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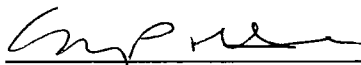
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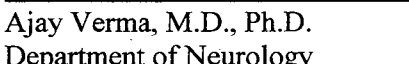
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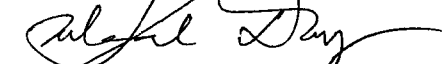
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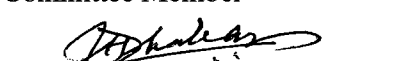
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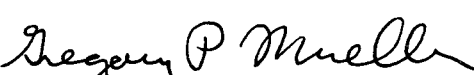
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A handwritten signature in black ink, appearing to read 'Jeremy Henriques', with a long, sweeping horizontal line extending to the right.

Jeremy Henriques
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**Branched-chain Amino- and Keto acid Biochemistry and Cellular Biology in an
in vitro Model of Central Nervous System Disease**

Jeremy Henriques, Doctor of Philosophy in Neuroscience, 2008

Supervised by Ajay Verma, MD, PhD, Former Associate Professor of Neuroscience

Abstract

Branched-chain amino acids (BCAAs: leucine, isoleucine, and valine) are essential components of many biochemical and biological processes. There are well-established pathways, such as fatty acid synthesis and oxidation, anabolic use to synthesize new proteins, and anaplerotic use to generate or sustain metabolic intermediate molecules, which we define as “classical” pathways here. Recently, new “non-classical” roles for BCAAs have been discovered, most notably for leucine. Leucine has been shown to initiate protein synthesis by increasing translational protein complex activities and to suppress feeding behaviors in rats; both phenomena are at least partially mediated through the mammalian target of rapamycin (mTOR) kinase cascade. Our lab previously identified another potential non-classical pathway independent of mTOR, acting through the hypoxia inducible factor (HIF) transcriptional regulatory protein. Our goal for the first line of research for this work was to validate and further elaborate that leucine promotes HIF transcriptional activation through its 2-oxoacid derivative, α -ketoisocaproic acid, using an *in vitro* glioma cell line transgenically altered to express a reporter protein for HIF- α degradative cycle activity. The 2-oxoacid was found to increase the half-life of the HIF-1 α component of the HIF-1 heterodimeric complex in normal oxygen conditions and induce secretion of a known HIF-1 transcriptional protein target

independent of mTOR activation. The effect of 2-oxoacid stimulation of HIF-1 transcriptional activation was inhibited by addition of ascorbic acid, a cofactor known to increase HIF degradation in normoxic conditions. Our goal for the second line of research was to investigate the interaction between classical and non-classical BCAA pathways, that is to say the interaction between metabolic and signaling roles in an *in vitro* model of cancer. BCAAs exclusively undergo irreversible decarboxylation and oxidation through the branched-chain α -ketoacid dehydrogenase complex (BCKDC), a multienzyme complex regulated by reversible phosphorylation. By knocking down the kinase, which normally decreases BCKDC activity, with a stable shRNA transfection in a glioblastoma cell line, the BCKDC exhibited decreased activity and phosphorylation signal compared to wild-type and control vector cell lines. We expected knockdown of the kinase would decrease the anabolic protein translational pathway to result in a decrease of cancerous phenotypes, such as proliferation, invasion, migration, and colony formation, but found the opposite. Further investigation indicated compensatory changes to glycolytic pathways such as decreased pyruvate dehydrogenase complex (PDC) activity and increased lactate production. These characteristics are known to increase the cancerous phenotypes investigated, a phenomenon called “The Warburg Effect”. These results implicate BCAAs as an underappreciated component in cancer biochemistry, but also relate to various other lines of research, such as clinical sequelae of a congenital genetic disorder known as maple syrup urine disease (MSUD), neurotransmitter homeostasis and disruption in epilepsy, and muscle wasting in patients with cancer known as cancer cachexia.

**Branched-chain Amino- and Keto acid Biochemistry and Cellular Biology in an
in vitro Model of Central Nervous System Disease**

By

Jeremy Henriques

Dissertation submitted to the Faculty of the Program in Neuroscience of the
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requirements for the Degree of Doctor of Philosophy, 2008.

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152

Abbreviations

153 ANOVA –analysis of variance

154 ATP – adenosine 5'-triphosphate

155 BCA – bicinchoninic acid

156 BCAA – branched-chain amino acid

157 BCAT – branched-chain amino acid transaminase

158 BCKA – branched-chain keto acid

159 BCKDC – branched-chain α -keto acid dehydrogenase complex

160 BDK – branched-chain α -keto acid dehydrogenase complex kinase

161 BDP – branched-chain α -keto acid dehydrogenase complex phosphatase

162 CCD – charge-coupled device

163 CIC – α -chloroisocaproic acid

164 CMA – cylindrical mirror analyzer

165 CNS – central nervous system

166 CSF – cerebrospinal fluid

167 CVC6 – shRNA control vector C6 glioma cells

168 DMEM – Dulbecco's Modified Essential Medium

169 DNA – deoxyribonucleic acid

170 ECM – extracellular matrix

171 ELISA – enzyme-linked immunosorbent assay

172 FBS – fetal bovine serum

173 FIH – factor inhibiting HIF

174 GBM – glioblastoma multiforme

175 GFP – green fluorescent protein
176 HIF – hypoxia inducible factor
177 HPH – HIF prolyl hydroxylase
178 HSD – honest significant difference
179 KIC – α -ketoisocaproic acid
180 LAT – L-type amino acid transporter
181 MSUD- maple-syrup urine disease
182 mTOR – mammalian target of rapamycin
183 ODD – oxygen-dependent degradation domain
184 PBS- phosphate buffered saline
185 PDC – pyruvate dehydrogenase complex
186 PDK – pyruvate dehydrogenase complex kinase
187 PHD – prolyl-hydroxylase domain
188 PLSD – protected least squared difference
189 RNA – ribonucleic acid
190 S6R – S6 ribosomal protein
191 SDS – sodium dodecyl sulfate
192 shK – BDK shRNA C6 knockdown cells
193 SPSS – statistical package for the social sciences
194 TCA – tricarboxylic acid
195 tRNA – transfer RNA
196 VEGF – vascular endothelial growth factor
197

Introduction

Background

Branched-chain amino acids (BCAAs: leucine, isoleucine, and valine) are essential components of many biochemical and biological processes. Unlike many amino acids, humans are unable to synthesize BCAAs *de novo* and must obtain them through dietary consumption. This fact is important when one realizes the many different roles the BCAAs play in human physiology; some well established pathways involving BCAAs are fatty acid synthesis and oxidation, anabolic use to synthesize new proteins, and anaplerotic use to generate or sustain metabolic intermediate molecules. These functions for BCAAs are the oldest and most widely known, which we define as “classical” pathways here. Recently, new “non-classical” roles for BCAAs have been discovered, most notably for leucine. As stated above, BCAAs are utilized, like other amino acids, as building blocks for protein synthesis. When there are insufficient amounts of BCAAs available for protein synthesis, functional proteins are unable to be synthesized. Until recently it was unclear how BCAA limitation effectively terminated protein synthesis. It was discovered that leucine acts to initiate the congregation of proteins necessary for protein synthesis, collectively referred to as ‘protein translational machinery’ in this work, by interaction with multiple signal transduction and regulatory molecules (Kimball and Jefferson 2006). Along the same line of research, it was discovered that leucine interaction with signal transduction molecules occurs in the hypothalamus of rats to increase protein translation locally, and also to decrease feeding behaviors (Cota and others 2006). Both actions were inhibited by blocking one of the signaling molecules,

221 mammalian target of rapamycin (mTOR), downstream of leucine. It becomes evident
222 from this prior work that leucine and the other BCAAs are important for *both*
223 biochemical and molecular biological functions. We became interested in both
224 aspects of BCAA function (ie, as metabolic and signaling, or signal initiation,
225 molecules).

226 *Rationale and Hypotheses*

227 Normal cells grow, perform necessary and regulated functions based on
228 expression of an appropriate set of genes, then die. Senescence and apoptosis are
229 mechanisms through which the body regulates its own function. However, when
230 genetic mutation leads to dysregulation of the cell cycle, the cell can lose its ability to
231 self-regulate its growth cycle. These new, abnormal cells outlive normal cells and
232 begin to populate tissue or an organ at a higher rate than normals cells, which can
233 cause cancer. Half of all men and one third of all women in the US will be diagnosed
234 with cancer within their lifetimes. Cancers can begin in many different parts of the
235 body, but different types of cancer have varying biochemical and cytological
236 characteristics. For example, lung cancer and breast cancer are very different
237 diseases. They grow at different rates and respond to different treatments. That's
238 why people with cancer need treatment that has shown to be effective for their
239 particular type of cancer. While there have been great advances in targeted
240 chemotherapy, investigations into basic biochemistry have led to exciting results that
241 could have implications for many, if not all, types of cancers. If a novel molecular
242 target can be identified that can change the way cells behave on a fundamental,

biochemical level, it could bring new insight into the therapeutic strategy against cancers.

Recently, our lab had identified pyruvate dehydrogenase complex (PDC) as one such metabolic enzyme whose activity could predictably and consistently be correlated to cancer phenotypes. In this work, we propose the branched-chain α -ketoacid dehydrogenase complex (BCKDC) as another metabolic enzyme target whose substrates and products act as signaling molecules and whose activity can be altered to change cancer phenotypes. Our hypotheses were:

1. α -ketoisocaproic acid, a substrate of the BCKDC and deaminated product of leucine, can act to decrease HIF degradation cycle activity thereby promoting neoplastic activity in an *in vitro* model of CNS cancer
2. cells genetically modified by small hairpin RNA for the BCKDC kinase will increase BCKDC activity and indicate a reciprocal relationship between BCKDC activity and cellular aggressiveness (eg, proliferation, migration, invasion, colony formation) in an *in vitro* model of CNS cancer.

Survival: The Essence of Evolution

Adaptability is the essence of evolution. Throughout the development of our modern cell, the environment has exerted pressure which allowed the emergence of advantageous traits. These changes result in progeny that are more adaptable to environmental stress. There have been hundreds of survival mechanisms discovered in the mammalian cell. Survival mechanisms also entail senescence and apoptosis, since an aberrant cell can lead to widespread dysregulation and system failure or death. When these mechanisms function normally, a cell is able to

withstand a certain threshold of stress greater than the cell would normally allow. However, when one of these mechanisms is dysregulated, the effects can be severe. Perhaps the most common life-threatening disease caused by mutations to genes encoding for regulatory proteins is cancer. Among the causes of cancer are: mutations to the so-called “tumor suppressor” genes; to the genes encoding for apoptotic pathway proteins; and to the genes encoding for cell cycle regulatory proteins. But cancer as an *in vitro* cellular phenotype and as a clinical disease is distinctly different. Much like a square is a rectangle but a rectangle is not a square, *in vitro* cancer is uncontrolled proliferation but this uncontrolled proliferation does not necessarily constitute a clinical diagnosis of cancer. Cancer disease is defined by Hanahan and Weinberg (2000) as having six hallmarks: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). Recent advances have determined that there are a handful of molecules involved in many, if not all, of these phenomena seen in cancer. One such protein is hypoxia inducible factor (HIF), an evolutionarily conserved heterodimeric transcription factor.

Another commonly observed characteristic of cancer cells, that some consider the seventh hallmark of cancer, is altered glucose metabolism, which is characterized by high levels of lactate in normal oxygen conditions when compared to normal tissue. HIF is known to regulate over 100 genes encoding for growth factors, metabolic enzymes, and signaling molecules, though its evolutionary roles

has been to respond to hypoxic, or low oxygen environments. The survival response induces, in particular, a rapid switch to glycolysis and subsequent angiogenesis.

HIF biology

The hypoxic response involves an evolutionarily conserved protein known to be regulated primarily by oxygen concentrations. HIF is a protein involved in signaling survival mechanisms in cells under hypoxic, or low oxygen conditions (Goldberg and others 1988; Semenza and others 1991). HIF is a heterodimeric transcriptional regulator that upregulates the expression of over 100 genes, including glycolytic enzymes and vascular growth factors (for review (Ke and Costa 2006)). Recent estimates using DNA microarray analyses indicate that as much as 2% of human genes in arterial endothelia are regulated by HIF-1 (Manalo and others 2005). HIF-1 was discovered by the identification of a hypoxia response element (HRE) present in the gene encoding for erythropoietin, the primary factor involved in the proliferation of red blood cells (Goldberg and others 1988; Semenza and others 1991). The active HIF complex is a heterodimer comprised of an α and a β subunit. The α -subunit of HIF is constitutively degraded under normoxia, or normal oxygen conditions, by the HIF prolyl hydroxylase (HPH, or alternatively prolyl hydroxylase domain proteins or PHDs) enzymatic cycle. The HPH enzymes affix an –OH group to proline-402 or -564 resulting in ubiquitin-mediated degradation of the subunit (Masson and others 2001; Srinivas and others 1999). In hypoxic conditions, the availability of oxygen becomes a limiting factor that reduces the constitutive hydroxylation of HIF- α necessary for targeted degradation. As a result, HIF- α levels are stabilized which enables association with HIF- β , a generic transcription cohort

(Wang and others 1995). Once HIF- α/β have dimerized, the HIF complex translocates to the nucleus to associate with other transcription factors for targeted transcriptional upregulation. Another regulatory protein, factor inhibiting HIF (FIH), hydroxylates an aspartate residue in the HIF- α subunit in the nucleus in normoxic conditions. This hydroxylation prevents association with p300, an essential interaction for transcriptional activation, which inhibits hypoxia-induced gene expression (Dalgard and others 2004; Lando and others 2002a; Lando and others 2002b).

The HRE sequence previously discussed provided for the discovery of HIF (Goldberg and others 1988; Semenza and others 1991). Genes with an imbedded HRE sequence (5'-RCGTG-3')¹ are targeted by HIF upon activation, primarily by hypoxia (although other factors have been shown to activate HIF [for review (Ke and Costa 2006)]). One such encoding sequence is the VEGF gene (Levy and others 1995).

Transcriptional activation of biochemical molecules (ie, glycolytic enzymes, metabolic regulators)

As stated in the previous section, HIF activation has been shown to upregulate the expression of over 100 transcripts (for review (Ke and Costa 2006)). Not only are molecular alterations necessary for protection and survival, but metabolism must also be affected in order to sustain physiological function. HIF is a known regulator of enzymes involved in glycolysis. Glucose transporter-1 and -3

¹ R = G or A

(Chen and others 2001), lactate dehydrogenase-A (Semenza and others 1996), glyceraldehyde phosphate dehydrogenase (Graven and others 1999), hexokinase-1 and 2 (Mathupala and others 2001), and pyruvate dehydrogenase kinase (PDK)-1 (Kim and others 2006; Papandreou and others 2006) and -3 (Denko and others 2003) are just some in the long list of glycolytic enzymes that have been shown to have increased expression in response to hypoxia and HIF activation. In normal conditions, glucose transporters import glucose into the cytosol where it undergoes glycolytic metabolism to produce pyruvate. Pyruvate can then be converted to lactate or transported into the mitochondria. In the presence of oxygen, most cells further degrade pyruvate through PDC, the committed step providing entry into the TCA cycle. The cell may then further catabolize pyruvate to enable oxidative phosphorylation and increased energy production. Full oxidation of carbons from glucose through oxidative phosphorylation generates a 17-fold increase in adenosine tri-phosphate (ATP) levels compared to anaerobic pathways. In 1857 Louis Pasteur recognized that in the presence of oxygen, cells metabolize glucose via cellular respiration in the mitochondria; however, in hypoxic environments, cells undergo fermentation. This is called the “Pasteur Effect” [(Pasteur 1857; Pasteur 1859) (reviewed in English (Racker 1974))]. Recent understandings of molecular biology and biochemistry indicate that in response to HIF activation, PDK is upregulated. PDK inhibits PDC activity by phosphorylation at three serine sites. PDC inhibition drives fermentation, the phenomenon identified over a century and a half ago. HIF activation and alterations to glucose metabolism have subsequently been shown in many pathologic states, including cancer.

HIF and the Warburg Effect

In 1924 Otto Warburg observed that cancer cells preferentially engaged glycolytic metabolism and terminated at the conversion of pyruvate to lactate (Warburg 1930; Warburg 1956). This occurred in the presence of oxygen, the opposite of the Pasteur Effect previously discussed where normal cells metabolize glucose through the TCA cycle and promote mitochondrial respiration under these conditions. The production of lactate via glycolysis in normoxic conditions has since been dubbed the “Warburg Effect”, also known as “aerobic glycolysis” in the literature, and is less efficient at producing ATP than complete metabolism through the TCA cycle and oxidative phosphorylation. This metabolic phenotype is commonly seen in cancer cells, as well as HIF activation in normoxic conditions (Kim and others 2007; Koukourakis and others 2005; McFate and others 2008; Robey and others 2005; Semenza 2007).

