The Ketogenic Diet in the Treatment of Malignant Glioma: Mechanistic Effects on Hypoxia and Angiogenesis

by

Eric C. Woolf

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved July 2014 by the
Graduate Supervisory Committee:

Adrienne C. Scheck, Co-Chair
Joshua LaBaer, Co-Chair
Douglas Lake

ARIZONA STATE UNIVERSITY

August 2014

ABSTRACT

Patients with malignant brain tumors have a median survival of approximately 15 months following diagnosis, regardless of currently available treatments which include surgery followed by radiation and chemotherapy. Improvement in the survival of brain cancer patients requires the design of new therapeutic modalities that take advantage of common phenotypes. One such phenotype is the metabolic dysregulation that is a hallmark of cancer cells. It has therefore been postulated that one approach to treating brain tumors may be by metabolic alteration such as that which occurs through the use of the ketogenic diet (KD). The KD is high-fat, low-carbohydrate diet that induces ketosis and has been utilized for the non-pharmacologic treatment of refractory epilepsy. It has been shown that this metabolic therapy enhances survival and potentiates standard therapy in mouse models of malignant gliomas, yet the anti-tumor mechanisms are not fully understood.

The current study reports that KetoCal® (KC; 4:1 fat:protein/carbohydrates), fed *ad libitum*, alters hypoxia, angiogenic, and inflammatory pathways in a mouse model of glioma. Tumors from animals maintained on KC showed reduced expression of the hypoxia marker carbonic anhydrase 9 (CA IX), a reduction in hypoxia inducible factor 1-alpha (HIF-1α) and decreased activation of nuclear factor kappa B (NF-κB). Animals maintained on KC also showed a reduction in expression of vascular endothelial growth factor receptor 2 (VEGFR2) and decreased microvasculature in their tumors. Further, peritumoral edema was significantly reduced in animals fed the KC and protein analysis showed significantly altered expression of the tight junction protein zona occludens-1 (ZO-1) and the water channeling protein aquaporin-4 (AQP4), both of which have been implicated in malignant processes in glioma, including the formation of peritumoral edema in patients. Taken together the data suggests that KC alters multiple processes involved in malignant progression of gliomas. A greater understanding of the effects of the ketogenic diet as an adjuvant therapy will allow for a more rational approach to its clinical use.

ACKNOWLEDGMENTS

Graduate Committee: Adrienne C. Scheck, Joshua LaBaer and Douglas Lake

Technical Training and Support: Mohammed G. Abdelwahab and Kathryn E. Fenton

Contributions: Kara Curley, Julie A. Charlton, Rajet Vatsa, Claire Woodrow, Greg Turner, Qingwei Liu, Mark C. Preul

Funding and Support: Students Supporting Brain Tumor Research (SSBTR), Arizona State University School of Life Sciences, Remi Savioz Glut 1 Foundation

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION AND BACKGROUND

Brain Tumors

Glioblastoma multiforme (GBM), the most aggressive type of brain tumor, represents one of the greatest challenges in the management of cancer patients worldwide. Despite aggressive surgery followed by radiation and chemotherapy, patients with newly diagnosed GBM have an average life expectancy of 12-18 months and less than 10% survive 5 years [1, 2]. Brain tumors are highly infiltrative, and surgery rarely removes all tumor cells, particularly from eloquent areas of the brain. Further, while radiation and chemotherapy can kill *most* of the remaining tumor cells, those that survive typically regrow. Thus, these tumors often recur within 2 years of their original diagnosis and in the same general area as the primary tumor. The proximity of the recurrent tumor to the primary tumor often precludes the use of additional standard radiation therapy because of toxicity concerns [3]. Once a GBM recurs following chemotherapy with temozolomide (TMZ), there are few additional chemotherapeutic agents with demonstrated efficacy for these tumors. The identification of new therapeutic targets for malignant gliomas has focused on molecular targets, often those found through global analyses done by The Cancer Genome Atlas consortium [4] and other groups [5-8]. This work demonstrated that there are approximately four molecular subtypes of GBMs based on genetic aberrations, gene expression profiling and protein expression. One goal of these studies is the identification of therapeutic targets and a better understanding of how to determine the patients most likely to benefit from a specific targeted agent [9]. However, solid tumors are heterogeneous, and these targets are typically not found on all cells in a tumor. The failure to successfully manage primary GBM and its recurrence remains a major challenge, and advances in survival and quality of life rely on new therapeutic approaches.

