXPRESSION LEVELS OF HIF-1β AT EACH HYPOXIA TIME POINT CONDUCTED BY MULTIPLE UNIVARIATE ANOVA ANALYSIS**

The following table depicts the effects that the hypoxia exposure time point had on the expression of HIF-1β. This chart does not include any effects from cell line, only the effects resulting from hypoxic exposure treatment. Between the following time periods there were significant changes in the expression of HIF-1β: 0-6 hours, 0-12 hours, 12-24 hours in hypoxia.

*Statistically significant values are highlighted and bolded in the figure below.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Mean Difference (i-j)</th>
<th>Std. Error</th>
<th>Sig.b</th>
<th>95% Confidence Interval for Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>03 Hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06 Hour</td>
<td>0.228</td>
<td>0.168</td>
<td>0.190</td>
<td>-0.123</td>
</tr>
<tr>
<td>12 Hour</td>
<td>0.341</td>
<td>0.178</td>
<td>0.071</td>
<td>-0.033</td>
</tr>
<tr>
<td>24 Hour</td>
<td>-0.060</td>
<td>0.168</td>
<td>0.725</td>
<td>-0.412</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-0.299</td>
<td>0.168</td>
<td>0.092</td>
<td>-0.651</td>
</tr>
<tr>
<td>06 Hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03 Hour</td>
<td>-0.228</td>
<td>0.168</td>
<td>0.190</td>
<td>-0.580</td>
</tr>
<tr>
<td>12 Hour</td>
<td>0.112</td>
<td>0.178</td>
<td>0.537</td>
<td>-0.261</td>
</tr>
<tr>
<td>24 Hour</td>
<td>-0.289</td>
<td>0.168</td>
<td>0.102</td>
<td>-0.640</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-.527*</td>
<td>0.168</td>
<td>0.005*</td>
<td>-0.879</td>
</tr>
<tr>
<td>12 Hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03 Hour</td>
<td>-0.341</td>
<td>0.178</td>
<td>0.071</td>
<td>-0.714</td>
</tr>
<tr>
<td>06 Hour</td>
<td>-0.112</td>
<td>0.178</td>
<td>0.537</td>
<td>-0.485</td>
</tr>
<tr>
<td>24 Hour</td>
<td>-.401*</td>
<td>0.178</td>
<td>0.037</td>
<td>-0.774</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-.639*</td>
<td>0.178</td>
<td>0.002*</td>
<td>-1.013</td>
</tr>
<tr>
<td>24 Hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03 Hour</td>
<td>0.060</td>
<td>0.168</td>
<td>0.725</td>
<td>-0.292</td>
</tr>
<tr>
<td>06 Hour</td>
<td>0.289</td>
<td>0.168</td>
<td>0.102</td>
<td>-0.063</td>
</tr>
<tr>
<td>12 Hour</td>
<td>-.401*</td>
<td>0.178</td>
<td>0.037*</td>
<td>0.028</td>
</tr>
<tr>
<td>Normoxia</td>
<td>-.239</td>
<td>0.168</td>
<td>0.172</td>
<td>-0.591</td>
</tr>
<tr>
<td>03 Hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06 Hour</td>
<td>.299</td>
<td>0.168</td>
<td>0.092</td>
<td>-0.053</td>
</tr>
<tr>
<td>12 Hour</td>
<td>.527*</td>
<td>0.168</td>
<td>0.005*</td>
<td>0.179</td>
</tr>
<tr>
<td>24 Hour</td>
<td>.639*</td>
<td>0.178</td>
<td>0.002*</td>
<td>0.266</td>
</tr>
</tbody>
</table>

*Statistically significant values are highlighted and bolded in the figure below.

Based on estimated marginal means

* The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).
The following table depicts the effects that the hypoxia exposure time point had on the expression of GLUT1. This chart does not include any effects from cell line, only the effects resulting from hypoxic exposure treatment. Between the following time periods there were significant changes in the expression of GLUT1: 0-3 hours, 0-6 hours, 0-12 hours, and 0-24 hours in hypoxia.

*Statistically significant values are highlighted in the figure below.

**Table 9: Multivariate Analysis Pairwise Comparisons of the Expression Levels of GLUT1 at Each Hypoxia Time Point Conducted by Multiple Univariate ANOVA**

The following table depicts the effects that the hypoxia exposure time point had on the expression of GLUT1. This chart does not include any effects from cell line, only the effects resulting from hypoxic exposure treatment. Between the following time periods there were significant changes in the expression of GLUT1: 0-3 hours, 0-6 hours, 0-12 hours, and 0-24 hours in hypoxia.