Our lab has recently shown that decreasing the lactate production by knocking down PDK translation via constitutively expressed small inhibitory hairpin RNA (shRNA) causes a shift from aerobic glycolysis (the “Warburg Effect”) to full oxidation of glucose and results in decreased metastatic qualities in tumor cell lines (McFate and others 2008). Therefore manipulation of a key metabolic pathway may be able to change metastatic phenotypes in cancerous tissues, thus identifying possible new therapeutic targets.

Cancer hypermetabolism

A diagnosis of cancer is reserved for uncontrolled cell growth with metastatic phenotypes like migration, invasion and metastasis into surrounding tissues

(Hanahan and Weinberg 2000). CNS cancers are among the most invasive and deadly observed. One type of CNS cancer known as glioblastoma multiforme (GBM) is an astrocyte-derived grade 4 cancer that results in death on average from six to 12 months following diagnosis (Burton and Prados 2000; Miller and Perry 2007; Visted and others 2003). GBMs are notoriously resistant to therapeutic intervention and, due to the compacted and highly specific organization of the brain, are difficult to fully extract through surgical procedures (Burton and Prados 2000; Miller and Perry 2007; Nieder and others 2005; Terzis and others 2006). GBMs do metastasize and become increasingly deadly, though this metastatic event is rarely observed (Medhkour and Chan 2005; Mujic and others 2006; Newton and others 1992; Saad and others 2007). The aggressive nature of GBMs also makes it difficult to identify and treat these diseased tissues before the cancer becomes established or metastasizes (Aldape and others 2003; Nieder and others 2005; Terzis and others 2006).

The metastatic, metabolic, and proliferative qualities of GBMs exert a high demand of resources on surrounding CNS tissues. These qualities also cause healthy tissue to become necrotic, engulfed and degraded, or compacted leading to dysfunction (Burton and Prados 2000; Miller and Perry 2007). These cancerous phenotypes are also studied *in vitro* through cell culture and xenograph studies to better understand the basic biochemistry and cellular biology of GBMs. One such experimental model is the C6 glioma rat cell line, derived in the late 1960s by exposure of rat astrocytes to N,N'-nitroso-methylurea (Benda and others 1968). In a review article of *in vitro* experimental models of cancer, data on C6 glioma cells were

compiled and analyzed as a model closely associated to GBMs with respect to:
morphology; adhesion protein expression and activity; metastatic behaviors; and
biochemistry (Grobbs and others 2002). It is for these reasons that the studies
described in this work uses genetic transformations in C6 cells.

Branched-chain amino acids in cancer

Cancerous tissues are in a state of hypermetabolism due to increased
catabolic and anabolic demands for producing double the amounts of energy,
protein, and lipids for daughter cells. While glucose metabolism is altered in
cancerous tissues [the theme of my colleague's work (McFate and others 2008)],
other essential metabolic and physiologic pathways are also dysregulated in
cancerous diseases. Branched-chain amino acids (BCAAs) are a subset of essential
amino acids, categorized as such due to human inability to synthesize these basic
building blocks *de novo*. Given their multipotent roles in catabolism, anaplerosis,
anabolism, and signaling, it may be no surprise that a disease such as cancer can
have direct and indirect effects on BCAA pathways.

Free amino acids circulate throughout the body and also accumulate in small
pools in the intracellular environment. Hypermetabolic cancerous tissue can deplete
the human body of these essential amino acids. Although BCAAs are involved in
many pathways, both catabolic and anabolic, studies have shown that tumor-bearing
physiology drives hypoanabolism and hypercatabolism of the skeletal muscle in
animal models (Lorite and others 1997; Strelkov and others 1989). When this occurs
in humans with cancer, called cachexia, muscle wasting is often severe (Lundholm

and others 1976). Likewise, the breakdown of BCAA-rich skeletal muscle in the tumor-bearing state is documented in both the clinical setting (Hunter and others 1989; Inculet and others 1987; Inui 2002; O'Keefe and others 1990) and animal models (Baracos and Mackenzie 2006; Tessitore and others 1993; Whitehouse and others 2001). While the breakdown of skeletal muscle elevates BCAA levels for use in catabolism, the general health of the organism is impaired making resilience and recovery to therapy immensely difficult.

The tumor-bearing state is taxing to the organism, and metabolism is elevated in cancers. BCAAs are estimated to contribute 2-5% of total energy production in normal tissues, while they compose almost 25% of new proteins (Harper 1989). The precise pathways utilized in the cancerous or tumor-bearing state are unclear. However, where cachexia is a resultant influence of the cancerous tissue on the organism, the cancerous tissue itself requires the use of BCAAs for catabolic, anaplerotic, and anabolic pathways. The additional necessity of anabolism in these tissues makes them our primary target for metabolic manipulation. The proliferative and invasive phenotypes of some cancers require that BCAAs are supplied for the production of new proteins to build daughter cells upon division.

Branched-chain Amino Acids

BCAAs are important to many physiologic processes and have unique qualities, particularly to the human metabolic and molecular biological systems. They belong to a group of amino acids called essential amino acids that cannot be

synthesized in humans², which indicates that they must be obtained by dietary consumption. Once introduced into the physiological system of humans, the essential amino acids are shuttled and shunted to various pathways; the BCAAs are no different. Due to recent discoveries, it may be beneficial to make a distinction between the classical and non-classical understandings of BCAAs where the classical refers to metabolic pathways and the non-classical refers to signaling pathways.

Metabolism through the BCKDC

BCAAs largely bypass hepatic metabolism and are circulated throughout the body. Once BCAAs are within the cell membrane, they can proceed through many different metabolic fates; however, they must first be deaminated and/or decarboxylated in order to enter the biochemical system. Amine groups may be transferred between the carbon skeletons of amino acids by a number of aminotransferases that usually operate at or near equilibrium. There are two isotypes of branched-chain amino acid transaminases (BCAT); one is localized to mitochondria (BCATm) and is widely expressed in most tissues, and one is localized to the cytosol (BCATc) and selectively expressed in the brain, ovary, and placenta (Bixel and others 1997; Hall and others 1993; Hutson and Hall 1993; Hutson and others 1992; Ogawa and others 1970). The kinetic constants for both species are similar, with the K_M values for leucine (1–1.3mmole/L) and α -ketoglutarate (~0.6 mmole/L) exceeding the endogenous brain concentration of either substrate (~0.2 mmole/L) (Erecinska and others 1984). Deamination liberates an amine group that

² other essential amino acids are lysine, methionine, phenylalanine, threonine and tryptophan

can be used in classical processes, such as urea synthesis, and non-classical processes, which are discussed in detail later. The resulting deaminated BCAA is a carboxylic acid, branched-chain α -keto acid (BCKA). The BCAT enzyme operates on LeChatlier's principle of equilibrium since deamination is an easily reversible process. As such, the decarboxylation of the BCKAs is the most important step for their catabolism in humans (reviewed in (Harper 1989; Harper and others 1984)).

Decarboxylation occurs through the BCKDC. This complex belongs to a family of three dehydrogenase complexes including pyruvate dehydrogenase complex (PDC) and 2-oxo-glutarate dehydrogenase complex. These dehydrogenase complexes share the same basic structure, perform the same basic reactions, and all require the same set of cofactors: thiamine pyrophosphate, FAD, NAD, lipoate, and coenzyme A (CoA). The BCKDC is organized around a cubic core consisting of 24 lipoate-bearing dihydrolipoyl transacylase (E2) subunits, associated with the branched-chain α -keto acid decarboxylase/dehydrogenase (E1), dihydrolipoamide dehydrogenase (E3), BCKDC kinase (BDK), and BCKDC phosphatase (BDP). The E1 is formed by the α and β proteins interacting to form the two most important structures of the complex, the binding pocket for BCKAs and a crucial exposed Ser293 residue. Phosphorylation status of Ser293 indicates the activity state of BCKDC and is targeted by a specific kinase (Popov and others 1992; Shimomura and others 1990) and a phosphatase (Damuni and others 1984; Damuni and Reed 1987), although recent evidence has suggested isolation of another phosphatase (Joshi and others 2007). It has been established that the most potent inhibitor of BDK is the endogenous transamination product of leucine, KIC, indicating

that these metabolites promote their own metabolism. Decarboxylation of the BCKAs is the committed step in the catabolism of these molecules. After processing through the BCKDC, the metabolic products can be converted to acetyl-CoA and degraded for energy production (catabolism), used for fatty acid synthesis and energy storage (anabolism), or converted into intermediates to sustain biochemical pathways (anaplerosis). The combination of BCAA transport, BCAT reversible deamination of BCAAs, and the decarboxylation of BCKAs through BCKDC justify the supposition that BCKDC activity and intracellular concentrations of BCAAs and BCKAs govern the direction of BCAT activity.

Complete catabolism of BCAAs and BCKAs occurs only part of the time. Once BCKAs have proceeded through the BCKDC, they result in ubiquitously produced small molecules involved in various metabolic pathways, such as gluconeogenesis, the citric acid cycle, and fatty acid synthesis. However, BCAAs and BCKAs are involved in alternate pathways prior to catabolism through BCKDC.

New signaling roles have emerged (the comprehensive model)

In contrast to the classical understanding of BCAAs as energy substrates, new discoveries have made way for an emergent non-classical signaling paradigm. A few notable examples occur in the CNS where BCAAs are transported across the blood-brain barrier by the LAT1 and 2 transporters. Once inside the neurochemical milieu, BCAAs are used for glutamate homeostasis. One-third of all amine groups incorporated into glutamate, the brain's primary excitatory neurotransmitter, were found to originate in BCAAs; leucine alone supplies 30-50% to both glutamate and

glutamine, an important glutamate precursor and biochemical shuttling molecule (Kanamori and others 1998; Yudkoff and others 1990; Yudkoff and others 1983).

Although BCAA biochemistry is extensive, in both its nature and the study of the different pathways, there are also signaling pathways involving the BCAAs. The BCAAs leucine and valine are known to play important roles in the regulation of protein synthesis through interactions with translational initiation factors, including the mammalian target of rapamycin (mTOR), although leucine has been shown to be the most potent in stimulating the mTOR pathway (Figure 1) (Anthony and others 2000a; Kimball and Jefferson 2006; Lynch and others 2003). mTOR is a global regulator of cellular and molecular processes concerning cell survival. Briefly, activation of mTOR, a protein kinase, begins a cascade of events promote protein translation, such as: phosphorylation of 4E-binding protein 1 (4E-BP1) for the release of eukaryotic initiation factor-4E (eIF-4E) (Brunn and others 1997; Kimball and others 1996; Xu and others 1998); phosphorylation of S6 ribosomal protein kinase (S6RK) to activate downstream targets (Kimball and others 1999; Long and others 2000); and ribosomal biogenesis, although this is still considered a hypothesis by many which is refuted by some (Stolovich and others 2002; Tang and others 2001).

The most recent data detailing leucine's non-classical signaling role has been presented which details a further role of leucine-mTOR signaling in the hypothalamus (Cota and others 2006). Cota *et al* (2006) reported that leucine, acting through mTOR and other translational regulators, was able to initiate signaling to

regulate fuel availability and usage via the arcuate and paraventricular nuclei in the rat brain. These nuclei are located near the medial eminence, a circumventricular organ in the brain with a direct “window” to the blood supply. Such gaps in the blood-brain barrier allow the careful monitoring and regulation of metabolites and hormones. As a result, hypothalamic signaling can occur adjusted to dynamic systemic needs based on feeding and fasting. The authors found that central administration of leucine resulted in increased hypothalamic mTOR signaling and decreased food intake and body weight (Cota and others 2006). This study further elaborated on leucine’s importance as a metabolic signaling molecule.

Summary and Segue

In light of the previously defined complex nature of BCAAs in physiological systems, we examined the role of BCAAs in HIF biology and cancer biochemistry. Our research questions were: what is the effect of increased BCAA levels in an organism with cancer (seen in rats and humans); and is there a greater role for BCAAs in cancer metabolism, especially in light of the fact that PDC is very clearly a metabolic switch? As previously stated, BCAA transaminated products (BCKAs) have been shown to stabilize HIF at high *in vitro* levels. Our first question is concerned with what occurs when BCAAs and BCKAs are present at lower levels and if HIF activation is robust enough to drive HIF-targeted transcriptional upregulation. Our second question is concerned with the hypermetabolic state present in cancer cells and if BCKDC can be manipulated in order to cause significant changes to cancer cell phenotypes.

Branched-chain α -ketoacids increase HIF-1 signaling independently of mTOR

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Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain α -

ketoacid; BCKDC, branched-chain α -ketoacid dehydrogenase complex; BCKDH,

branched-chain α -ketoacid dehydrogenase; BDK, BCKDH kinase; GFP, green

fluorescent protein; FIH, factor inhibiting HIF; HIF, hypoxia inducible factor; KIC, 2-

ketoisocaproate; KIV, 2-ketoisovalerate; KMV, 2-keto-3-methyl valerate; mTOR,

mammalian target of rapamycin; ODD, oxygen-dependent degradation domain;

PDH, pyruvate dehydrogenase; PHD, prolyl hydroxylase domain proteins; S6R, S6 ribosomal protein; VEGF, vascular endothelial growth factor

Summary

Branched-chain amino acids and ketoacids participate in several biochemical pathways and are emerging as novel signaling molecules. Leucine activates the mammalian target of rapamycin (mTOR) kinase, while its deaminated metabolite alpha-ketoisocaproic acid (KIC) can stabilize the hypoxia-inducible transcription factor, HIF-1. Since mTOR has been implicated in HIF-1 stabilization, we investigated whether the ability of KIC to stabilize HIF-1 involved mTOR activity. In rat C6 glioma cells, KIC treatment promoted the accumulation of HIF-1 α as well as a stably transfected green fluorescent protein containing the HIF-1 α oxygen-dependent degradation domain. Inhibition of the HIF-1 decay mechanism by KIC was correlated with increased VEGF secretion by C6 cells. KIC also increased phosphorylation of the mTOR target protein S6R. However, while rapamycin treatment inhibited KIC-induced S6R phosphorylation, it did not affect KIC-induced HIF-1 stabilization or VEGF secretion. Instead, these rapamycin-insensitive KIC effects were selectively reversed by ascorbate, a cofactor required by the proly-hydroxylase domain proteins, which control HIF-1 decay. KIC also promoted ascorbate sensitive HIF-1 α accumulation and VEGF secretion in primary rat astrocytes. These results suggest that KIC may reversibly inactivate proly-hydroxylase domain proteins independently of mTOR activity.

Introduction

The branched-chain amino acids (BCAAs: leucine, isoleucine, and valine), whose side chains contain a branched methyl group, are essential amino acids known to be involved in several biochemical pathways. Leucine, isoleucine, and valine are deaminated by a specific branched chain amino acid transferase to their respective branched chain α -ketoacids (BCKAs) 2-ketoisocaproate (KIC), 2-ketoisovalerate (KIV), and 2-keto-3-methylvalerate (KMV) (Hutson and others 1988). BCKAs are then catabolized through the mitochondrial branched-chain α -keto acid dehydrogenase complex (BCKDC), which is composed of multiple subunits, including the regulatory branched chain ketoacid dehydrogenase E1- α subunit (BCKDH- α) (Danner and others 1978; Parker and Randle 1978; Pettit and others 1978; Roberts and Sokatch 1978). BCKDC is regulated by reversible inhibitory phosphorylation of BCKDH- α through the actions of a branched chain dehydrogenase kinase (BDK) (Popov and others 1992; Shimomura and others 1990) and an unidentified phosphatase (Damuni and others 1984; Damuni and Reed 1987). The catabolism of BCAAs via the transaminase and the BCKDC generates acyl-CoA intermediates which undergo dehydrogenation. Ultimately, leucine is converted to acetyl-CoA and acetoacetate; isoleucine to acetyl-CoA and succinyl-CoA; and valine to succinyl-CoA. In some tissues, these final products can be fully oxidized via the citric acid cycle, while in others these are directed toward the synthesis of ketone bodies (acetoacetate and acetyl-CoA) and glucose (succinyl-CoA) (Greenberg and Reaven 1966; Noda and Ichihara 1974; Noda and Ichihara 1976).