Tumor Metabolism

Cancer cells must meet the demands of rapid proliferation, thus cellular energy metabolism is one of the main processes affected during the transition from normal to cancer cells. Otto Warburg first described this shift in what we now call aerobic glycolysis or the “Warburg Effect” in 1924 [10, 11]. The

Warburg Effect describes the tumor cell's use of glycolysis to provide energy and biomolecules regardless of the availability of oxygen. Under adequate oxygenation, normal cells rely on mitochondrial oxidative phosphorylation to generate ATP and switch to the less favorable anaerobic pathway of glycolysis when exposed to hypoxia. However, many types of cancer cells survive and proliferate by generating ATP via glycolysis rather than oxidative phosphorylation even when oxygenated; a process that occurs very early in tumorigenesis, prior to hypoxia [12]. We now know that cancer metabolism is much more complex than just a higher rate of glycolysis. Mitochondrial biogenesis is also altered, and the cancer cell's fate becomes reliant on the balance between the availability of energy, sufficient macromolecular synthesis for increased growth, and the modulation of reactive oxygen species (ROS)[13, 14].

Since Warburg's discovery, metabolism has been of interest in the cancer field, but it often seemed overshadowed by discoveries of oncogenes, tumor suppressor genes, growth factor pathways, molecular subtypes of cancers, etc. There is a resurgence of interest in metabolism as a central theme in cancer, and we continue to find that metabolic pathways intersect and often regulate key components of tumor initiation, progression and therapy response [15, 16]. Many pathways long known to be associated with tumor cell growth, escape from apoptosis, aggressive blood vessel formation (angiogenesis) and therapy resistance have now been linked to cellular metabolism [17]. For example, p53 is a tumor suppressor encoded by *TP53* which is frequently mutated in cancer. P53 promotes a variety of cellular responses to hypoxia, DNA damage and oncogene activation; however, recently it has been found to regulate glycolysis and assist in maintaining mitochondrial integrity [18]. The overactivation of the stress responsive PI3K/AKT signaling pathway, typical in many cancers, has also been closely linked to metabolism and under low glucose conditions results in rapid tumor cell death [17, 19, 20]. Hypoxia, a common occurrence in the tumor microenvironment, induces hypoxia inducible factor 1 (HIF-1), which regulates the uptake of glucose and the expression of a number of genes involved in glycolysis and energy metabolism [21]. Myc, an oncogene long known to be involved in malignant cell transformation has also been shown to play a role in metabolic regulation, particularly in response to changes in the tumor microenvironment [22]. These connections, and others, suggest that targeting metabolic changes can and should be considered in the context of other, more classic therapeutic targets.

The Ketogenic Diet

The ketogenic diet (KD) is a medically regimented, high-fat low protein/carbohydrate diet used to treat refractory pediatric epilepsy [23, 24]. It simulates fasting, thus increasing ketones and decreasing glucose in the blood, leading to high rates of fatty acid oxidation and an increase in the production of acetyl-CoA. When the amount of acetyl-CoA exceeds the capacity of the tricarboxylic acid cycle to utilize it, there is an increase in the production of the ketone bodies β–hydroxybutyrate (βHB), acetoacetate (ACA) and acetone which can be used as an energy source in the brain [25-28]. While the mechanisms are not fully understood, the neuroprotective effects of a KD on the brain have led to interest in using it for the treatment of a host of neurological disorders including Alzheimer's disease, traumatic brain injury and amyotrophic lateral sclerosis [29, 30].

It has been postulated that the KD may be useful as a therapeutic strategy in exploiting tumor metabolism and the Warburg Effect. Unlike normal brain cells, many tumor cells cannot utilize ketones effectively due to their various genetic and mitochondrial defects, and must rely on glucose as their primary energy source [14, 31-36]. In addition, evidence suggests that ketone bodies may also be directly toxic to some human tumor cells [37-39]. So by reducing the glucose availability to cancer cells and providing ketones as an alternative energy source for normal cells, the KD may offer an approach to targeting the Warburg Effect in highly glycolytic tumors, such as malignant gliomas. However, as we have learned primarily from the epilepsy literature, the action of the KD is more complex and its anti-tumor actions are likely to extend beyond the effects of reduced blood glucose. For example, the KD has been shown to reduce reactive oxygen species (ROS) production in the brain [23, 40]. ROS are multi-faceted effector molecules involved in numerous cellular pathways, including those regulating autophagic/apoptotic responses to genotoxic stress, inflammation, hypoxia and nutrient deprivation. Cancer cells often have increased levels of ROS [41] and they have been implicated in angiogenesis induction and tumor growth through the regulation of the vascular endothelial growth factor (VEGF) pathway and HIF-1 [42].