*Statistically significant values are highlighted in the figure below.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Mean Difference (±J)</th>
<th>Std. Error</th>
<th>Sig. b</th>
<th>95% Confidence Interval for Differenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>03 Hour GLUT1 Log</td>
<td>06 Hour</td>
<td>-0.155</td>
<td>0.453</td>
<td>0.735</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>-0.303</td>
<td>0.480</td>
<td>0.535</td>
</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>0.507</td>
<td>0.453</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>-2.097*</td>
<td>0.453</td>
<td>0.000*</td>
</tr>
<tr>
<td>06 Hour GLUT1 Log</td>
<td>03 Hour</td>
<td>0.155</td>
<td>0.453</td>
<td>0.735</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>-0.148</td>
<td>0.480</td>
<td>0.761</td>
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<td></td>
<td>24 Hour</td>
<td>0.663</td>
<td>0.453</td>
<td>0.160</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>-1.942*</td>
<td>0.453</td>
<td>0.000*</td>
</tr>
<tr>
<td>12 Hour GLUT1 Log</td>
<td>03 Hour</td>
<td>0.303</td>
<td>0.480</td>
<td>0.535</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>0.148</td>
<td>0.480</td>
<td>0.761</td>
</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>0.811</td>
<td>0.480</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>-1.794*</td>
<td>0.480</td>
<td>0.001*</td>
</tr>
<tr>
<td>24 Hour GLUT1 Log</td>
<td>03 Hour</td>
<td>-0.507</td>
<td>0.453</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>-0.663</td>
<td>0.453</td>
<td>0.160</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>-0.811</td>
<td>0.480</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>-2.604*</td>
<td>0.453</td>
<td>0.000*</td>
</tr>
<tr>
<td>Normoxia GLUT1 Log</td>
<td>03 Hour</td>
<td>2.097*</td>
<td>0.453</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>1.942*</td>
<td>0.453</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>1.794*</td>
<td>0.480</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>2.604*</td>
<td>0.453</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Based on estimated marginal means

* The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).
**Table 10: Multiple univariate ANOVA pairwise comparisons of expression levels of HIF-1α, HIF-1β and GLUT1 in U87MG cells and MSU1.1 cell lines.**

The following table depicts the significance of the effects seen in the individual gene expression levels of HIF-1α, HIF-1β, and GLUT1. Each gene was analyzed separately along with a concordant independent variable, cell line. Below it shows that HIF-1α expression is significantly different in the two cell lines and it is visualized in Figures 11 and 14. In this analysis, HIF-1β expression was not significantly affected by cell line, which was also found in the multiple univariate ANOVAs depicted in Table 5. GLUT1 expression was significantly affected by the cell line it was being expressed in, which was also shown in Table 5.

*Statistically significant values are highlighted in the figure below.

<table>
<thead>
<tr>
<th>Pairwise Comparisons</th>
<th>Dependent Variable</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.b</th>
<th>95% Confidence Interval for Difference b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>HIF-1α Log</td>
<td>MSU1.1</td>
<td>U87</td>
<td>0.540*</td>
<td>0.144</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>U87</td>
<td>MSU1.1</td>
<td>-0.540*</td>
<td>0.144</td>
<td>0.001*</td>
</tr>
<tr>
<td>HIF-1β Log</td>
<td>MSU1.1</td>
<td>U87</td>
<td>0.002</td>
<td>0.109</td>
<td>0.982</td>
</tr>
<tr>
<td></td>
<td>U87</td>
<td>MSU1.1</td>
<td>-0.002</td>
<td>0.109</td>
<td>0.982</td>
</tr>
<tr>
<td>GLUT1 Log</td>
<td>MSU1.1</td>
<td>U87</td>
<td>2.294*</td>
<td>0.293</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>U87</td>
<td>MSU1.1</td>
<td>-2.294*</td>
<td>0.293</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*Based on estimated marginal means

* The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).
**Table 11: Estimated Marginal Means of HIF-1α, HIF-1β, and GLUT1 in Both U87MG and MSU1.1 Cell Lines**