623 BCAAs are also used as anabolic building blocks for de novo protein
624 synthesis. Whereas all three BCAAs serve equally prominent roles in these
625 biochemical pathways, leucine has emerged as an important signaling molecule as
626 well. Leucine has been shown to stimulate protein synthesis via mobilization of the
627 protein translation machinery through activation of mammalian target for drug
628 rapamycin (mTOR) activity (Kimball and others 1999). Leucine also promotes insulin
629 synthesis and secretion (Lambert and others 1986), and inhibits autophagy (Mordier
630 and others 2000). In biochemical pathways restricted to central nervous system
631 tissue leucine plays an important role in maintaining homeostasis of glutamate, the
632 brain's major excitatory and most abundant neurotransmitter (Yudkoff and others
633 1996; Yudkoff and others 1990; Yudkoff and others 1983). More recently, signaling
634 actions of leucine in the hypothalamus have been implicated in the regulation of
635 feeding behavior (Cota and others 2006).

636 Accumulation of BCAAs and BCKAs has also long been known to contribute
637 to the pathogenesis of BCKDC deficiency, which is commonly known as maple
638 syrup urine disease (Dancis and others 1960). Symptoms of this condition begin in
639 early infancy and include poor feeding, vomiting, dehydration, lethargy, hypotonia,
640 seizures, ketoacidosis, and neurological decline (Chuang and others 2006).
641 However, the pathophysiology underlying the nervous system effects remains
642 unclear and has not been linked to the signaling roles identified for BCAAs. A better
643 appreciation of BCAA signaling roles may thus help clarify the regulation of
644 important physiological and pathological processes.

645 Novel signaling actions of many other metabolic intermediates have also
646 become elucidated in recent years. Glycolytic metabolites and tricarboxylic acid
647 (TCA) cycle intermediates have also been shown to promote insulin secretion
648 (MacDonald and others 1989), regulate hypothalamic hunger signals (Lam and
649 others 2005), induce angiogenesis (Murray and Wilson 2001), and promote
650 stabilization of the hypoxia inducible transcription factor HIF-1 (Isaacs and others
651 2005; Lu and others 2005; Lu and others 2002; Pollard and others 2005; Selak and
652 others 2005). Cell survival in hypoxic environments is critically dependent upon HIF-
653 1 in both normal and neoplastic tissues (Dalgard and others 2004; Lu and others
654 2005; Lu and others 2002). Our recent study evaluating the effect of BCAAs on HIF-
655 1 α stabilization showed that BCKAs could also stabilize HIF-1 α levels (Lu and others
656 2005).

657 Under normal oxygen conditions (or normoxia) HIF- α is constitutively
658 synthesized but rapidly degraded by specific HIF prolyl hydroxylases, referred to as
659 prolyl hydroxylase domain proteins 1-3 (PHD 1-3) (Wang and others 1995). The
660 PHD enzymes require the cofactors iron, ascorbate (Knowles and others 2003) and
661 the tricarboxylic acid [TCA] cycle intermediate α -ketoglutarate (Bruick and McKnight
662 2001; Ivan and others 2001; Jaakkola and others 2001), for sustained enzymatic
663 activity. PHDs transfer hydroxyl (-OH) groups derived from dissolved O₂ onto two
664 proline residues located in the oxygen-dependent degradation (ODD) domain of the
665 HIF- α protein (Hon and others 2002; Masson and others 2001; Min and others 2002;
666 Srinivas and others 1999). This oxygen dependent post-translational modification
667 acts as a recognition signal for the ubiquitin-mediated degradation of HIF- α subunits.

In the absence of atmospheric oxygen, HIF- α protein levels increase, dimerize with HIF- β (Gradin and others 1996; Kallio and others 1997; Wood and others 1996), translocate to the nucleus, and activate transcription of many genes that promote hypoxic survival. Another HIF- α hydroxylase known as the factor inhibiting HIF (FIH) governs the hydroxylation of an asparagine residue on HIF- α (Hewitson and others 2002; Lando and others 2002a; Lando and others 2002b; Sang and others 2002), thus regulating its association with other transcriptional cofactors.

Along with hypoxia, PHD enzyme activity can be inhibited by reducing the interaction of cofactors with the enzyme. Thus, iron chelators, α -ketoglutarate analogues (Bruick and McKnight 2001; Ivan and others 2001; Jaakkola and others 2001), and ascorbate deficiency (Knowles and others 2003) can blunt PHD activity and enhance HIF-1 accumulation. In fact, the effect of on BCKAs on HIF-1 stabilization was discovered through a screen of biological α -ketoacids with structural similarity to α -ketoglutarate (Lu and others 2005). However, given the wider emerging signaling roles of BCAAs, it is possible that the BCKA effect we observed on HIF-1 also involved other pathways. In particular, mTOR has been shown to be both an upstream regulator of HIF signaling in cancer cells (Hudson and others 2002) and a regulatory target potentially influenced by leucine (Anthony and others 2000b). Thus it remains unknown whether the BCKA effect on HIF-1 regulation is mediated selectively via the PHDs or via mTOR. Given the potential widespread impact of α -ketoacid signaling mechanisms, the present study further investigated the role of BCKAs (and BCAAs) in the stabilization of HIF- α to clarify this question.

691

692 **Experimental Methods**

693 All chemicals were purchased from Sigma-Aldrich and cell culture products were
694 purchased from GIBCO, unless otherwise stated.

695 **Transgenic ODD-GFP C6 Glioma Cell Line**

696 The generation of the ODD-GFP C6 glioma cell line has been previously
697 described (D'Angelo and others 2003). The ODD is the portion of the HIF-1 α protein
698 that is hydroxylated and subsequently degraded by the ubiquitin ligase molecular
699 machinery. Fusion of the HIF-1 α ODD to a GFP molecule allowed GFP
700 immunodetection to serve as a probe for the PHD mediated degradation of HIF-1 α .

701 **Cell culture and Chemical Treatments**

702 Cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Invitrogen)
703 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and
704 1.5 mg/mL G418 (Gibco). G418 was used to select transgenically altered cells. Cell
705 lines were maintained in 21% O₂, 5% CO₂, and 74% N₂ in a humidified cell incubator
706 at 37°C. Chemical treatments were performed in Krebs Saline Buffer and incubation
707 times are as indicated. Each chemical treatment was performed by dilution from
708 100X stock in Krebs saline buffer. For cell hypoxia treatment, the culture dishes
709 were sealed in a modular incubator chamber, flushed with gas containing 1% O₂,
710 5%CO₂, and 94% N₂ for 5 min, and incubated in this environment at 37°C for the
711 indicated times.

712 **Primary Astrocyte-enriched Cell Cultures**

713 Primary cultures from derived from rat postnatal day 2 cerebral cortex were
714 prepared as described (Armstrong 1998). Briefly, brains were surgically extracted,
715 digested with protease, and plated in poly-D-lysine coated tissue culture flasks.
716 Cultures were maintained in DMEM with 10% fetal bovine serum (FBS): DMEM (Life
717 Technologies) supplemented with 1 mM sodium pyruvate (Sigma) and 25 mg/ml
718 gentamicin (Life Technologies). Following 10 days in culture the flasks were placed
719 on a rotary shaker (190rpm) for 18h to dislodge immature oligodendrocyte lineage
720 cells and microglia. The remaining cells were astrocyte-enriched populations.
721 Astrocyte cultures were refreshed every 72h with DMEM medium containing 10%
722 FBS supplemented with 1mM sodium pyruvate and 25mg/ml gentamicin.

723 **Western Blot and Densitometry Analyses**

724 Cells were washed three times with cold PBS. Appropriate amount of lysis
725 buffer containing Radio Immuno Precipitant Assay (RIPA) buffer (Tris-HCL, pH 7.4
726 [50mM], NaCl [150mM], NP-40 [1%], Sodium deoxycholate [0.5%], SDS [0.1%], and
727 EDTA [5mM]; Bioworld), 1% SDS, and 1X protease inhibitor cocktail (Roche) then
728 scraped. Cell remnants were then collected in 1.5 ml microcentrifuge tubes. The cell
729 material was sonicated for 25 seconds at 50 Hz, then placed in rack at room
730 temperature. Cell lysates were then spun for 5 minutes at 12,000 g and the
731 supernatants were transferred to fresh tubes. Protein levels were determined by the
732 BCA method of analysis (Pierce). Proteins were separated on 4-12% Bis-Tris SDS-
733 polyacrylamide gradient gels (Invitrogen) and transferred to nitrocellulose
734 membranes (Invitrogen). Membranes were blocked using 5% goat or horse serum
735 (Vecotr Labs) in Tris buffered saline with 0.1% Tween-20. Antibodies used were

anti-HIF-1 α 1:500 (Biossource, Novus), anti-HIF-1 β 1:1000 (Biossource, Novus), anti-phospho-Ser292-E1 α BCKDH (generous gift from C. Lynch, 1:20,000) (Lynch and others 2003), anti-E1 α BCKDH (generous gift from C. Lynch, 1:1000) (Lynch and others 2003), anti-GFP 1:1000 (Roche), anti-phospho-S6R 1:1000 (Cell Signaling), and anti- β actin 1:10,000 (AbCam). Protein bands were visualized by enhanced chemiluminescence (Pierce) using either Kodak film and developer or CCD luminescence camera (Fuji Film). Signals were quantified using densitometry in ImageJ (Wayne Rasband, NIH). Phosphoproteins and HIF-1 α were normalized to total protein amounts and HIF-1 β , respectively, unless otherwise stated. The β -actin levels of non-phosphoproteins were statistically compared. If β -actin levels differed, the data were not used in the subsequent analysis. Graphs indicate arbitrary units on the y-axes derived from signals normalized to non-treated or wild type controls by direct comparative ratios. These normalizations were necessary to control for protein loading.

Enzyme Linked Immunosorbent Assay

Cells were cultured to 100% confluency in 6-well cell culture plates. Experimental conditions were applied and the cells were incubated for 12 hours under culture conditions. 50 μ L of media was extracted and used in a Quantikine Rat VEGF (vascular endothelial growth factor) ELISA assay kit (R&D Systems Inc.) Absorbance was read at 405nm generating pg/mL VEGF protein. Cells were trypsinized and counted using the ViCell Trypan Blue automated cell counter subsequent to media extraction for ELISA. Cell numbers were used to normalize the

data to account for differential plating and proliferation confounders. Data is represented as pg VEGF protein per mL media per hundred cells.

Cell counting

Cell were washed and trypsinized for cell counting procedures for ELISA normalization. Trypsin was inactivated by adding equal amounts of media with 10% FBS. Five hundred μ L of suspensions of each preparation were then placed in a Vi-Cell Trypan Blue automated counter for cell counting.

Statistical Analyses

Statistical analyses ANOVA, Tukey's HSD and Fisher's PLSD post hoc tests were performed using STATview statistical software. Where it was necessary to compare treatment only to control groups, ANOVA and Dunnett's post hoc analyses were performed in SPSS statistical software.

Results

KIC induced HIF-1 stabilization is independent of mTOR activity

KIC can be reversibly transaminated to produce leucine, which can activate mTOR, a kinase that regulates protein translation via phosphorylation of proteins such as S6R. In some studies, mTOR activation has been implicated in increasing HIF-1 α (Hudson and others 2002) and VEGF protein levels (Humar and others 2002), suggesting that this could be one mechanism for the previously observed HIF-1 α stabilization by KIC. Alternatively, stabilization of HIF-1 α by α -ketoacids has been proposed to result from a reversible inactivation of PHD enzymes (Lu and others 2005). This conclusion is supported by the rapid reversibility of α -ketoacid mediated HIF-1 α accumulation by ascorbate and Fe²⁺ (Lu and others 2005; Lu and

others 2002). To determine which of these mechanisms is involved in KIC induced HIF-1 α accumulation we sought to determine the relative sensitivity of the KIC-induced HIF-1 α accumulation to either ascorbate or rapamycin, a well known mTOR inhibitor (Brown and others 1994; Sabatini and others 1994; Sabers and others 1995). We employed immunodetection of pSer^{235/236}S6R to assess mTOR activity and ODD-GFP stabilization to assess PHD activity.

As shown in figure 1A and 1B, when ODD-GFP transfected C6 glioma cells were treated with hypoxia (1% O₂) vs. KIC (2mM) for 3h, only KIC treatment demonstrated significant increase of pSer^{235/236}S6R immunoreactivity in cell extracts. Moreover, rapamycin treatment (0.1ng/ml) completely prevented S6R phosphorylation while ascorbate treatment (100 μ M) did not. In the same cell extracts, we also examined changes in HIF-1 α and HIF-1 β immunoreactivity. As shown in figure 1A and 1C, both hypoxia and KIC treatment led to a significant accumulation of HIF-1 α with no change in HIF-1 β . The KIC induced HIF-1 α accumulation was clearly far more sensitive to inhibition by ascorbate than by rapamycin. ODD-GFP accumulation was also stimulated by KIC and this effect was selectively inhibited by ascorbate but not by rapamycin (figure 1A, 1D).

Functional HIF-1 induced gene expression requires nuclear translocation of the HIF-1 α /HIF-1 β heterodimer and interaction with the transcriptional coactivator CBP/P300 (Arany and others 1996). This latter step is normally kept inhibited by the asparagine hydroxylase FIH (Lando and others 2002a; Lando and others 2002b; Sang and others 2002). Full gene expression also requires DNA transcription and mRNA translation to produce functional protein. In order to assess whether the KIC

induced HIF-1 α accumulation that we observed was effective in activating gene expression, we measured the media accumulation of VEGF protein, a well known target of HIF-1 α following 12h culture. As shown in figure 1E, both hypoxia and KIC significantly increased media VEGF levels and the KIC effect was selectively blunted by ascorbate and not by rapamycin. Similar results were also seen with leucine treatment (figure 1F). These results supported the notion that KIC induced HIF-1 α accumulation is mediated by reversible inactivation of PHD activity and is not significantly impacted by mTOR activation.

HIF-1 α stabilization and BCKD α phosphorylation show reciprocal dose responses to KIC

The BCKAs KIC, KIV, and KLV have been shown to increase HIF-1 α levels in normoxic conditions at high doses. At these high doses these α -ketoacids may have antioxidant and other non-specific actions. Moreover, in living cells, these ketoacids may also be rapidly metabolized through BCKDC activity. BCKDC normally regulates the BCKDC through inhibitory phosphorylation of the BCKDH α subunit at Ser292. KIC is the chief endogenous inhibitor of BCKDC, a mechanism that normally allows feed-forward activation of the BCKDC to maintain low levels of KIC. These complex fuel routing mechanisms may thus impact the selectivity of signaling functions for KIC. We therefore determined whether the state of inhibitory BCKDH α phosphorylation at Ser292, correlated with the effect of KIC on HIF-1 α stabilization. To accomplish this, C6-ODD-GFP cells were treated with concentrations of KIC ranging from 1 to 3000 μ M. The treated cells were then harvested for analysis of HIF-1 α levels and phosphorylation status of BCKDH- α Ser292. A representative

dose-response curve for KIC effect on HIF-1 α accumulation and BCKDH- α pSer292 levels via Western blotting is shown in Fig. 2A. Densitometric analysis of blots is shown in Fig. 2B with standardization to non-treated controls. Increased HIF-1 α signal relative to non-treated controls could be seen at the 10 μ M dose of KIC ($p=0.001$) and all concentrations from 30 μ M KIC to 3000 μ M KIC showed significant HIF-1 α accumulation ($p<0.0001$). Phosphorylation of BCKDH- α Ser292 is shown as the ratio of phosphorylated to total protein for internal standardization. Treatments of cells with 1, 10 and 30 μ M KIC resulted in significantly decreased phosphorylation ratios of BCKDH- α with respect to non-treated controls ($p<0.0001$) with no BCKDH- α phosphorylation being observed at higher doses. These data indicated that despite inhibition of BCKDH phosphorylation, BCKAs such as KIC can still promote HIF-1 stabilization. We did however observe a complex dose response curve for KIC in stabilizing HIF-1 which suggested the interaction of more than one process to the overall effect.

KIC and leucine induced VEGF production displays a biphasic response

Given our demonstration of the ability of KIC to participate in distinct signaling and metabolic pathways in the same cell type, we wondered whether the overall production of HIF-1 mediated gene expression by this α -ketoacid would show a linear dose response or one that was more complex. We therefore investigated the dose response of KIC in promoting an increase in media VEGF. C6-ODD-GFP cells were treated with increasing concentrations of KIC as well as leucine ranging from 100 to 5000 μ M. Positive and negative controls were also included using hypoxia (1% O₂) and vehicle-treated conditions, respectively. The treated cell media was

then harvested after 12h and analyzed by the ELISA technique. Cells were then counted with the ViCell Trypan blue automated cell counter. We found increased secretion of VEGF protein in hypoxia positive-control condition (Figs. 2C and D; $p < 0.0001$). However, the dose-response curves for KIC and leucine were bimodal (Fig. 2C and D). Thus while 0.1mM KIC produced significant increase in VEGF production, 0.5mM KIC did not. However, subsequently increasing concentrations of KIC did produce a significant increase in VEGF. A similar biphasic dose response was observed with leucine although the required effective dose range was higher than that for KIC.