The KD in Preclinical Studies

Recent studies have shown that the KD causes a reduction in blood glucose, an elevation of blood ketones and extends life in an immunocompetent mouse model of malignant glioma [43, 44]. Results demonstrated that increasing blood ketones affects a number of tumor-related gene networks. This includes alteration in the expression of genes like cyclooxygenase-2 (COX-2) and others involved in the cellular response to oxidative stress in tumor tissue. There was also a concomitant reduction in reactive oxygen species (ROS) [43]. A separate study using the same model found that the KD plus radiation therapy also reduced expression of COX-2 while reducing the production of ROS [45] *in vivo*. Additional changes in gene expression suggest that the KD may inhibit insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF) and epidermal growth factor receptor (EGFR) signaling pathways [39] as has also been shown in various caloric restriction (CR) and restricted ketogenic diet (RKD) studies [46-48].

The KD not only targets specific aspects of tumor biology as described above, but may also exert a global effect on the aberrant genetic landscape found in tumors. Results using cDNA Array technology demonstrated that overall gene expression in tumor from animals fed the KD was shifted more towards the gene expression found in non-tumor containing tissue from animals fed either the KD or standard diet [43] (Figure 1). While the mechanism(s) through which this global shift in gene expression as a result of the KD is not known, one hypothesis is that the KD may be altering the tumor epigenome. The epigenome describes the collection of abnormal, heritable changes in gene activity that are not caused by changes in the DNA sequence [49]. These modifications include chromatin remodeling, histone modifications, DNA methylation, and microRNA pathways; all of which have now been linked to metabolism in many cancers, including brain tumors [50, 51]. Epigenetic changes in cancer are now being looked at as potential therapeutic targets. New therapies such as histone deacetylase (HDAC) inhibitors are actively being tested for their ability to reverse the abnormal gene expression patterns inherent to the cancer epigenome and for their ability to enhance other anti-tumor therapies [52, 53]. A recent study suggests that β-hydroxybutyrate (βHB), the major ketone that is increased in the blood in animals and patients on the KD can alter the epigenetic landscape in mammalian cells by inhibiting HDAC [54]. Another study showed that

the KD reversed the major epigenetic modifications found in the brains of epileptic rats [55]. While the effect of the KD on tumor epigenetics has yet to be studied directly, evidence warrants further exploration.

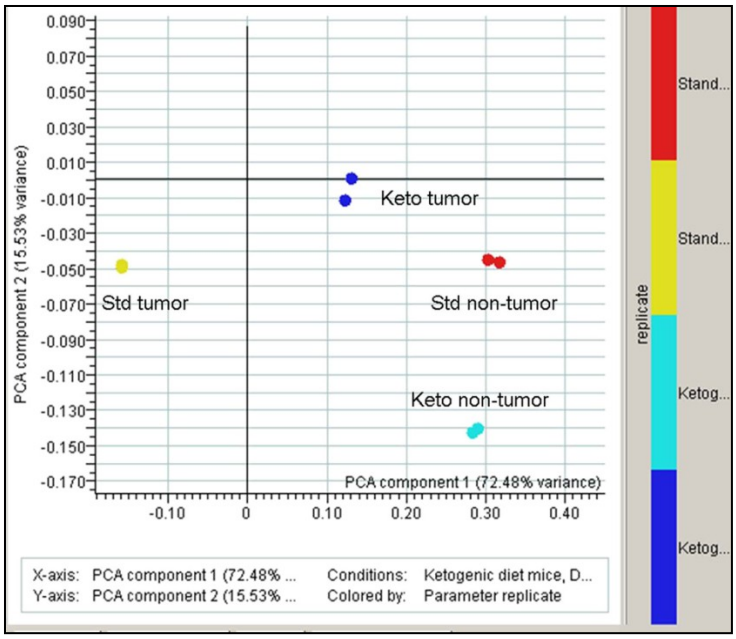

Figure 1: cDNA Microarray Data [43]. Eight microarrays were analyzed by 2-way ANOVA for interaction effects, using standard Bonferonni multiple testing correction. There were strong interactions between ketogenic diet and normal diet in non-tumor classes, especially in context of standard diet non-tumor. The trend is that many expression profiles in tumor mice on a ketogenic diet seem to trend back to a profile seen with mice living on a standard diet having no tumor.