The following table shows the estimated marginal means of the measured effects that each cell line and hypoxia time point have on the gene being expressed (HIF-1α, HIF-1β, and GLUT1). These values are visualized in Figures 14-16 and their significance is discussed above in the results section.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Mean</th>
<th>Std. Error</th>
<th>95% Confidence Interval</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α Log</td>
<td>03 Hour</td>
<td>0.835</td>
<td>0.222</td>
<td>0.371</td>
<td>1.299</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>1.035</td>
<td>0.222</td>
<td>0.450</td>
<td>1.499</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>0.893</td>
<td>0.222</td>
<td>0.490</td>
<td>1.357</td>
</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>1.283</td>
<td>0.222</td>
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<td>1.747</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>1.335</td>
<td>0.222</td>
<td>0.870</td>
<td>1.799</td>
</tr>
<tr>
<td></td>
<td>03 Hour</td>
<td>0.418</td>
<td>0.222</td>
<td>-0.046</td>
<td>0.882</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>0.605</td>
<td>0.222</td>
<td>0.140</td>
<td>1.069</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>0.472</td>
<td>0.272</td>
<td>-0.097</td>
<td>1.041</td>
</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>0.248</td>
<td>0.222</td>
<td>-0.217</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>0.936</td>
<td>0.222</td>
<td>0.472</td>
<td>1.400</td>
</tr>
<tr>
<td>HIF-1β Log</td>
<td>03 Hour</td>
<td>2.530</td>
<td>0.168</td>
<td>2.178</td>
<td>2.882</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>2.567</td>
<td>0.168</td>
<td>2.215</td>
<td>2.919</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>2.562</td>
<td>0.168</td>
<td>2.210</td>
<td>2.914</td>
</tr>
<tr>
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<td>24 Hour</td>
<td>2.765</td>
<td>0.168</td>
<td>2.413</td>
<td>3.117</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>2.396</td>
<td>0.168</td>
<td>2.044</td>
<td>3.348</td>
</tr>
<tr>
<td></td>
<td>03 Hour</td>
<td>2.920</td>
<td>0.168</td>
<td>2.568</td>
<td>3.272</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>2.425</td>
<td>0.168</td>
<td>2.073</td>
<td>2.777</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>2.206</td>
<td>0.206</td>
<td>1.775</td>
<td>2.637</td>
</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>2.805</td>
<td>0.168</td>
<td>2.453</td>
<td>3.157</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>3.051</td>
<td>0.168</td>
<td>2.699</td>
<td>3.403</td>
</tr>
<tr>
<td>GLUT1 Log</td>
<td>03 Hour</td>
<td>2.159</td>
<td>0.453</td>
<td>1.211</td>
<td>3.106</td>
</tr>
<tr>
<td></td>
<td>06 Hour</td>
<td>2.397</td>
<td>0.453</td>
<td>1.449</td>
<td>3.345</td>
</tr>
<tr>
<td></td>
<td>12 Hour</td>
<td>2.516</td>
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<td>1.688</td>
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</tr>
<tr>
<td></td>
<td>24 Hour</td>
<td>2.259</td>
<td>0.453</td>
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</tr>
<tr>
<td></td>
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<td>2.758</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>Normoxia</td>
<td>3.158</td>
<td>0.453</td>
<td>2.210</td>
<td>4.106</td>
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</table>
This chart illustrates the statistical analysis data shown in Table 10. Overall, HIF-1α expression was significantly correlated to the cell line it was expressed in: U87MG versus MSU1.1 (p = .001). However, the hypoxia treatment did not lead to significant changes in HIF-1α expression (p = .215) and there was no significance when the effects of cell line and hypoxia treatment were combined during statistical analysis (p = .558).
This chart illustrates the data shown in Table 10. Overall, HIF-1β expression was not significantly correlated to the cell line it was expressed in: U87MG versus MSU1.1 (p = .911). However, HIF-1β expression was significantly correlated with hypoxia treatment (p = .018). When the effects of cell line and hypoxia treatment were combined during statistical analysis, the interaction was not significant (p = .332). Overall, hypoxia treatment significantly affected HIF-1β expression.
**Figure 16: Multivariate Analysis Estimated Marginal Means Plot depicting how hypoxia exposure over time leads to GLUT1 relative expression level changes.**

This chart illustrates the data shown in Table 10. Overall, GLUT1 expression was significantly correlated to the cell line it was expressed in: U87MG versus MSU1.1 (p = .000). GLUT1 was also significantly correlated with hypoxia treatment (p = .000). When the interacting effects of cell line and hypoxia treatment were combined during statistical analysis, the interaction was also significant (p = .002).


**Discussion**

The aim of this study was to determine whether hypoxia has a role in regulating the expression of HIF-1α, HIF-1β, and GLUT1 in a GBM cancer cell line. We hypothesized that the expression of these genes is proportional to *in-vitro* hypoxic conditions. The *in-vitro* model consisted of the U87MG (GBM) cell line as well as MSU1.1 (fibroblast) cell line and relative gene expressions of HIF-1α, HIF-1β, and GLUT1 were characterized in these lines. Although MSU1.1 cells were used as a control and therefore not the focal point of the study, the limited number of studies characterizing MSU1.1 cells make findings from this study potentially novel. Contrastingy, the expressions of HIF-1α, HIF-1β, and GLUT1 as well as the direct relationship that HIF-1α and HIF-1β expression have on downstream targets such as GLUT1, have been well characterized in many studies. However, the relationship of acute versus chronic hypoxia on the expression of these genes has not been fully elucidated.