KIC stabilizes HIF-1 α and promotes VEGF expression in primary astrocyte cultures

We also addressed whether the effects of KIC that we observed in the C6 glioma cell line could be observed in primary cultures of non-transformed cells. We thus treated astrocyte-enriched primary rat brain cultures with hypoxia, or with KIC [0.1 and 1.0 mM] with and without ascorbate [100 μ M]. Media from treated cells were analyzed by ELISA (Fig. 3C). Treated cells were harvested and analyzed by Western blotting for HIF-1 α and - β . A representative Western blot of analyses is shown in Fig. 3A. Densitometry of these blots is plotted in Fig. 3B. The positive-control hypoxia treatments resulted in significant increases from all other treatments ($p < 0.001$ for all comparison). Both the 0.1 mM and 1.0 mM KIC treatments resulted in increased HIF-1 α/β ratios relative to non-treated controls ($p < 0.05$ for both comparisons). The results of the two KIC alone treatments did not differ statistically from one another. The KIC treatments showed significantly increased HIF-1 α/β

ratios vs. treatment with KIC and ascorbate ($p < 0.05$ for both comparisons). The two KIC treatment doses with ascorbic acid did not differ from each other. These data indicate that KIC indeed stabilizes HIF-1 α protein levels at normoxia in normal non-neoplastic cells as well. KIC-induced, ascorbate-reversible VEGF protein expression was also observed in media taken from the primary astrocyte cultures (Fig. 3C). The non-treated controls showed much lower VEGF protein compared to the positive-control hypoxia ($p < 0.0001$) and the two doses of KIC ($p < 0.0001$). No differences were seen however between non-treated controls and either of the KIC treatments when ascorbic acid was included.

Discussion

BCAAs and BCKAs are known to be involved in multiple biochemical pathways and are also becoming appreciated for their signaling functions. Previous work from our lab identified BCKAs as being among a few naturally occurring α -ketoacids that were capable of stabilizing HIF-1 α levels (Lu and others 2005). Among the BCKAs this effect was greatest for KIC. In this report, we have elaborated on this previous work to show this action to be independent of mTOR. KIC was shown to activate signaling via mTOR as indicated by its enhancement of rapamycin sensitive S6 phosphorylation. KIC was also able to decrease phosphorylation of BCKDH α . However, the ability of KIC to promote HIF-1 α stabilization and produce VEGF elaboration appeared to result from an interference of PHD activity. This was supported by the ability of ascorbate to reverse both of these actions and also by the ability of KIC to promote ODD-GFP accumulation in an

896 ascorbate-reversible manner. The latter tool is driven by constitutive expression
897 under a CMV promoter and the protein product is degraded specifically by O₂-
898 dependent hydroxylation via the PHD enzymes (D'Angelo and others 2003).
899 Interestingly ascorbate treatment of C6 transgenic cells led to a decreased level of
900 ODD-GFP fusion protein as compared to non-treated controls (Figure 1D). This is
901 possibly due to the normal homeostatic relationship between ODD-GFP expression
902 and PHD activity which results in a basal level of protein (evidenced by the former)
903 and the addition of ascorbate may increase the PHD degradation cycle turnover
904 (evidenced by the latter), thus reducing the normal basal level to a non-detectable
905 signal.

906 In addition to these actions, BCKAs participate in transamination events,
907 possess antioxidant capabilities and also influence the bioenergetic status of cells.
908 Thus, it is not surprising that we observed unusual dose response curves for VEGF
909 elaboration by KIC and leucine. Although a clear explanation for this effect requires
910 further experiments, the differential dose dependent actions of KIC on the distinct
911 biological effects demonstrated and described above may account for this
912 phenomenon. It is possible that KIC and leucine are capable of inactivating PHDs at
913 low doses. However, BCKDC activation by higher doses of KIC due to inhibition of
914 BCKDH α phosphorylation may lower the effective concentration of KIC for
915 performing this action. Although other explanations may be possible, our results do
916 clearly demonstrate the ability of KIC to stabilize HIF-1 in living cells and show for
917 the first time the ability of KIC and leucine to promote VEGF production. This latter
918 action may have clinical relevance.

High accumulation of BCAAs and BCKAs is a hallmark of maple syrup urine disease (Dancis and others 1960). One of the most ominous presentations of this disease is cerebral edema (Brismar and others 1990; Riviello and others 1991). Our demonstration that KIC could induce HIF-1 α stabilization and VEGF elaboration in primary astrocytes suggests that this signaling mechanism may play a role in the pathogenesis of cerebral edema in maple syrup urine disease. This is because VEGF is well known to be a key contributor to edema through its action on vascular permeability (Josko and others 2000). Moreover, our demonstration that ascorbate can reverse KIC-induced VEGF elaboration from astrocytes suggests a possible simple treatment that can be tested in this clinical condition. Finally, given the prominent role of HIF-1 biology in cancer progression, our results suggest that a role for altered BCKDC biology in cancer should be further evaluated.

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Legends to Figures

Figure 1. BCAAs and BCKAs stabilize HIF-1 α in normoxia, reversible by ascorbate treatment.

A. Representative blots loaded with 20 μ g of whole-cell protein extracts from C6-ODD-GFP cells were analyzed for HIF-1 α , ODD-GFP, HIF-1 β , and phospho-S6R proteins (time=3hrs, n=4). **B, C, D.** Densitometric analyses of signals of pSer^{235/236}S6R, HIF-1 α / β , and ODD-GFP, respectively, are displayed in densitometry units normalized to non-treated controls. The specific conditions for treatments presented in the figure are hypoxia [1% O₂, 5% CO₂, and 94% N₂], KIC [2.0 mM], rapamycin [0.1 ng/mL], and ascorbate [100 μ M]. Statistical significances were determined by Tukey's HSD post hoc analysis (*p<0.05 compared to control; **p<0.001 compared to control; +p<0.05 compared to ascorbate treatment; ++p<0.001 compared to ascorbate treatment; ##p<0.001 compared to rapamycin treatment). **E, F.** C6-ODD-GFP cells were analyzed for VEGF protein by ELISA. Secreted protein was normalized to viable cell number determined by trypan blue uptake. The specific conditions for treatments presented in the figure are hypoxia [1% O₂, 5%CO₂, and 94% N₂], KIC [2.0 mM] (**E.**), leucine [2.0 mM] (**F.**), rapamycin [0.1 ng/mL], and ascorbate [100 μ M]. (time=12hrs, n=4). Statistical significances were determined by Tukey's HSD post hoc analysis (*p<0.05 compared to control; **p<0.001 compared to control; +p<0.05 compared to ascorbate treatment; ++p<0.001 compared to ascorbate treatment).

Figure 2. Dose-response for BCAAs and BCKAs on HIF-1 α level stabilization and functional roles in gene upregulation

A. Representative blots loaded with 20 μ g of whole-cell protein extracts from C6-ODD-GFP cells are shown for treatments corresponding to 0.5 increment increases on the log scale (time=3hrs, n=3). **B.** Densitometric analyses of HIF-1 α and phosphorylation status of Ser292-E1 α BCKDH. Data are normalized to non-treated control. **C.** Graph indicating non-treated control, positive control hypoxia [1% O₂, 5%CO₂, and 94% N₂], and dose-response of the BCKA KIC at 0.1, 0.5, 1.0, 2.5, and 5.0 mM (time=12hrs, n=4). **D.** Graph indicating non-treated control, hypoxia positive control, and dose-response of the BCAA leucine at 0.1, 0.5, 1.0, 2.5, and 5.0 mM (time=12hrs, n=4). Protein secretion levels shown in **C.** and **D.** are normalized to cell number. Statistical significances were determined by Dunnett's post hoc analysis (*p<0.05 compared to control; **p<0.001 compared to control).

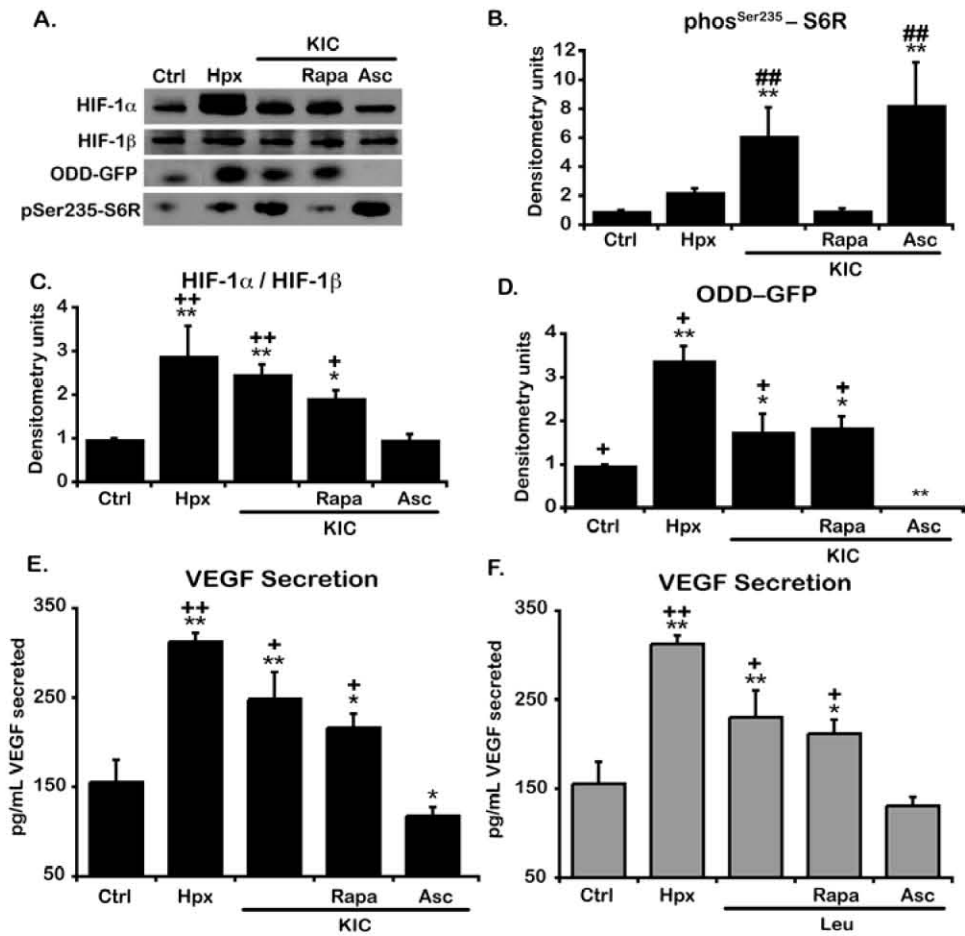
Figure 3. KIC induces HIF-1 α stabilization in primary astrocyte-enriched rat cultures, reversible by ascorbate treatment.

Representative blots loaded with 20 μ g of whole-cell protein extracts from primary astrocyte-enriched cell cultures were analyzed for HIF-1 α and HIF-1 β proteins for non-treated control, hypoxia [1% O₂, 5%CO₂, and 94% N₂], KIC [0.1 and 1.0 mM], and KIC treatments with ascorbate [0.1 mM] (time=3hrs, n=4). **B.** Densitometric analyses of HIF-1 α / β signals displayed in densitometry units normalized to non-treated controls showing statistical significances as determined by Tukey's HSD post hoc analysis (*p<0.05 compared to control; **p<0.001 compared to control; *p<0.05

compared to 0.1 mM KIC ascorbate treatment; [#]p<0.05 compared to 1.0 mM KIC with ascorbate treatment). **C.** Primary astrocyte-enriched cultures were analyzed for VEGF protein secretion by ELISA for non-treated control, hypoxia [1% O₂, 5%CO₂, and 94% N₂], KIC [0.1 and 1.0 mM], and KIC treatments with ascorbate [0.1 mM] (time=12hrs, n=4). Protein levels were normalized to cell number for each sample prior to statistical analyses. Statistical differences were determined by Tukey's HSD post hoc analysis (***p<0.0001 compared to control; ⁺⁺⁺p<0.0001 compared to 0.1mM KIC with ascorbate treatment; ^{###}p<0.0001 compared to 1.0mM KIC with ascorbate treatment).

1174 **Figures**

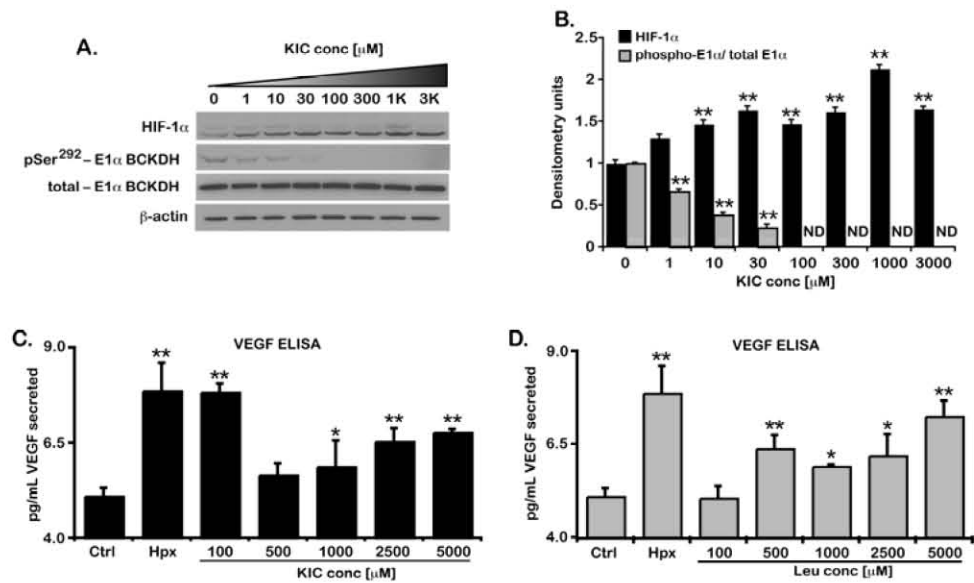
1175 **Figure 1. BCAAs and BCKAs stabilize HIF-1α in normoxia, reversible by**
1176 **ascorbate treatment**



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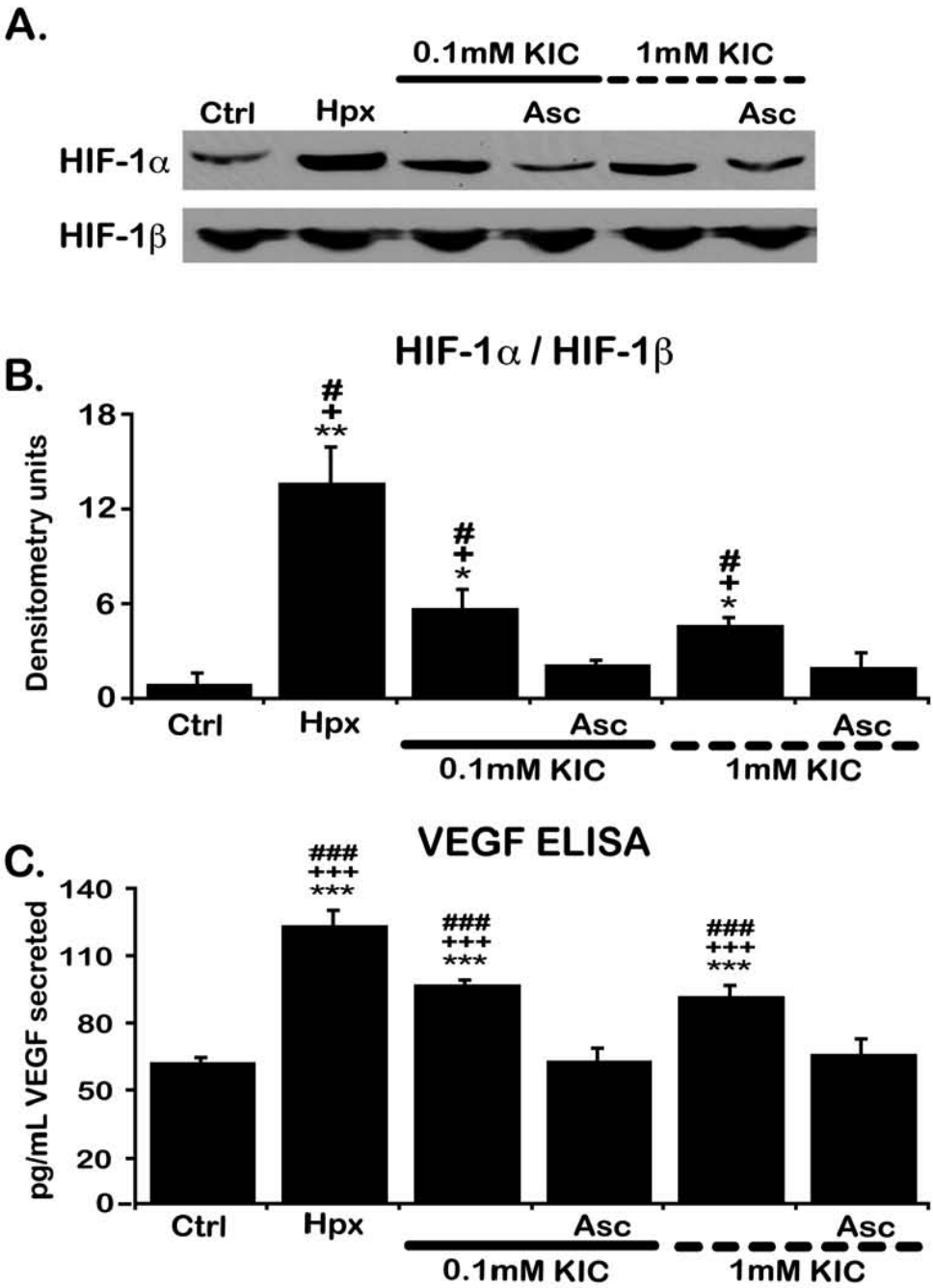
1179 **Figure 2. Dose-response for BCAAs and BCKAs on HIF-1 α level stabilization**
1180 **and functional roles in gene upregulation**