There appear to be two factors modulating HIF differential regulation: the severity of the hypoxic environment and time spent in hypoxia (acute versus chronic). It appears that HIF-1α expression is more responsive to severe hypoxia (1-3% O2) than the other HIF proteins, primarily HIF-2α, whose expression has been characterized in less hypoxic conditions (5% O2) (Holmquist-Mengelbier et al., 2006). Additionally, it has been postulated that HIF-2α rather than HIF-1α mediates responses to chronic hypoxia (12+ hours), while HIF-1α only mediates responses to acute episodes of hypoxia. In two
neuroblastoma cell lines grown in 1% O₂, expression of HIF-1α climbed rapidly in the first
couple of hours in hypoxia, but then gradually decreased after 72 hours. Contrasting,HIF-2α protein expression consistently climbed in 1% O₂ for all 72 hours of the study.
When taken together it suggests that HIF-2α and not HIF-1α is likely responsible for
changes in chronically hypoxic areas at physiological O₂ levels, at least in a neuroblastoma
model (Holmquist-Mengelbier et al., 2006).

It is worth noting that in the aforementioned neuroblastoma model, HIF-2α mRNA
levels increased in both 1% and 5% O₂ conditions despite a concurrent rise in PHD2 and
PHD3 protein levels, whose role is to inactivate HIFs. This data suggests that HIF-2α grows
less sensitive to PHDs over time or that high protein synthesis of HIF-2α can counteract
PHD degradation (Holmquist-Mengelbier et al., 2006). With this study in mind, it is a good
reminder that any relative expression changes noted in this study could be due to a
decreased sensitivity to other protein regulators rather than simply being upregulated.
Acute versus chronic hypoxia in the context of HIF-1 versus HIF-2 expression was not
investigated in this study, aside from the confirmed presence of HIF-2α, HIF-2β, and HIF-3β
were confirmed in U87MG and MSU1.1 cells in both normoxia and hypoxia using end-point
PCR. Further studies investigating the relative expression levels of the various HIFs in
various tissues needs to be done. A particularly underdeveloped area of HIF research is
regarding the temporal differences of the HIFs as well as their general sensitivity to O₂
conditions (Holmquist-Mengelbier et al., 2006) in the tumor microenvironment and in
normal tissues. However, studies on the genes downstream of HIFs, such as GLUT1, are
needed to understand the GBM microenvironment at the various proximities to
vasculature.
This study aimed to confirm that HIF-1α, HIF-1β, and GLUT1 are all expressed in U87MG cells, using the largely uncharacterized MSU1.1 fibroblast cells as a ‘novel’ control cell line. The expression of HIF-1α, HIF-1β, HIF-2α, HIF-2β, HIF-3β, GLUT1, and GLUT3 were confirmed in U87MG and MSU1.1 cells (Figures 6-10). However, the presence of HIF-3α in either cell line was not successfully characterized in this study, very likely due to primer design issues. It is possible that the primer could be effective, but that the conditions needed to induce expression of HIF-3α might not have been ideal in the design of this study. However, that is unlikely since ‘moderate’ hypoxia has been shown to induce the expression of some HIF-3α variants (Heidbreder et al., 2003). To test this in an in-vitro model, cells could be exposed to 1% and 5% O₂ levels for time periods spanning a few minutes to 72 hours could elucidate more about HIF-3α relative expression.

The NCBI Gene Database and Blast were utilized to design the primers and used to test the sequences of HIF-3α primers that were used in past studies. Upon researching, it was discovered that many of the published HIF-3α primer sequences did not successfully target HIF-3α when analyzed. Therefore, the characterization of HIF-3α published thus far might be more inconclusive than thought prior to this study. Due to the issues designing a suitable HIF-3α primer in this study and or its lack of expression and amplification, its characterization was not continued after the EP-PCR phase of this study. However, it remains a highly contentious aspect of hypoxia medicated expression changes in GBM.
General Effects of Cell Line and Hypoxic Exposure Treatment on Lumped Expression of HIF-1α, HIF-1β, and GLUT1

The qPCR data acquired only investigated the expression of HIF-1α, HIF-1β, and GLUT1. As mentioned previously, (p. 43, 49-50), two types of statistical analyses were conducted: MANOVA and multiple Two-way ANOVAs. MANOVA was conducted to determine the general effect that each independent variable (IV) or the interaction of the two IVs (Cell line and hypoxia exposure), had on the expression of all the following genes lumped together: HIF-1α, HIF-1β, and GLUT1 (Table 4). These results show that cell line, hypoxic exposure, and the interaction of the two factors each independently led to significant general effects in the expression of the group of target genes (HIF-1α, HIF-1β, and GLUT1) and are shown in Table 4. Since a general effect from the cell lines and hypoxic treatment had significant effects in gene expression, additional statistical tests were conducted to determine the effects that each IV had on the expression of each individual gene of interest.
Specific Effects of Cell Line and Hypoxic Exposure Treatment on Gene Expression

Effect of Cell Type on Individual Genes Expressed

Multiple two-way ANOVAs identified significant correlations between cell type and each of the following genes (DV): HIF-1α and GLUT1. It was expected that both genes would be upregulated in the U87MG GBM cells compared to the MSU1.1 cells since GLUT1 has been shown to be overexpressed in cancerous tissues, including GBM (Ma et al., 2015) (J. Liu et al., 2015) (Asmaa Gaber Abdou, MD et al., 2015) (Labak et al., 2016) (Nes, Johannes AP, et al, 2015) (Bache et al., 2015). GLUT1 is a downstream target gene of HIF-1, but not solely of HIF-1α and is over-expressed in cancerous tissues, whether resulting from hypoxia or oncogenic activation, or can just result from hypoxic normal tissues. In this study, the differential expression of HIF-1α in the two cell lines was higher in GBM cells than in the control MSU1.1 fibroblasts used in this study, which aligns with the general knowledge of the field. The lack of effect seen in HIF-1β was surprising because of its concordant expression with HIF-1α, which is upregulated in many tissues and in malignant tumors. Therefore, U87MG and MSU1.1 fibroblast cells likely have differential signaling of hypoxia induced HIF-1α stability and the downstream GLUT1 upregulation. More specifically, other HIFs could be at play in this scenario, such as HIF-2β, and this requires future study to better understand this phenomenon.
Effect of Hypoxia Exposure Treatment on Individual Genes Expressed

Two-way ANOVA also found that hypoxia exposure treatment affected HIF-1β and GLUT1 expression, but surprisingly not HIF-1α expression except within the first three hours of hypoxic exposure. It was expected that GLUT1 would be responsive to relative oxygen levels as well as to the cell line in which it was expressed. Many studies have reported that GLUT1 is enriched in areas of the brain, especially in endothelial cells lining the blood brain barrier. Since GLUT1 is upregulated in healthy brain tissues, it isn’t surprising that GLUT1 is overexpressed in GBM cells since they occur in the brain. This upregulation of GLUT1 seen is adaptive in light of the Warburg Effect. Studies have shown that cancer cells display a tendency to metabolize glucose more than normal cells. Cancer cells perform aerobic glycolysis as their main method of ATP generation rather than using mitochondrial respiration.

It was expected that HIF-1α would be responsive to hypoxia treatment in the first few hours of the assay, which was seen in this study in the first three hours of hypoxic exposure (G. L. Wang, Jiang, Rue, & Semenza, 1995). HIF-1α expression has been shown to be dependent on relative oxygen concentration, but there are some reports that HIF-1α is only present by acute hypoxia which could explain why it was overexpressed in the first three hours as opposed to after 24 hours of hypoxic exposure. As discussed, the PHD enzymes that regulate HIF stability are not active during hypoxia, but eventually stabilize in chronic hypoxia and then start to again degrade HIF-1α proteins quickly as they do in normoxia. However, if this post-translational HIF-1α protein degradation is occurring, it is possible that HIF-2α protein might be elevated in the (5% O2) experimental conditions of
this study, but the expression of HIF-2α likely wouldn’t have been activated until the relative oxygen was decreased further to around 3% O₂. Regardless, it does not account for why the transcription of HIF-1α is only increased within the first three hours of hypoxic exposure when the above changes to HIF-1α occurred after translation.