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1183 Figure 3. KIC induces HIF-1 α stabilization in primary astrocyte-enriched rat
1184 cultures, reversible by ascorbate treatment



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1188 **Alterations to branched-chain amino acid metabolism increase *in vitro***
1189 **malignant characteristics of C6 glioma cells**

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1211

1212 Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain α -
1213 ketoacid; BCKDC, branched-chain α -ketoacid dehydrogenase complex; BCKDH,
1214 branched-chain α -ketoacid dehydrogenase; BDK, BCKDH kinase; BDP, BCKDH
1215 phosphatase; HIF, hypoxia inducible factor; KIC, 2-ketoisocaproate; KIV, 2-
1216 ketoisovalerate; KMV, 2-keto-3-methyl valerate; mTOR, mammalian target of
1217 rapamycin; PDH, pyruvate dehydrogenase; PDK, PDH kinase

1218

1219 **Summary**

1220 Branched-chain amino acids (BCAAs) participate in several biochemical pathways
1221 and are emerging as novel signal-initiating molecules. BCAAs are exclusively
1222 metabolized through the branched-chain α -ketoacid dehydrogenase complex
1223 (BCKDC), a mitochondrial macroenzyme complex, which is regulated through
1224 reversible phosphorylation. Cancer cells are in a state of hyperactivity utilizing
1225 metabolic substrates for energy (catabolism), for synthesis of new molecules
1226 (anabolism), and/or replenishing pools of intermediary metabolites (anaplerosis).
1227 Here we sought to better understand the impact of BCAA metabolism on cancer
1228 cells by altering the activity state of the BCKDC. We successfully created a shRNA
1229 protein knockdown system for the BCKDC kinase (BDK) in C6 glioblastoma cells,
1230 thus preventing phosphorylation and increasing activity of BCKDC. We found BDK
1231 knockdown cells had greater proliferation, invasion, and migration rates compared
1232 with control vector and wild type cell lines. We also found an increased lactate
1233 output for the BDK knockdown cells and pyruvate dehydrogenase complex (PDC)

1234 phosphorylation, both are hallmarks of “The Warburg Effect”. These data indicate
1235 compensatory changes occur to retain metabolic homeostasis as cancer cells are
1236 forced to promote BCAA oxidation. The result is increased progressive *in vitro* cell
1237 behavior. Our conclusion is that these two metabolic systems, BCKDC and PDC,
1238 may not act independently of one another in cancer cells.

Introduction

Branched-chain amino acids (BCAAs: leucine, isoleucine, and valine) comprise a subgroup of essential amino acids that cannot be synthesized *de novo* and have multipotent roles in both biochemistry and signaling biology. Leucine is particularly potent, if not singularly effective, in many signaling pathways (eg, translational activation (Mordier and others 2000), insulin synthesis and secretion (Lambert and others 1986), inhibition of autophagy (Mordier and others 2000), and hypothalamic satiety signaling (Cota and others 2006)). BCAAs can also be deaminated to α -keto acids and metabolized for fatty acid synthesis and β -oxidation (Greenberg and Reaven 1966; Noda and Ichihara 1974; Noda and Ichihara 1976). Owing to these many biological pathways open to BCAAs, it is peculiar that their irreversible metabolism is known to be governed by one multi-enzyme complex, the branched-chain α -keto acid dehydrogenase complex (BCKDC).

The BCKDC is composed of E1, E2 and E3 subunits, similar to other dehydrogenases, pyruvate dehydrogenase complex (PDC) and α -ketoglutarate dehydrogenase complex (Harris and others 1995). First step metabolism proceeds through the heterodimeric E1 subunit comprised of two α subunits and two β subunits (Danner and others 1978; Parker and Randle 1978; Pettit and others 1978; Roberts and Sokatch 1978). The BCKDC is active when the serine residue 292 on the E1 α subunit is dephosphorylated and is inactive upon phosphorylation. This reversible phosphorylation is regulated by a specific kinase (BDK) (Popov and others 1992; Shimomura and others 1990) and phosphatase (BDP) (Damuni and others 1984; Damuni and Reed 1987). The biochemical processes occurring through

the BCKDC are irreversible. Therefore, the careful regulation of the BCKDC is the most important step in BCAA metabolism.

In normal tissues, BDK and BDP carefully control the activity of the BCDKC. This control regulates the amount of free BCAAs available not only for catabolic energy production, but also anabolic and signaling requirements. Anabolic demands for BCAAs include fatty acid synthesis and single amino acids used in the synthesis of proteins. In the absence of sufficient amounts of BCAAs, the translation is unable to occur. This is not only owing to the lack of BCAAs, but also the signaling roles BCAAs have in initiating translation. Leucine in particular has been shown to initiate translational machinery indirectly by stimulating insulin secretion and directly via the mammalian target of rapamycin (mTOR) (Kimball and others 1999). The fate of BCAAs amidst these different pathways is carefully controlled in the normal state. However, it has been shown that when tissues and cells are in a state of hypermetabolism (ie, cancer (Baracos 2000; Baracos and Mackenzie 2006)), the free pool of BCAAs is used up rapidly for energy and protein synthesis requirements. Increasing energy demands of tumor-bearing rats have been shown to increase leucine oxidation measured by radio-labeled CO₂ release (Costelli and others 1995), indicating a systemic depletion of leucine in the host organism through BCKDC activity. As a compensatory mechanism to refill the rapidly depleting free BCAA pool in patients with severe, invasive cancer, skeletal muscle, which is rich in BCAAs, is degraded.

The compensatory increases in free BCAAs, high protein turnover rates, and high oxidation rates cause increased BCKA levels, at least transiently. Our lab has

recently shown high levels of BCKAs have signaling properties through the hypoxia inducible factor (HIF) (manuscript in submission). This survival factor is known to play an important role in cancer, particularly in altering transcription of essential proteins for proliferation, invasion, and compensatory metabolism (reviewed in (Ke and Costa 2006; Semenza 2007)). HIF specifically alters glucose metabolism by increasing glucose transporters, PDC kinase (PDK), and lactate dehydrogenase (LDH), to name just a few. These actions drive anaerobic glycolysis which is advantageous since it has been shown that tumors exist in a hypoxic environment. However, this effect has been shown in the presence of oxygen, an observation known as the Warburg Effect (Warburg 1930; Warburg 1956). Regardless of the many changes that occur as a result of HIF activation in cancer, our lab has recently shown that PDK activity is directly responsible for increasing cancerous phenotypes (McFate and others 2008). PDK acts to shut down PDC activity the same way BDK acts to shut down BCKDC. Given that PDK activity can be manipulated to alter cancer phenotypes, we hypothesized that altering BDK activity would also alter cancer phenotypes.

In this publication, we show that utilizing stably transfected shRNA knockdown system of BDK in cancer cells, the phenotypes of the cells are altered. However, these data also suggest that by altering the BCKDC system, metabolic compensation occurs to the point where PDC activity is affected. These data indicate that the dehydrogenases PDC and BCKDC may not act independently of one another.

Experimental Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Cell culture products were purchased from GIBCO.

Cell culture and Chemical Treatments

Cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1.5 mg/mL G418 (Gibco). G418 was used to select transgenically altered cells. Cells were cultured under 37°C incubation and in 21% O₂, 5%CO₂, and 74% N₂. Chemical treatments were performed in Kreb's Saline Buffer and incubation times are as indicated. Each chemical treatment was performed by dilution from 100X stock in Kreb's saline buffer. For cell hypoxia treatment, the culture dishes were sealed in a modular incubator chamber, flushed with gas containing 1% O₂, 5%CO₂, and 94% N₂ for 5 min, and incubated in this environment at 37°C for the indicated times.

Generation of Transgenic C6 Glioma Cell Lines for BDK Knockdown

An shRNA knockdown vector for transfection into C6 glioma cells specific to BDK was obtained from SuperArray. The cells with shRNA BDK are referred to as shK (shRNA BCKD-Kinase) from hereafter. The SuperArray kit provided a standard control vector consisting of a scrambled DNA sequence cloned into the identical vector for quality and specificity of effects that we also used and is referred to as CVC6 (control vector for C6 cells) hereafter. CVC6 and shK cells were treated identically through the transfection procedures. The cells were transfected as described in the product manual. Briefly, vectors were grown in *E.coli* JM109 (Stratagene). DNA was purified by a commercial miniprep kit (Qiagen). DNA was

then transfected into C6 cells with Lipofectamine 2000 reagent (Invitrogen) and incubated in fresh DMEM with 10% FBS following transfection. After 48 hrs recovery time, transfected cells were selected by adding 1.5 mg/mL G418 (Gibco) in complete DMEM. Media was changed as cells died and colonies were allowed to grow until large enough to pluck and grow independently. Once cells were grown and verified through Western blotting of BDK, they were used as stable cell stocks to ensure clonal cell lines. shK and CVC6 cell lines were incubated as previously described for wild type in the “**Cell Culture and Chemical Treatments**” section.

Cell counting

Cell were washed and trypsinized for cell counting procedures for ELISA normalization. Trypsin was inactivated by adding equal amounts of media with 10% FBS. Five hundred μ L of suspensions of each preparation were then placed in a Vi-Cell Trypan Blue automated counter for cell counting.

Western Blot and Densitometry Analyses

Cells were washed three times with cold PBS. Appropriate amount of lysis buffer containing RIPA buffer (Bioworld), 1% SDS, and 1X protease inhibitor cocktail (Roche) then scraped. Cell remnants were then collected in 1.5 ml microcentrifuge tubes. The cell material was sonicated for 25 seconds at 50 Hz, then placed in rack at room temperature. Cell lysates were then spun for 5 minutes at 12,000 g and the supernatants were transferred to fresh tubes. Protein levels were determined by the BCA method of analysis (Pierce). Proteins were separated on 4-12% Bis-Tris SDS-polyacrylamide gradient gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked using 5% goat or horse serum

(Vecotr Labs) in Tris buffered saline with 0.1% Tween-20. Antibodies used were anti-phospho-Ser292-E1 α BCKDH (generous gift from C. Lynch, 1:20,000), anti-E1 α BCKDH (generous gift from C. Lynch, 1:1000), anti-BDK 1:2000 (generous gift from Dr. R. Harris), E1 β /E2 antisera 1:2000 (generous gift from Dr. R. Harris), anti-phospho-Ser293-E1 α PDC 1:5000 (Novus), anti-E1 α PDC 1:2000 (Novus) and anti- β actin 1:10,000 (AbCam). Protein bands were visualized by enhanced chemiluminescence (Pierce) using either Kodak film and developer or CCD luminescence camera (Fuji Film). Signals were quantified using densitometry in ImageJ (Wayne Rasband, NIH). Phosphoproteins were normalized to total protein amounts unless otherwise stated. Graphs indicate arbitrary units on the y-axes derived from signals normalized to non-treated or wild type controls by direct comparative ratios. These normalizations were necessary to control for protein loading.

Proliferation assays

One hundred thousand cells were plated in 6-well culture dishes to a volume of 2 mL of DMEM media solution with 10% FBS and 1% penicillin-streptomycin. At time points, cells were counted as described in the “*Cell Counting*” procedure. The data are presented as either raw cell numbers or relative proliferation rates normalized to wild type controls.

Migration and Invasion Assays

Migration experiments were performed using BD Falcon cell culture inserts with 8 micron pores (BD Biosciences). Cell invasion experiments were performed using 24-well Biocoat Matrigel™ invasion chambers with an 8- μ m pore polycarbonate filter

according to the manufacturer's instructions (BD Biosciences) Fifty thousand cells were plated in migration and invasion inserts to a volume of 0.5 mL of DMEM solution. The inserts were then placed in 24-well culture dishes containing 0.75 mL DMEM solution with 1% FBS and 1% penicillin-streptomycin. Cells were incubated at 37 degrees Celsius, 1% O₂, 5.0% CO₂ and 94% N₂ for 72 hr. Cells that remained inside the insert after 48 hours were thoroughly wiped with a cotton swab and invading cells were fixed and stained using Diff-Quick Stain Solution (Dade Behring). Images were then taken with a Canon CoolSNAP digital camera in Metamorph imaging program under light microscopy. Cell number was determined by marking individual cells with spots in the nucleus in ImageJ imaging software and using the quantifier for spot counting. Five images were taken, counted and averaged from each well insert to ensure adequate sampling.

Colony Formation Assay

Ten thousand cells from each cell line were placed in 1 ml of DMEM with 0.3% low-melting agarose (soft agar) and 10% FBS, and overlaid onto 1 ml/well of DMEM with 0.5% agarose and 10% FBS. Cells were then incubated for 18 days at 37 C in 5.0% CO₂. At the endpoint, wells were placed under light microscope with COOLSnap Kodak camera. Five images were taken, counted and averaged from each well insert to ensure adequate sampling. Colonies were counted by identification with exclusion parameters at ~10 nm. Colony diameters were determined by importing tiff images in ImageJ software. A line was then drawn from end to end of colony going through the center and ImageJ quantified diameter.

CMA Analysis of Lactate

Cells cultured and samples of media were taken at indicated times and frozen at -80°C. Cell numbers were then obtained as described previously in the "*Cell Counting*" section. Media samples were thawed, vortexed and centrifuged to eliminate air bubbles. 50 µL of media samples were used to measure metabolite levels. Lactate was measured using a CMA 600/microdialysis analyzer (CMA Microdialysis AB). Resulting data were normalized to cell number for each sample.

BCKDC Activity Assay by Radiolabeled CO₂ Capture

The following analysis was performed by collaborators (Jeoung and others 2006). Briefly, cells were plated in culture flasks (1 x 10⁶ cell/flask for wild type cells, 0.75 x 10⁶ cell/flask for CVC6 and shk cells because growth rates were different.) Cells were cultured until growth reached 90% confluency. Cells were then washed twice with PBS (room temperature). Five mL of Krebs-Henseleit buffer containing 5 mM glucose, 1 mM ¹⁴C-valine (specific activity; 150 uCi/mmol), 0.2 % BSA, and 1 mU/mL insulin. The air was flushed with 95% O₂ and 5% N₂, then the bottle was closed with a rubber stopper. Experimental incubation time was 1 hr at 37°C. Three-hundred µL of 60% perchloric acid was added to stop the reaction process. ¹⁴CO₂ was collected into the center well. In order to check for KIV production from valine, 1 mL of stopped solution was taken out and put into a scintillation vial. The rubber stopper was then replaced and 350 µL of 30% H₂O₂ was added and incubated for 30 min. Radioactivity in the center well was then counted. Data are expressed as nmol of oxidized valine determined to be in equimolar proportions with captured ¹⁴CO₂ and normalized to cell number.