**Interaction Effect of Cell Type and Hypoxia Exposure Treatment on Individual Genes Expressed**

Analysis using two-way ANOVA found that GLUT1 expression was significantly affected when both cell line and hypoxia exposure were used as factors (Table 4). Specifically, hypoxic exposure led to a significant upregulation of HIF-1β (Figure 12) and GLUT1 transcription (Figure 13) in U87MG (GBM) cells compared to control MSU1.1 fibroblast cells. These results led us to reject the null hypothesis that GLUT1 and HIF-1 expression changes are directly correlated with *in-vitro* hypoxic conditions because there were significant changes seen in the genes of interest due to hypoxic exposure as well as between cell lines. The upregulation of HIF-1β was surprising because numerous other studies have shown that HIF-1β is constitutively made and requires the hypoxia dependent HIF-1α subunit to elicit any changes to the cell. Without HIF-1α hetero-dimerization with the HIF-1β subunit, there is no translocation into the nucleus and no HIF initiated genetic changes within the host cell.

As could be expected based on the above reasoning, the combined effects of cell line and hypoxia exposure led to a significant interactional effect in GLUT1 expression. It was expected that GLUT1 would be responsive to relative oxygen levels as well as to the cell
line in which it was expressed. Many studies have reported that GLUT1 is enriched in areas of the brain, especially in endothelial cells lining the blood brain barrier (Zuchero et al., 2016). The Warburg Effect also hypothesized that cancer cells tend to metabolize glucose more than normal cells. Cancer cells perform aerobic glycolysis as their main method of ATP generation rather than using mitochondrial respiration. Therefore, the upregulation of GLUT1 in U87MG cells compared to MSU1.1 cells in this study is characteristic of typical brain cancer cells.

**Potential Effect of Chronic versus Acute Hypoxia**

Perhaps the stabilization of HIF-1α is more easily accomplished in malignant cells than in healthy cells. This could be accomplished by altering the various mechanisms by which HIF-1α is normally degraded. As there are multiple pathways that can degrade HIFs, it is possible that some of the hypoxia regulating signaling cascades are specific to cell type and malignancy status, as well as to the specific level of oxygen in the tumor microenvironment. Several studies have suggested that there are also differences in HIF family expression levels based on the percentage of oxygen in the environment (Holmquist-Mengelbier et al., 2006). Some would argue that the 5% O₂ used in this study wasn’t low enough and would have recommended 1% O₂ or lower to stabilize HIF-1α.

Since the longest hypoxia time point in this study was 24 hours, a larger sample size and longer treatment times might be needed to get a comprehensive understanding of HIF-1α/β expression and regulation. A larger number of biological replicates as well as more technical replicates would help to yield a more comprehensive understanding of this
phenomenon. Overall, this study found that HIF-1β and GLUT1 were responsive to changes in low relative oxygen levels, and HIF-1α was not.

**Summary**

Hypoxia has been shown to lead to GLUT1 upregulation in various cancer types including: endometrial (Ma et al., 2015), gastric (J. Liu et al., 2015), squamous cell carcinoma (Asmaa Gaber Abdou, MD et al., 2015), ovarian (Labak et al., 2016), meningioma (Nes, Johannes AP, et al, 2015), and glioblastoma (Bache et al., 2015) and has been implicated in metabolic remodeling, which is one of the hallmarks of cancer. The positive correlation of GLUT1 and HIF-1α with an acutely hypoxic GBM microenvironment has been described in this study in a GBM and fibroblast cell line model. This provides further confirmation that these two proteins are interrelated. However, this is needs to be investigated further, as the hypoxia severity was not directly tested. Additionally, GLUT1 is also upregulated in normal tissues including the cortex, hippocampus, and the liver after short periods (2 hours) of hypoxia (Heidbreder et al., 2003). Its upregulation has been induced by oncogenic transformation (C. Chen et al., 2001, p. 9519) as well as by hypoxic conditions modulated by HIF-1α expression. The presence of GLUT1 in GBMs and other tumors makes it a fair candidate for cancer therapies, but systemic treatments targeting GLUT1 might be detrimental due to the to its widespread distribution throughout the body, especially in the brain (Denko, 2008)(Ozbudak et al., 2008)(Airley & Mobasher, 2007).

However, GLUT3 has been shown to be hypoxia modulated via HIF-1α as well (Y. Liu et al., 2009a)(Rooj et al., 2016), and may be a better target for cancer therapies, as both
GLUT1 and GLUT3 have been shown to have increased expression in the hypoxic tumor microenvironment (Y. Liu et al., 2009a) and CSC self-renewal (Christensen et al., 2015, p. 4). By extension, the HIF-modulated tumor microenvironment selects for the intra-tumoral CSC population which is thought to indirectly confer a myriad of selective advantages to the tumor such as tumor aggression, resistance to treatment, tumor recurrence, and invasion of other tissues (Labak et al., 2016). Additionally, differences in glycolytic metabolism have been found in CSC populations (Zhou et al., 2011)(Hanahan & Weinberg, 2011) which further supports the argument that CSCs, the GBM microenvironment, and metabolic remodeling are interrelated. A study confirmed that GLUT3 was expressed more in highly malignant gliomas as opposed to low-grade gliomas, and since it was the predominant GLUT in the tumor it was a good prognostic factor for patients (Y. Liu et al., 2009a). Despite the need to study GLUT3, its relative expression was not measured in this study due to time and budget constraints.

The direct relationship of GLUT1 and GLUT3 and their roles in GSCs are still debated, but they appear to be very good targets for the GSC population in the GBM(Y. Liu et al., 2009a)(Shibuya et al., 2015). Specifically, in mice, GLUT1 was identified as a major protein in the blood brain barrier (Zuchero et al., 2016) and as the blood brain barrier is an obstacle to drug treatment, GLUT1 being highly expressed could help identify future mechanisms to treat GBM and other CNS malignancies. As both GLUTs 1 and 3 have been found within GBM (Y. Liu et al., 2009b), it seems that there is a likely correlation with tumor grade, intra-tumor cell populations, and potentially the level of hypoxia and thus HIF-1 versus HIF-2 regulation. Exclusively HIF-2α and not HIF-1α has been shown to regulate Oct4 expression by binding to the Oct4 promoter depicted in a mouse embryo in-
vivo model (Covello et al., 2006, p. 4). These studies that have found HIF-2α regulates Oct4 indicates that HIF-2α is related to the stem cell subpopulation of the GBM that has been shown to confer treatment resistance. However, knockdown of HIF-1α expression has been linked with abrogation in CD-133 positive GSCs, which indicates also that HIF-1α has a role with stem cells in GBM. This finding could prove useful, as it answers another question about which proteins modulate GSCs and consequently tumor aggression, immune suppression, and tumor recurrence. Overall, the roles of HIF-1α, HIF-2α, GLUT1, and GLUT3 are still unclear, especially in clarifying which of these proteins plays a major role in the GSC population in glioma. It is evident that hypoxia mediates the roles of them all, at least indirectly, and that they play roles in many of the properties that make GBM difficult to treat and thus are important for future study.

**Future Studies**

Additional studies would need to be conducted to further validate the data found in this study but confirmed our overall hypothesis: hypoxia induced changes in the genes of interest when viewed as a group. However, when studied individually, the patterns found in this study are somewhat different than the literature has described, necessitating an expansion of the scope of the experimental design. Future studies should include healthy non-cancerous control cells, GSGs, normal neural stem cells, more varied in-vitro relative oxygen levels (<5%O₂), and shorter (30 seconds) and longer time periods (24-48 hours) to analyze the mRNA expression and protein expression of these genes. There is significant data from this study and others warranting continued studies of the relationship between
the HIFs and GLUT1 using an in-vitro GBM model. Although, budget and time constraints didn’t allow for exploration into GLUT-3, HIF-2α, HIF-2β, HIF-3α or HIF-3β expression and regulation, they all seem to be very interesting targets to study for future therapeutic approaches to GBM. Specifically, future studies could determine the protein levels of the HIFs as well as the GLUTs in a GBM model with the goal of then pursuing in-vivo studies. Specifically, the differential regulatory roles of HIF-2 and HIF-3 in a GBM model: and especially in the subpopulations of the GBM. HIF-2 would likely be a very interesting target to investigate and an experimental design like used in this study could be utilized. It would also be worth also investigating how 1% hypoxia induced regulation differs from 5% oxygen, especially when applied to the role of CSCs in the GBM model. Additionally, expanding this study to examine actual protein levels of these genes rather than just the relative expression level via mRNA expression, would be very important. Since these genes elicit functions at the protein level it is necessary to evaluate them as such.

Resource Availability

Funding was sought from internal NMU sources over the course of the winter 2016 semester. The Excellence in Education Grant and the Graduate Research Scholarly Activities Fund were received in May 2017. Any costs that exceeded the $1500 from the Excellence in Education grant were graciously provided by the Upper Michigan Brain Tumor Center.
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Methods

Direct Cell Lysis

Cells were lysed directly in the cell culture dish to prevent any reversal of possible gene expression changes induced by the treatment conditions. As it is still contested whether brief normoxia exposure is long enough to reverse hypoxia induced gene expression changes, this precaution was taken. Cell cultures had the media aspirated and were then washed with 2-3 mL of cell culture grade 1xPBS.