ATP bioluminescence assay

Cells were grown under culture conditions. Cells were detached from the culture plates with trypsin and suspended in media for counting. Media volumes were adjusted to a range of 10^5 to 10^8 cells/mL. Boiling 100 mM Tris, 4 mM EDTA, pH 7.75 was added (9:1 ratio to sample volume) and incubated for 2 min at 100°C. Samples were then centrifuged at 1000g for 60 sec. The supernatants were transferred to clean tubes. Luciferase reagent was then added to the samples and bioluminescence was recorded after 10, 15, and 20 seconds. The highest values were taken and normalized to viable cell number established from left over cell suspensions for each sample.

Statistical Analyses

Statistical analyses ANOVA, Tukey's HSD and Fisher's PLSD *post hoc* tests were performed using STATview statistical software. Where it was necessary to compare treatment only to control groups, ANOVA and Dunnett's *post hoc* analyses were performed in SPSS statistical software.

Results

A specific BDK knockdown using stably transfected shRNA in C6 glioma cells proves viable in reducing BCKDC phosphorylation and activity

C6 glioma cells have a high basal level of BCKDC phosphorylation. It is unclear what role the metabolism of BCAAs and BCKAs has in cancer biochemistry. Previous reports are perfunctory and contradictory occurring mostly in tumor-bearing animals, but also in humans, leaving the question open to further and more specific investigation. We chose to manipulate an *in vitro* system of cancer to identify any key differences observed when the BCKDC activity state was constitutively active.

These C6 cells were used to generate stable-shRNA BDK knockdown cells (shK) and stable-control vector (CVC6) cell lines. The shK and CVC6 cells generated were analyzed via Western blot for BDK and phosphorylated Ser292-BCKDC E1 α to verify that there had reduced and comparable signals, respectively, as compared to C6 wild type (Fig. 1A). Densitometry of Fig. 1A Western blots are also shown in Fig. 1B. The shK cell line had a decreased BDK signal compared with both wild type ($p<0.0001$) and CVC6 ($p<0.0001$). There were no differences between wild type and CVC6 cell lines in BDK signal. Phosphorylated Ser292-BCKDC E1 α levels for shK cells were non-detectable, whereas wild type and CVC6 cell lines had comparable signals. For normalization of phosphorylated signals of proteins, the phosphorylated signal is divided by the total protein signal and expressed as a ratio. The shK cells had a decreased ratio of phosphorylated Ser292-BCKDC E1 α from wild type ($p<0.0001$) and CVC6 ($p<0.0001$), whereas no differences were detected between wild type and CVC6 cells. The activity of the E1 α -BCKDC was generously performed by Nam Ho Jeung, a collaborator, and shows shK cells have an increased activity of the E1 α subunit as compared to wild type and CVC6 ($p<0.05$ for both comparisons; Fig. 1B).

E1 β and E2 Subunits of BCKDC in the Different Cell Lines

BCKDC subunits E1 β and E2 were analyzed by Western blot and densitometry (Fig. 2A and B, respectively) to see if any disruption to normal levels of these proteins resulted from the transgenic alterations to BDK translation. The E1 β subunit is the heterodimeric partner to the E1 α that composes the E1 subunit. There were no statistical differences of E1 β signal between shK cells when compared to

either the wild type or CVC6. No differences were observed between wild type and CVC6. However, the signals from the blot of the E2 subunits of each cell line showed increased signals for shK cells compared with wild type ($p<0.05$). No differences were present between either the shK and CVC6 cell lines, or the wild type and CVC6 cell lines.

BDK Knockdown Cell Line Shows Increased Proliferation from Controls

Cell proliferation was compared for the cell lines over 72 hrs, the data from which are depicted in Fig. 3A. The number of viable shK cells was significantly increased compared with wild type for 24 ($p<0.001$), 48 ($p<0.0001$) and 72 hrs ($p<0.0001$). The number of viable shK cells was also significantly increased compared with CVC6 for 24 ($p<0.001$), 48 ($p<0.0001$) and 72 hrs ($p<0.0001$). The average proliferation rate of shK cells across 24, 48 and 72 hr time points was 137.63% of wild type and 136.82% of CVC6 cells. There were no differences between wild type and CVC6 cells for any time points. To investigate if the increased proliferation was due to growth factor signaling, we replicated the proliferation assay in serum-free media (Fig. 3B). The results show increased shK cell proliferation with respect to wild type at 48 ($p<0.05$) and 72 hrs ($p<0.05$), but not at 24 hrs. The number of shK cells was also significantly increased from CVC6 at 48 ($p<0.01$) and 72 hrs (0.05), but not 24 hrs. The average proliferation rate of shK cells across 24, 48, and 72 hr time points was 119.65% of wild type and 122.08% of CVC6 cells. There were no differences between wild type and CVC6 cells for any time points.

Metastatic Behaviors Increase in BDK Knockdown Cells Relative to Controls

To investigate if the increased rates of proliferation observed in the BDK knockdown cells were indicative of other malignant phenotypes, we measured the migration of cells through perforated culture inserts. This tests the mobility of cancer cells commonly seen in *in vivo* systems. We observed increased migratory rates for shK cells as compared to wild type ($p<0.0001$) and CVC6 ($p<0.0001$) cells, as shown in Fig. 4A and quantified in 4D.

Cell invasiveness, another metastatic phenotype commonly found in malignant cancerous tissue, was also analyzed. Invasion assays differed from migration only in that the cells were cultures in perforated culture inserts filled with extracellular matrix matrigel to mimic the extracellular environment. The results show increased invasiveness for shK cells as compared to wild type ($p<0.0001$) and CVC6 ($p<0.0001$) cells, as shown in Fig. 4B and quantified in 4E.

Cell lines were mixed with a soft agar DMEM solution and plated in 6 well dishes. Incubation of cells consisted of 3 weeks under culture conditions. Upon termination of the experiment, images were taken of cell colonies and analyzed as described in the *Experimental Methods* section. There were no differences in colony numbers between the three cell lines (data not shown). However, the size of the shK cell colonies was significantly increased with respect to wild type ($p<0.0001$) and CVC6 ($p<0.0001$) colonies, as shown in Fig. 4C and quantified in 4F.

Phosphorylated Characteristics of PDC, a Family member of the Heterodimeric Multimer Complex also Implicated in Metastatic Cancers

We next wondered if other mitochondrial dehydrogenases were affected by the BDK knockdown. Biochemical pathways in metabolism are tightly regulated

according to energy demands of the cell; alterations to one pathway may affect the another. Our lab had previously investigated PDC activity and its relation to cancerous phenotypes, classically termed “the Warburg Effect” (McFate and others 2008). The more aggressive cancerous phenotypes presented here were unexpected, leading us to investigate PDC metabolic activity. Cells were harvested and analyzed through Western blotting for E1 α PDC phosphorylation state. The phosphorylation state of Ser 293-E1 α PDC is shown with the total PDC E1 α signal beneath as loading controls (Fig. 5A). Densitometry of Western blots in Fig. 5A is represented in Fig. 5B. The PDC E1 α phosphorylation ratios are increased for the shK cell line when compared to both wild type ($p<0.05$) and CVC6 ($p<0.05$) cell lines.

Lactate Production as an Indicator of Warburg Characteristics

If the Warburg hypothesis is correct, then the BDK knockdown cells, which exhibit a more aggressive metastatic phenotype, should release more lactate into the culture medium. To test this, media samples were taken at 6, 12, 24, and 48 hrs for lactate analysis using CMA. Cell number was then determined as previously described. CMA analysis of the cultured media showed differences in lactate produced for the cell lines (Fig. 5C). The shK cells had increased lactate production with respect to wild type cells at 12 ($p<0.01$), 24 ($p<0.05$) and 48 hours ($p<0.05$). The shK cells also had increased lactate production with respect to CVC6 cells at 12 ($p<0.01$), 24 ($p=0.01$) and 48 hours ($p<0.05$).

Discussion

BCAA metabolism has been underappreciated in cancer biochemistry. Here we propose that shunting BCAA metabolism through the BCKDC affects overall cellular biochemistry in cancer cells. Using stably transfected shRNA, we successfully knocked down the translation of the BDK (shK cells), the kinase responsible for inactivation of the BCKDC, thus leaving the complex in a constitutively active state. Both phenotypical and biochemical changes were observed in the transgenically altered cancer cells. We found that shK cells were more proliferative, migratory, invasive, and able to form colonies in soft agar, an *in vitro* technique which shows cellular ability to form cancerous nodules. We also found that increased BCKDC activity in shK cells resulted in decreased activation of the PDC, another key mitochondrial metabolic multi-enzyme complex, and consequential increased lactate secretion from these cells. These data suggest that enhancing BCAA and BCKA metabolism through the BCKDC acts to modify cellular biochemistry so that PDC activity is decreased, which results in increased lactate production and increased invasive cancer phenotypes, historically dubbed the “Warburg Effect”.

Cancerous cells are in a constant state of hypermetabolism and accelerated growth. As such, their metabolic needs greatly differ from normal cells. Changes to their intracellular biochemistry cause appreciable differences to their behaviors and extracellular signaling. When cellular processes become dysregulated, like in cancer, the cell then has an altered transcriptional milieu from its genes in response.

1556 These changes are largely indicative of evolutionarily preserved survival and
1557 adaptation mechanisms.

1558 Recently, our lab has shown that increased levels of BCAAs and BCKAs can
1559 stimulate signaling mechanisms involving HIF, an evolutionarily conserved
1560 transcription factor which is normally activated by hypoxic stress conditions
1561 (manuscript in submission). Activation of the HIF pathway has been shown in cancer
1562 cells, where the level of increase above baseline corresponded to the degree to
1563 which invasive and metastatic characteristics were increased. These increased
1564 cancerous phenotypes have been shown to involve specific factors including VEGF,
1565 GLUT1, etc (reviewed in (Ke and Costa 2006)). However, increased activation of
1566 HIF has also been shown to be directly stimulated by increased levels of glycolytic
1567 metabolites, particularly pyruvate, OAA, succinate, and fumarate (Isaacs and others
1568 2005; Lu and others 2005; Selak and others 2005). HIF activation has been shown
1569 to increase glycolysis and decrease mitochondrial respiration, primarily through
1570 increased PDK expression, which leads to decreased PDC activity, a buildup of
1571 pyruvate, and, consequently, an increase in lactate production (Koukourakis and
1572 others 2005).

1573 Our lab has recently published data increasing the activity of PDC, the
1574 metabolic regulator of aerobic metabolism at the crux between glycolysis and
1575 cellular respiration, by PDK knockdown. These data showed increasing PDC activity
1576 through knockdown of PDK, HIF levels decreased, as did cancerous phenotypes of
1577 invasion, migration, and tumor size (McFate and others 2008).

1578 Our data show a decrease in presence and activity of BDK results in
1579 increased malignancy. These findings are contrary to our original hypothesis.
1580 However, perhaps a more comprehensive understanding has been reached in that
1581 the regulation of just one metabolic pathway is not always sufficient, as was shown
1582 by McFate et al (2008) and others (McFate and others 2008). Biochemistry of
1583 cancerous, as well as normal, cells requires a coordination of metabolic complexes.
1584 Our intent was to investigate whether altered BCKDC activity was capable of
1585 singularly altering the behavior of cancerous cells and cancer biochemistry in a way
1586 that would make it a new target for cancer therapy research, similar to PDC.
1587 However, it seems that there is no direct translation of metabolic complex to
1588 therapeutic target.

1589 In summary, BCAAs are metabolized through one multienzyme complex,
1590 BCKDC. In this report, we used an shRNA knockdown approach of BDK to cause
1591 hyperactivity of BCKDC. Our hypothesis was that by increasing BCKDC activity, the
1592 free amino acid pools would be depleted in cancer cells, which would limit their
1593 growth rates. The data show a successful knockdown of BDK to result in increased
1594 activation of BCKDC. However, the behavior of these cells increased in cancerous
1595 characteristics, such as proliferation, migration, invasion, and colony size. We further
1596 investigated whether these results were due to a coordination of metabolic
1597 complexes, namely BCDKC and PDC. These data show that alterations to BCKDC
1598 activity does indeed have an effect on PDC activity. The nature of this effect is one
1599 that drives PDC to become less active, a characteristic shown by our lab and others
1600 as causing increased cancerous behaviors. It seems that BCAA control is not solely

responsible for cancer behaviors, but these unexpected findings do, however, warrant a closer look at BCAAs in cancer biochemistry.

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Legends to Figures

Figure 1. Viability and functionality of BCKDC-kinase knockdown *in vitro* system.

A, Western blot analysis showing sufficient knockdown of BDK corresponding to inhibition of phosphorylation and increased activity of BCKDC. C6 wild type (w/t), shK C6 and CVC6 cell lines are shown for the BCKDC-kinase, phospho-Ser292-E1 α BCKDC and total-E1 α BCKDC (n=6). Densitometric analyses of Western blots are depicted in densitometry units with standard errors normalized to wild type control. **B**, shows sufficient increased BCKDC activity resulting from decreased phosphorylation from BDK knockdown. Statistical differences were determined by Tukey's HSD *post hoc* analysis (*p<0.05, **p<0.001, and ***p<0.0001 with respect to C6 wild type; +p<0.05, ++p<0.001, and +++p<0.0001 with respect to CVC6; ND – non-detectable signal).

Figure 2. Analysis of BCKDC additional subunits

A, Representative Western blot analysis of additional BCKDC subunits, E1 β and E2, for C6 wild type (w/t), shK and CVC6 cells (n=6). **B**. Densitometric analyses of Western blots are depicted in densitometry units with standard errors normalized to wild type control. Statistical differences were determined by Tukey's HSD *post hoc* analysis (*p<0.05 with respect to C6 wild type).

Figure 3. Cell proliferation in serum and serum-free media show differences between shK cells and wild type and CVC6 control cells.

A, indicates cell proliferation is increased in shK cells with and without serum growth factors. Cell proliferation is shown in millions of cells for 24, 48, and 72 hr time points for viable cells only (n=6 for each time point). **B**. Cell proliferation in serum-free media is shown in millions of cells for 24, 48, and 72 hr time points for viable cells only (n=6 for each time point). Statistical differences were determined by Tukey's HSD *post hoc* analysis (*p<0.05 and ***p<0.0001 with respect to C6 wild type; +p<0.05, ++p<0.01, and +++p<0.0001 with respect to CVC6).

Figure 4. Metastatic phenotype analyses indicate differences between shK cells and wild type and CVC6 control cells.

A, shows increased migration for shK cells. Migrating C6 wild type(w/t), shK C6, and CVC6 cells were fixed and stained following 48 hour incubation (n=6). Representative images indicating qualitative differences are shown in **A** and quantitative cell counting is displayed in **D** with standard errors. **B**, shows increased invasion for shK cells. Invading C6 wild type(w/t), shK C6, and CVC6 cells were fixed and stained following 48 hour incubation (n=6). Representative images indicating qualitative differences are shown in **B** and quantitative cell counting is displayed in **E** with standard errors. **C**, shows increased colony diameter for shK cells indicating higher proliferation and invasion characteristics. Pictures of C6 wild type(w/t), shK C6, and CVC6 were taken following 3-week incubation in soft agar and analyzed for colony diameter. Representative images indicating qualitative differences are shown in **C** and quantitative colony diameter size is shown in **F** with standard errors. Statistical differences were determined Tukey's HSD *post hoc*

analyses ($***p<0.0001$ with respect to C6 wild type; $+++p<0.0001$ with respect to CVC6).