RNA Isolation

A mixture of the Qiagen RLT buffer and a 1:1000 β-Mercaptoethanol was added to the cell culture dish to lyse the cells. In preparation, the RLT-β-Mercaptoethanol aliquots were housed in tin foil covered microcentrifuge tubes to minimize light induced degradation. 606 μL of the RLT mixture was added to each culture dish. The cells were scraped to one side of the cell culture dish using a cell scraper. The lysate was transferred to a QiaShredder and was centrifuged for two minutes. This was repeated until all the
lysate had gone through the column. 606 μL of 70% Ethanol was added to the lysate in the bottom of the QiaShredder tube and was mixed well by pipetting. Of this mixture, 700 μL at a time was transferred to an RNeasy spin column and was centrifuged for 15 seconds at 8000xg. The flow through was discarded and this was repeated until all the homogenized lysate had gone through the RNeasy spin column.

*RNA Purification*

700 μL of the Qiagen RW1 buffer was added to the RNeasy spin column and the column was spun for 15 seconds at 8000xg before discarding the column flow through. 500 μL of Qiagen RPE was added to the column and was spun for 15 seconds at 8000xg before discarding the flow through. An additional 500 μL of the RPE buffer was added and the column was spun down for 2 minutes at 8000xg and the flow through was discarded. The column was transferred to a new collection tube and was spun for 1 minute at 8000xg. The column was switch to another tube and then 10-20μL of RNAse free water was added to the column. The column rested for 1 minute and was then spun for 1 minute to elute the RNA from the spin column. To increase the yield of RNA eluted from the column, the eluted RNA at the bottom of the column was pipetted and added to the top of the column again to get more RNA from the column. The 1 minute rest and spin were both repeated. The final eluent was pipetted into a microcentrifuge tube and was immediately transferred to -80°C freezer until first strand cDNA synthesis was conducted.
**First Strand cDNA Synthesis**

RNA concentration was determined using the ThermoScientific Nanodrop 2000c. The RNA was then converted to cDNA via reverse transcription. Reverse transcription was conducted on the isolated RNA using either the avian myeloblastosis virus reverse transcriptase (AMV-RT) and the protocol suggested by the manufacturer (Promega, Cat # M5108) or the ThermoFisher Maxima Reverse Transcriptase (Cat # EP0741). The Promega protocol was utilized in reverse transcribing the RNA that was used in the End-Point PCR experiments. The ThermoFisher Maxima Reverse Transcriptase was used to make the cDNA for the qPCR experiments. The switch to the Maxima reverse transcriptase reagent was done due to increased efficiency and cost reduction.

**Promega AMV-Reverse Transcriptase Protocol**

1.0 µg of Oligo (dT)$_{15}$ primer was added to 2 µg of RNA in a microcentrifuge tube and the volume of water present did not exceed 11 µL. The tube was heated at 70°C for 5 minutes and were then chilled on ice for 5 minutes. The tube was briefly centrifuged in the MyFuge 12 Mini Centrifuge (Cat# 681725) to collect all the solution at the bottom of the tube.

The following components were added in the following order:

- 5µl AMV Reverse Transcriptase 5X Reaction Buffer (Cat# M5108)
- 2.5µl dNTP mix (Cat# U1511)
AMV RT 30 units (Cat# M5108)
Nuclease-Free Water to final volume 25μl

The tube was mixed by gently flicking the tube. If needed, the tube was briefly centrifuged again to ensure that all the solution was at the bottom of the tube. The tube was incubated for 60 minutes at 42°C. The tubes were stored at -20°C for the duration of the End-Point PCR studies.

ThermoFisher Maxima Reverse Transcriptase Protocol

All reagents were centrifuged briefly in the MyFuge (Cat# Z681725) and kept on ice for the duration of the protocol. The following components were added to a sterile micro-centrifuge tube on ice in the following order:

- 2 μg of template RNA
- 1.0 μL of (100 pmol) of oligo(dT)₁₅ primers (Cat# C1101)
- 1.0 μL of (10 mmol) dNTP Mix (Cat# U1511)
- 10.5 μL Nuclease-free water (Cat# EP0741)

The following reaction components were then added in the following order:

- 4 μL 5x Maxima RT Buffer (Cat# EP0741)
- 0.5 μL Nuclease-free water (Cat# EP0741)
*when RNAse inhibitor was not available

1.0 μL Maxima Reverse Transcriptase (Cat# EP0741)

The total volume of the reaction was 20 μL. The mixture was centrifuged briefly and immediately placed in the programmed Bio-Rad T100 Thermal Cycler. The reactions incubated for 30 minutes at 50°C. The reaction was terminated by heating the tubes to 85°C for 5 minutes. The cDNA product was stored in the -20°C freezer for the duration of the qPCR studies.