Figure 5. Differential phosphorylation status of the mitochondrial metabolic complex PDC for shK and wild type and CVC6 controls

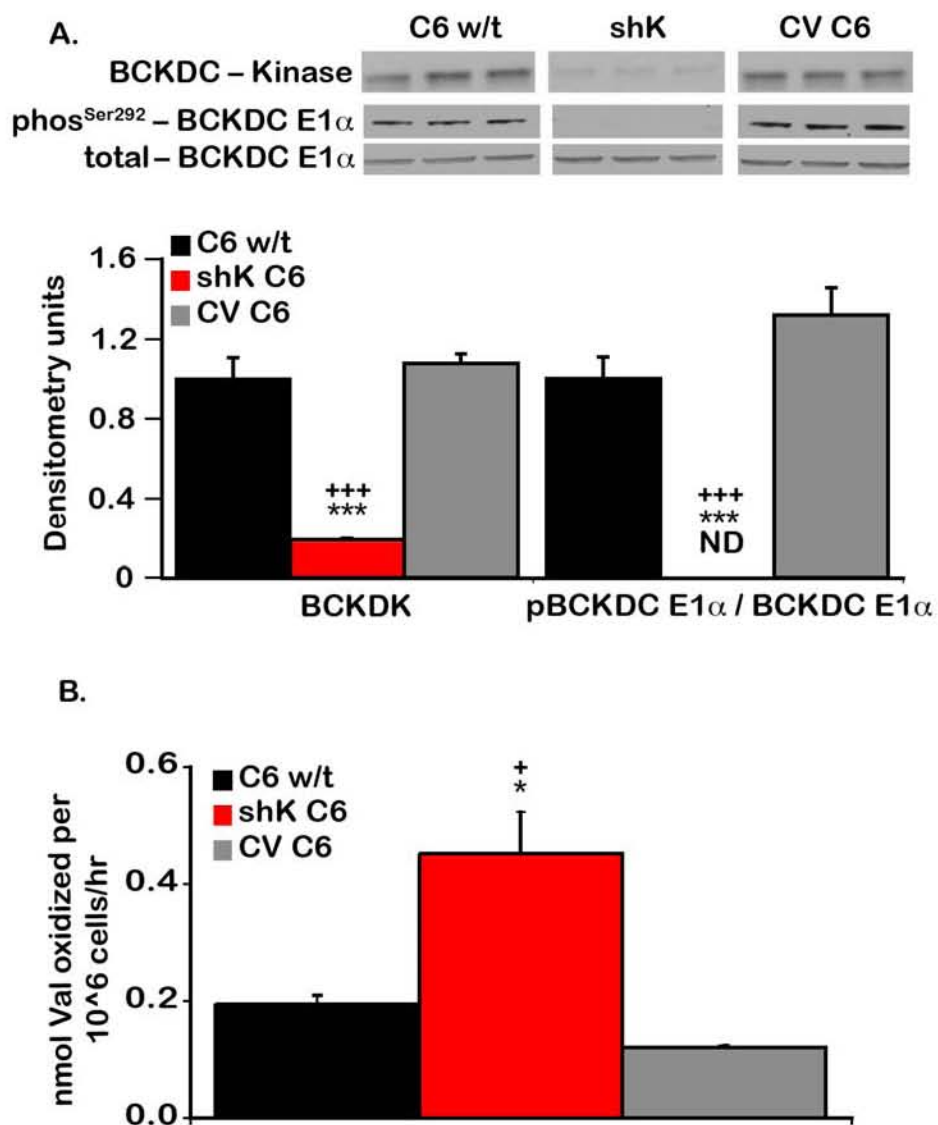
A, representative Western blot analysis shows phosphorylation status of Ser293-E1 α PDC for C6 wild type (w/t), shK C6 and CVC6 cells (n=6). **B**, indicates increased PDC phosphorylation ratios for shK cells. Densitometric analyses of Western blots are shown in densitometry units with standard errors normalized to wild type control. **C**, shows increased lactate levels for shK cells, which indicates an increased Warburg phenotype. Lactate production was measured from media by a CMA microdialysis analyzer at 6, 12, 24, and 48 hours (n=6 for each time point) normalized to cell number. Statistical differences were determined by Tukey's HSD *post hoc* analysis ($*p<0.05$ with respect to C6 wild type; $+p<0.05$ with respect to CVC6).

Table 1. Free ATP levels in C6 wild type, shK, and CVC6 cells

No significant difference between ATP levels was found. Data were gathered for shK, C6, and CVC6 cells by a luciferase bioluminescence assay. Data are displayed as nM ATP normalized by millions of viable cells ($\pm$ SEM). Statistical analysis showed no significant difference between any of the cell lines (Tukey's HSD *post hoc* analysis, n=6).

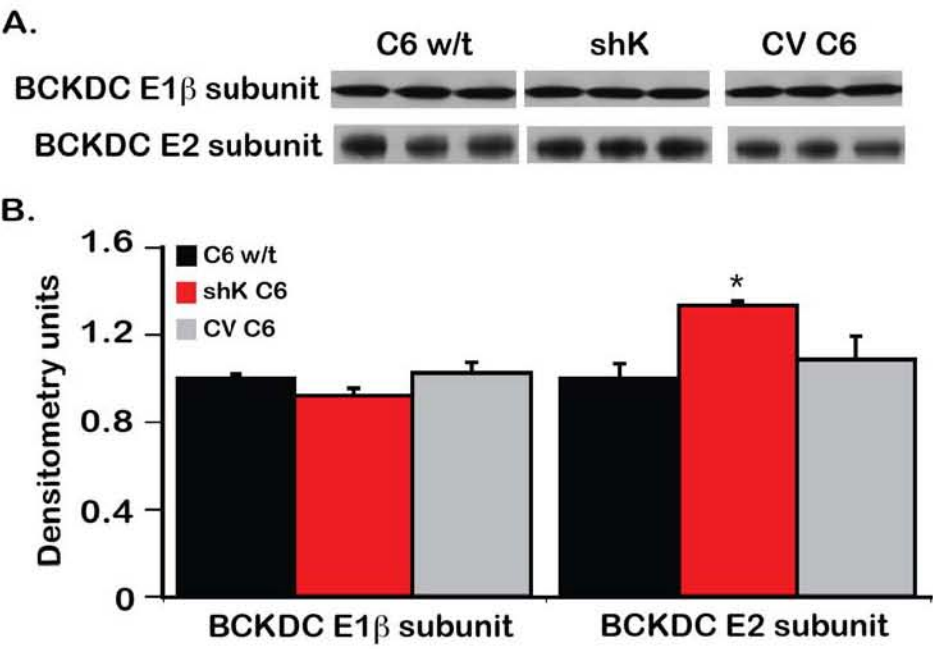
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Figure 4. Viability and functionality of BCKDC-kinase knockdown in vitro system



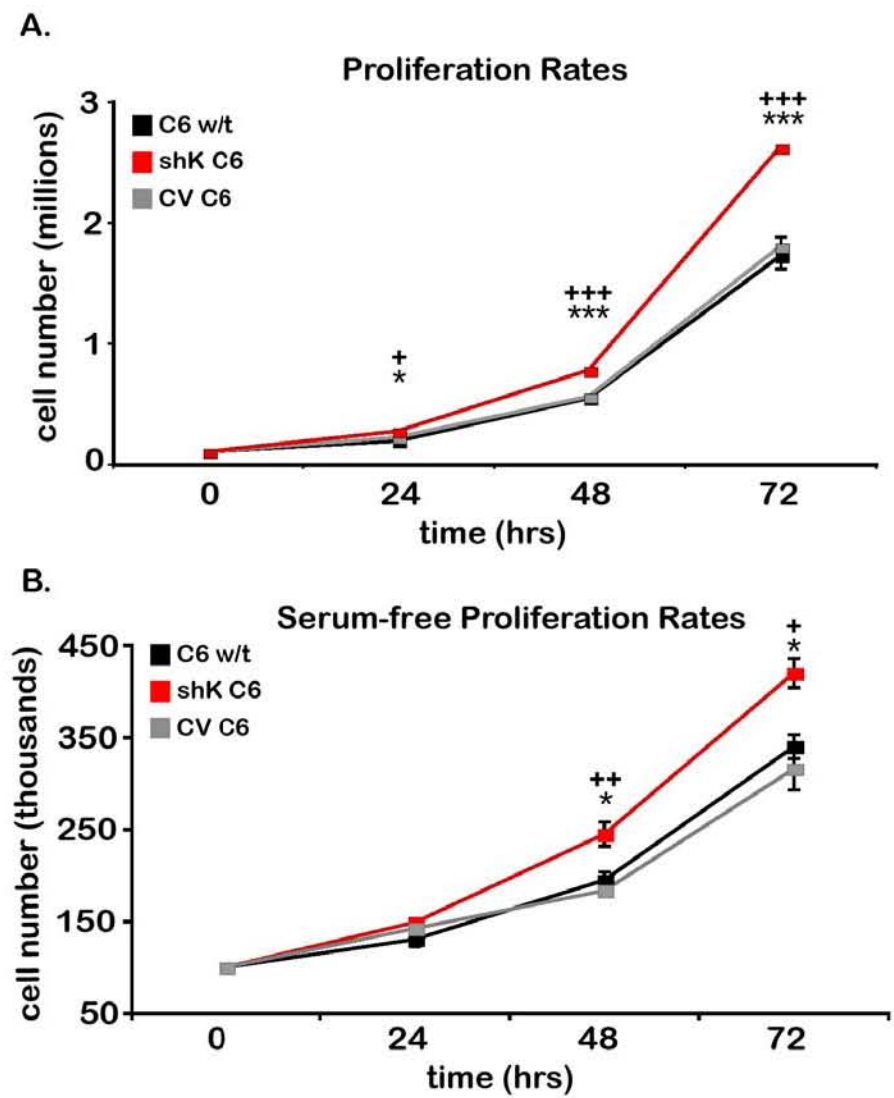
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Figure 5. Analysis of BCKDC additional subunits



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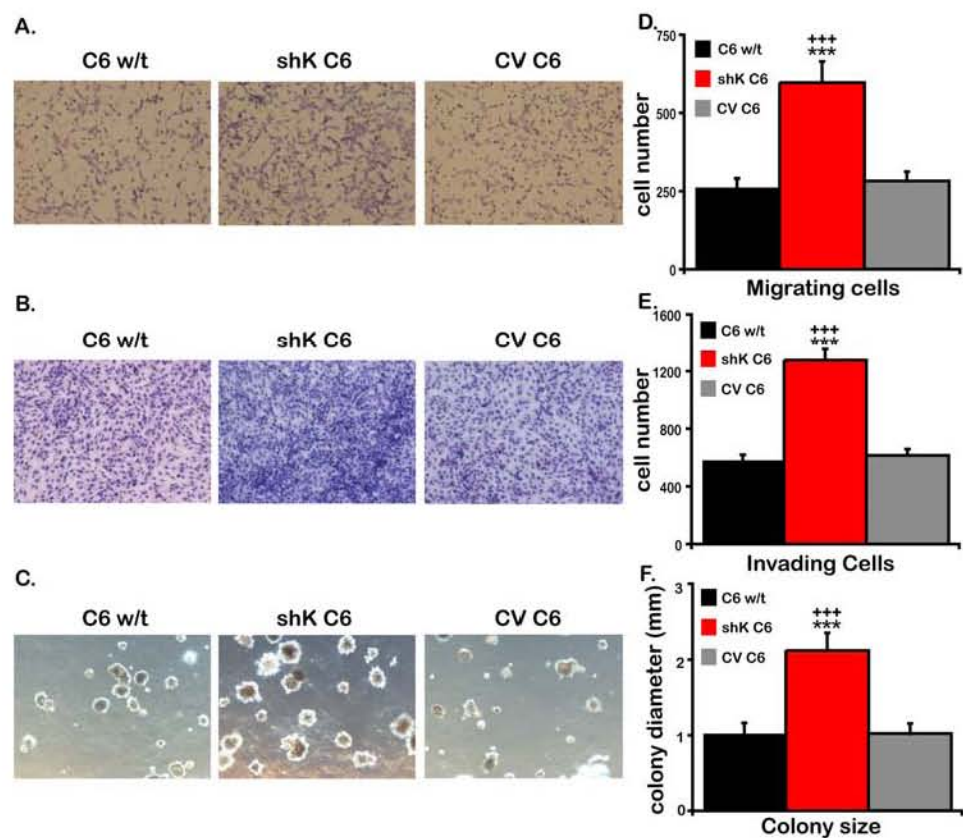
1769 Figure 6. Cell proliferation in serum and serum-free media show differences
1770 between shK cells and wild type and CVC6 control cells



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1773 **Figure 7. Metastatic phenotype analyses indicate differences between shK**
1774 **cells and wild type and CVC6 control cells**

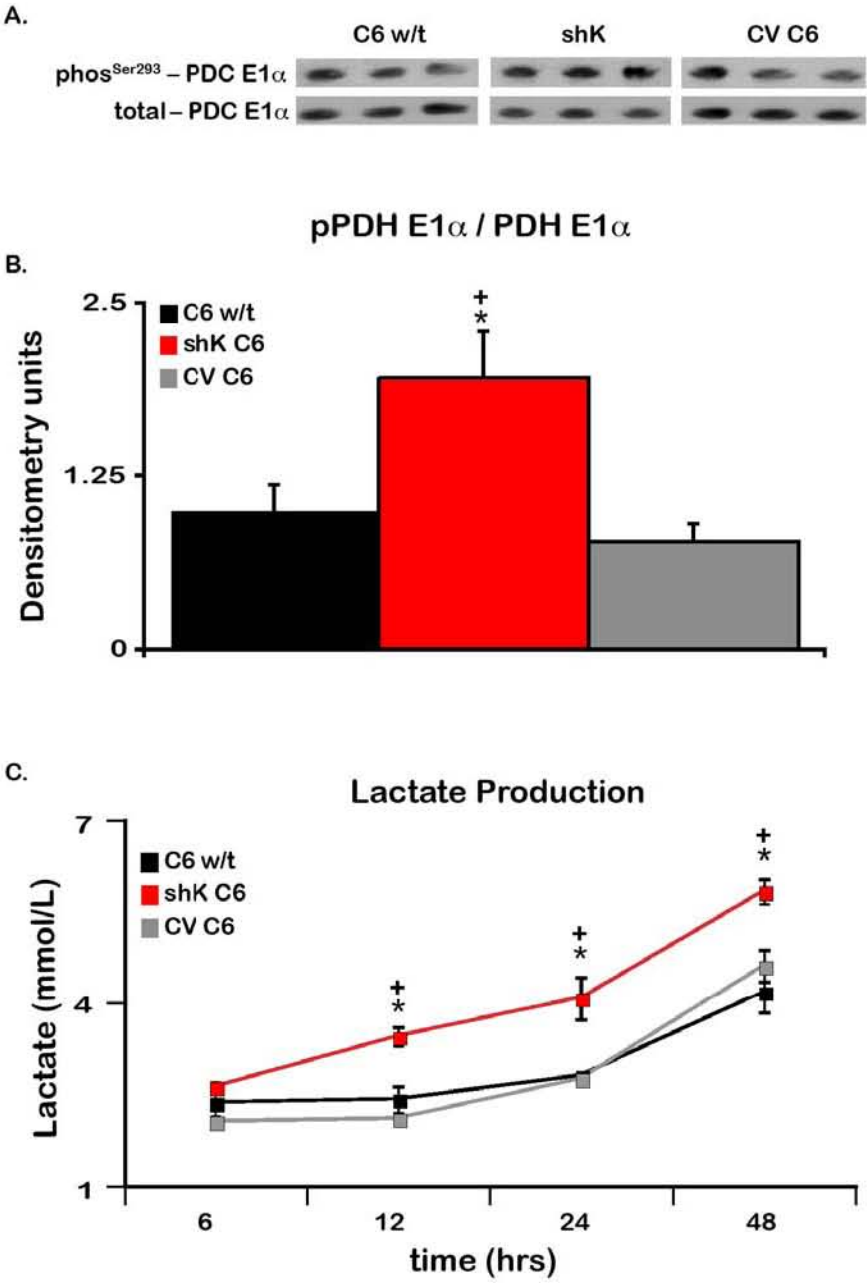


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1778 **Figure 8. Differential phosphorylation status of the mitochondrial metabolic**
1779 **complex PDC for shK and wild type and CVC6 controls**



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Table 1. Free ATP levels in C6 wild type, shK, and CVC6 cells	
Cell Type	nM ATP/million cells (means ± SEM)
C6 wild type	150 ± 23.4
shK C6	149 ± 2.5
CV C6	142 ± 7.3

General Discussion

The focus of this thesis is the role of BCAAs in cancer signaling and biochemistry. These data demonstrate a putative, novel signaling pathway involving HIF-1 α and that BCAA catabolism is not an independent regulator of *in vitro* cancer phenotypes. It has been known that the metabolism of cancer cells is aberrant in that they preferentially consume glucose anaerobically, even in the presence of oxygen levels sufficient for mitochondrial respiration. Recent findings from our lab have shown that manipulating PDC activity to be constitutively active (forcing aerobic respiration to occur) can reduce cancerous tumor formation and migration in a xenograph animal model. This work attempted to elaborate on the interest of another biochemical pathway, that of the BCAAs, in cancer biology. We set out to answer two research questions:

1. Does α -ketoisocaproic acid, a substrate of the BCKDC and deaminated product of leucine, act to decrease HIF degradation cycle activity thereby promoting neoplastic activity in an *in vitro* model of CNS cancer?
2. Do cells genetically modified by small hairpin RNA for the BCKDC kinase increase BCKDC activity and indicate a reciprocal relationship between BCKDC activity and cellular aggressiveness (eg, proliferation, migration, invasion, colony formation) in an *in vitro* model of CNS cancer, as similarly seen for PDC?

Chapter 1 focuses on the first question regarding BCKAs in HIF biology. It was first discovered in our lab that the BCKAs had a positive affect on HIF-1 α levels

in an *in vitro* system (Lu and others 2005). This work attempted to elaborate and further demonstrate that BCKAs can alter HIF regulation. Not only is HIF one of the most studied molecules in cancer biology, but, also, data have been shown that circulating BCAA levels are increased in patients with cancer. Increased circulating BCAA levels implies increased circulating BCKA levels, which, when taken together with our hypothesis for BCKA interference in HIF regulation, would have new implications in cancer biology.

Chapter 1, figure 1 shows dose-dependent curves for HIF-1 α protein and VEGF secretion in contrast to BCKDC phosphorylation status. It is clear from these data that BCKAs are able to affect HIF-1 α levels and VEGF secretion at relatively low levels, which fall in the range of BCAA levels found circulating in patients with cancer. Chapter 1, figure 2 shows BCKAs can inhibit the HPH cycle by sustaining HIF-1 α (endogenous HPH target) and ODD-GFP (exogenous reporter molecule of HPH cycle activity) levels. This figure also indicates that BCKA-induced HIF-1 α level stabilization is reversible. The mTOR molecule has been shown to be activated by leucine (Kimball and Jefferson 2004) and also to increase HIF-1 α levels (Hudson and others 2002). Chapter 1, figure 2 also shows increased HIF-1 α levels are not the result of mTOR activity.

Our research focused on the metabolism of BCAAs and BCKAs in two situations of potential clinical importance. The first involved the aberrant metabolism of BCKAs in maple syrup urine disease (MSUD), which leads us to propose ascorbate as a possible benign therapeutic intervention for treating MSUD-induced edema. Future work in this area could potentially extend these *in vitro* findings to a

physiological model of MSUD. The literature is still sparse with regards to mechanisms leading to the edematous phenotypes seen in MSUD patients in acute crisis. If the putative BCKA-HIF-VEGF pathway presented here is validated (either through *in vitro* models of MSUD or transgenic animals with inactive BCKDC), it may be beneficial to begin testing in MSUD patients. Ascorbate has long been known to either passively diffuse (Lam and Daniel 1986) or be transported via a specific Na-dependent transporter in the choroid plexus. Ascorbate levels have been shown to be 500 μ M in CSF (Stamford and others 1984), concentrated in the ventricular system (Spector and Lorenzo 1973). These levels are five-fold higher than necessary for activity *in vitro* (Knowles and others 2003).

The second project examined the effects of manipulation to BCAA metabolism on the resultant malignant phenotypes in cancer cell lines. While our initial aim was to identify BCKDC activation as a potential therapeutic mechanism, the research suggests that, at worst, the metabolic pathways of BCAAs are too intertwined with others to be clearly effective. In the best of circumstances, perhaps a line of research initiated by Doering and colleagues where BDK overexpression is used in an *in vitro* setting could indicate if artificially inactive BCKDC could lead to decreased malignant phenotypes, the inverse of what we have shown here.

The major finding shown in Chapter 2 was that a reduction of BDK activity, and thus an increase in BCKDC activity, increased *in vitro* aggressive and metastatic characteristics in C6 cells. This finding is surprising and the opposite of what was expected according to our rationale proposed where local depletion of

1855 essential amino acids would cause protein translation and *de novo* synthesis rates to
1856 decrease.

1857 Here we show short hairpin RNA interference successfully knocked down the
1858 translation of BDK. This kinase has been shown to phosphorylate Ser292 of the
1859 BCKDC E1 α subunit, causing deactivation (Harris and others 1997; Popov and
1860 others 1992; Shimomura and others 1990). This would allow the phosphatase to act
1861 unopposed, resulting in increased activation of BCKDC (Harris and others 2004).

1862 Through collaboration, the hypothesized increase in activation was verified through a
1863 BCKDC E1 α subunit activity assay. However, the increased activity was not to the
1864 proportions one would expect from the Western blots. Perhaps there is another
1865 kinase which phosphorylates a residue other than Ser292, leading to a decrease in
1866 BCKDC activity which would not be apparent in the Western blots. This seems
1867 unlikely due to the fact that various groups have consistently reported the isolation of
1868 one, and only one, kinase specific for BCKDC [reviewed in (Harris and others 2004;
1869 Harris and others 2005)]. There is another site phosphorylated by BDK (Ser³⁰²),
1870 although this has been shown to be a modification inconsequential to the activity of
1871 BCKDC (Cook and others 1984; Cook and others 1983; Li and others 2007). A more
1872 likely explanation is that, over time, the transfected cells have either rejected the
1873 plasmid, or the plasmid has mutated since the activity assays were the last
1874 chronological experiments performed. Both of these situations would result in the
1875 observed discrepancy, however, the latter is more likely due to the fact that the shK
1876 cells retained resistance to G418, the selection agent. A large body of evidence has
1877 shown tumor phenotypes such as proliferation, migration, invasion, and colony

formation are indicators of metastatic and aggressive cancers (Gao and others 2005; Ke and Costa 2006; Le Jeune and others 2006; Lin and others 2007; Martens and others 2006; Mohyeldin and others 2005; Mueller and others 1999; Zhang and others 2000). We performed experiments to analyze these characteristics to discover the effects of BDK knockdown on *in vitro* cancer phenotypes. Our hypothesis was that a reduction in BDK would increase BCKDC activity.

Consequently this would act

to deplete free BCAA from the pools necessary for other pathways, such as translational activation and anabolism for building new proteins *de novo*. The results of experiments testing these four metastatic phenotypes of our *in vitro*, transgenic GBM model

showed increased metastatic phenotypes, the opposite of what was expected. A summary of our

expected and actual results is shown in Fig 9. Interestingly, Nakai *et al.* showed a decrease in proliferation rates in C2C12 cells using transient transfection of small

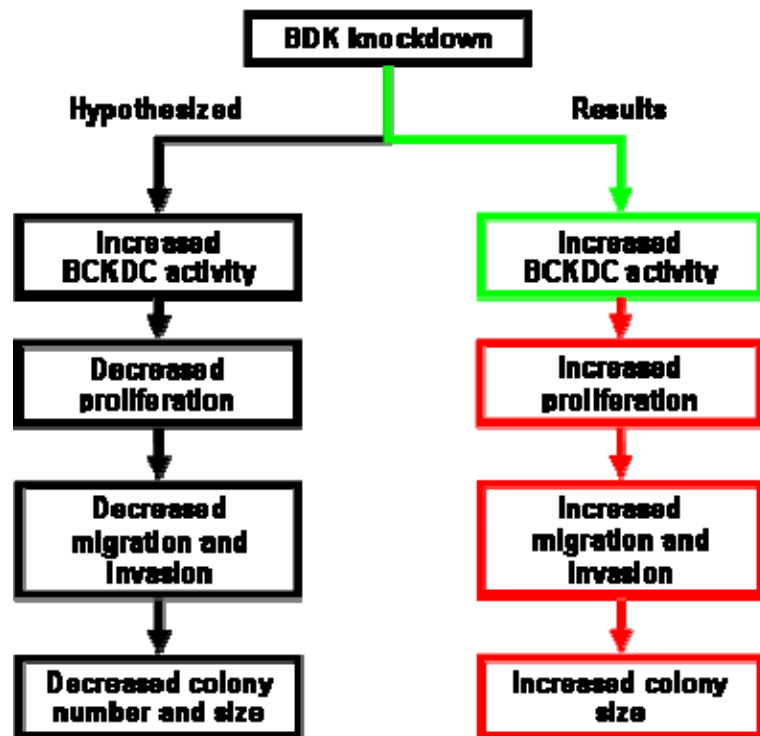


Figure 9. Hypothesis and results summary for BDK knockdown experiments.

Diagram indicates our experimental objective to generate a BDK knockdown with our hypothesized results (left arm) and our experimental results (right arm). In the right arm, results obtained in accordance with our original hypothesis are encased in green lines; results counter to our original hypothesis are encased in red lines.

1901 inhibitory RNA to knockdown BDK. There are distinct differences in the Nakai *et al*,
1902 methods and those used in the present work. For instance, their use of transient
1903 transfection may have resulted in a smaller population of cells contain the
1904 knockdown plasmid. This would provide results that are representative of a mixed
1905 population of BDK expressing cells (Nakai and others 2006). The authors also
1906 indicated that cells were used for assays at 50% confluency. It is unclear from the
1907 methods whether they began the proliferation assays at such high confluency, but
1908 was probably necessary to achieve maximal transfection efficiency. Our proliferation
1909 assays began at closer to 25% confluency, allowing for more growth space and less
1910 inhibitory autoregulation of cellular growth. Also, differences in C2C12 and C6 cell
1911 phenotypes have not been defined, which may add to complications in the
1912 seemingly conflicting data. C2C12 are myogenic cells, whereas C6 are astrocyte-
1913 derived glioma cells. It is unclear what differences there are, if any, in the protein to
1914 lipid ratios in these cell types, which may yield variant results due to the unique
1915 aspects of BCAA metabolism discussed throughout this work. In addition, Nakai *et al*
1916 showed increased insulin secretion was sustained in the BDK siRNA-treated cells.
1917 Insulin has long been known to increase cellular viability and proliferation. Recent
1918 reports (Akhtar and others 2009; Kai and others 2009; Probst and others 2008) have
1919 shown that inhibiting the activity of insulin and insulin-like growth factors lead to
1920 decreased proliferation of cancerous cells. Therefore it seems plausible that there
1921 are other unidentified mechanisms at work in the differences in cellular proliferation
1922 showed by Nakai *et al* and the present work.

Our second hypothesis was based on research performed in our laboratory where altering another mitochondrial dehydrogenase, PDC, reduced *in vitro* proliferation, invasion, migration, and colony formation (McFate and others 2008). Previous work had found that altering the phosphorylation status of Ser293-E1 α PDC (site 1) had effects analogous to what was originally hypothesized in this study, i.e. increasing PDC activation by interfering with the expression of pyruvate dehydrogenase complex kinase (PDK) decreased the metastatic phenotypes (Kim and others 2007; Koukourakis and others 2005; McFate and others 2008; Roche and Hiromasa 2007). The original research concerning PDC and cancer is related to the “Warburg Effect”, established in 1960 (Warburg 1930; Warburg 1956), and the “Pasteur Effect”, established in 1857 (Pasteur 1857; Pasteur 1859) [reviewed in English (Racker 1974)]. Warburg found that cancerous cells preferred glycolytic metabolism over cellular respiration, even in the presence of oxygen. His theory fell out of favor for 60 years until recently it has been revisited by researchers exploring the possibility that the metabolic changes actually cause cancer. Pasteur had focused more on the production of lactic acid as a byproduct of glycolytic metabolism in bacteria. These data combined formed the basis of PDC-linked cancer theories reported in the literature.

We investigated whether there was a change in the phosphorylation state of E1 α PDC in shK cells, which could possibly explain the unforeseen increased metastatic phenotypes. We found a higher phosphorylation ratio of phosphorylated to total E1 α PDC for shK cells with respect to both wild type and CVC6 control cells typical of increased metastatic tendencies (Koukourakis and others 2005; McFate

and others 2008; Roche and Hiromasa 2007). When we analyzed “the Warburg Effect” by lactate analysis, we found that lactate production was increased for the shK cells compared to both wild type and CVC6 cells. These data compliment our previous data showing that PDC has more phosphorylation in shK cells. With PDC activity reduced, the cells accumulate pyruvate by conversion to lactate. These data showed that shK cells had decreased BDK levels, which may have caused a permanent shift in metabolism from PDC to the BCKDC in those cells.

New evidence has shown that “the Warburg Effect” may not be entirely about glucose metabolism. DeBerardinis *et al*, showed that SF188 glioma cells grown in culture consumed glutamine beyond levels required to accommodate nitrogen demand. In fact, the authors found glutamine metabolism primarily provided anaplerotic metabolites to the TCA cycle. The driving factors for this were the requirements of NADPH and TCA cycle intermediates used in fatty acid synthesis (DeBerardinis and others 2007). Since BCAA catabolism through BCKDC produces acetyl-CoA and other CoA derivatives, it is possible that fatty acid synthesis is also increased in the shK cells as a result of increased BCKDC activation through BDK knockdown. We focused, perhaps too narrowly, on the requirements of BCAAs for the *de novo* synthesis of proteins. However, dividing cells also require lipids to encase daughter cells after mitosis is complete. The relevant lipid/protein ratios are not clearly defined, however, it is known that mitochondrial inner membranes and pig epidermal cells have a protein to lipid ratio of 3:2, human red blood cell membranes and mouse liver ratios are 1:1, and myelin-producing cells have ratios closer to 1:5. These are just some examples of the variation of protein to lipid ratios found in

tissues. Joshi et al, report on BDK knockout mice that organ tissues have differential growth rates in these animals. Particularly, BDK knockout mice had decreased brain, muscle, and adipose tissue weights, similar heart weights, and increased liver and kidney weights when compared to wild type animals with respect to wild type. The authors posit the differential endogenous activity of BCKDC in these tissues for the inconsistent differences in tissue weight (Joshi and others 2007). For instance, the liver is known to have high endogenous BCKDC activity in wild-type mice. Therefore knocking out the BDK would not alter the metabolic pathways that may be more highly regulated in other tissues (eg, brain) so that the natural metabolic network is not shifted in the opposite direction, but rather in an accelerated manner.

To indulge in further speculation, a recent report by Singh et al, studying *Staphylococcus aureus* where the investigators disrupted the transcription of BCKDC-akin genes in the bacterial genome led to decreased branched-chain fatty acid production. This alteration led to increased susceptibility to stresses and, therefore, decreased the viability of the cells (Singh and others 2008). This research agrees with another recent report showing inhibition of LAT1 transporters in eukaryotic cancer cells decreases cell proliferation and increases apoptosis through caspase 3 and 7 activities. These data indicate that shutting down the availability of BCAAs to the metabolic milieu of the cell decreases the viability of the cell. We elected to investigate if the role of the BCKDC could affect proliferation rates in a similar vane. Our results are contrary to our hypothesis about decreasing the availability of BCAAs for anabolic pathways, but it does seem that limiting BCAA entry into the metabolic milieu upstream of the BCKDC-involved processes does

1992 result in decreased proliferation. Perhaps further analyses on branched-chain fatty
1993 acid production, carbon skeleton tracking, and anabolic protein turnover would
1994 provide evidence to explain the unforeseen phenotypes seen in the shK cells and
1995 how alternate compensatory biochemical changes seem to have occurred.

1996 GBMs are difficult to treat, partially due to their location in the highly
1997 organized and compacted brain tissue. However, since these cells are
1998 hypermetabolic, it is possible that they may be preferentially susceptible to selective
1999 metabolic attack. The goal of this research was to investigate the potential of the
2000 BCKDC to be such a therapeutic target. Advances in targeted strategies for brain
2001 cancers have shown some interesting and promising results [reviewed in (Fine 2007;
2002 Lukas and others 2007)]. However, a major drawback of targeted therapies is their
2003 specificity for cancers with narrowly defined etiologies. Our strategy was an attempt
2004 to identify a more global target for cancer therapeutic agents. We hoped to find that
2005 BCKDC would be a potential target for further research and perhaps rational drug
2006 design for new, global cancer treatments. In light of the research supporting that
2007 cancer cachexia is the result of increased BCAA uptake in cancerous tissues,
2008 causing catabolic breakdown of skeletal muscle, we hypothesized that BCKDC
2009 would indeed lead to a novel therapeutic target. However, the data presented here
2010 indicate there is more to the story than BCAA shunting, but rather that this
2011 biochemical pathway may be regulated by other pathways that effect cancer cell
2012 phenotypes.

2013 It became apparent in the early stages of this research that the area of BCAA
2014 involvement in metabolic and signaling pathways pertinent to cancer biology lacked

2015 a seminal work to bring a comprehensive direction and strategy to research in this
2016 field. The rationale for the hypotheses which lay the foundation for this thesis was
2017 composed of research investigations in exercise physiology, bacterial and
2018 mammalian biochemistry, cancer cell metabolism, cancer's impact on muscular
2019 physiology and biochemistry, and genetic disorders of BCAA metabolism, to name a
2020 few. Recent reports discussed in this section do, however, begin examining the
2021 impact of BCAA metabolic changes in cancer cells. It is my opinion that this field of
2022 research has been building momentum and may be an important topic in cancer
2023 research in the near future.

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