KD as an Adjuvant Therapy

While the studies described above show that the KD and/or CR provides various anti-tumor benefits on their own, evidence suggests that they may also enhance other therapies for brain tumors by either protecting normal tissue, working in synergy with other treatments, or both. Gene expression changes in the tumors from animals fed the KD were not the same as those in the non-tumor containing contralateral side of the brain [39, 43]. This allows for the hypothesis that the neuro-protective activity of blood ketones may also function to reduce the deleterious side effect of cranial radiation on normal brain. A recent publication showed that fasting, which elevates blood ketones, not only sensitizes many types of cancer cells to standard therapies but may promote the protection of normal tissue from the toxicity associated with radiation and chemotherapy [56].

The KD as an adjuvant enhanced the anti-tumor effects of both chemotherapy and radiation. KD greatly enhanced survival in a mouse model of glioma when combined with TMZ when compared to either treatment alone [57]. In addition, a separate study showed that 9 out of 11 animals maintained on the KD and treated with radiation had complete and sustained remission of their implanted tumors, even after being switched back to a standard rodent diet [44] (Figure 2). Another recent study found that combining the KD with radiation and chemotherapy resulted in decreased tumor growth rate and increased survival in a lung cancer xenograft model [58]. Studies also show that CR and fasting may act in synergy with other anti-cancer therapeutics [56, 59-62].

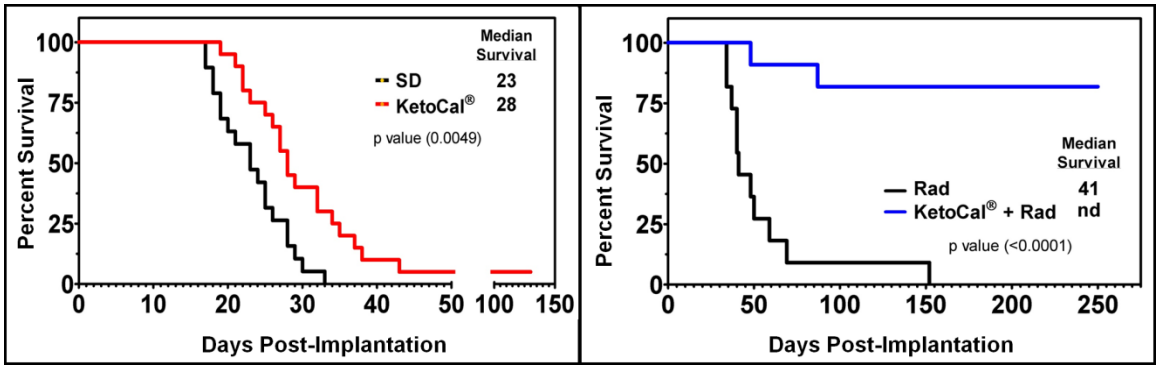

Figure 2: Kaplan-Meier Survival Analysis of Tumor-Bearing Mice [44]. Kaplan-Meier plot of survival in KC versus SD (left), radiation versus KC plus radiation (right). Animals on KC survived significantly longer when treated with KC alone (p,0.005), or when combined with radiation (p,0.0001). Results are a combination of (left) 4 separate experiments and (right) 2 separate experiments.

It has been suggested that the KD and/or caloric restriction may be more effective in combination with other agents targeting metabolism and specifically glucose. It has been shown that a KD given in combination with the glycolysis inhibitor, 2-deoxy-D-glucose (2DG), reduced the growth of a mouse CT-2A astrocytoma to a greater extent than either therapy administered alone [63]. Metformin, a therapy for diabetes mellitus, and the analog phenformin are becoming a focus in the cancer metabolism research community due to their antitumor activity in a variety of *in vitro* and *in vivo* cancer models, including brain tumors [64]; however they have not been investigated in combination with CR or the KD. Hyperbaric oxygen is another experimental anti-cancer therapy that works by reversing tumor hypoxia, which can contribute to a tumor's dependence on glycolysis [65] and it has recently been demonstrated that the KD showed a

synergistic effect when used with hyperbaric oxygen therapy, prolonging survival in a mouse model of metastatic cancer over either therapy alone [66].

The Ketogenic Diet in Humans

The first use of the KD for the treatment of human malignant brain tumors was in 1995 by Nebeling and colleagues [67]. The patients in this study were two female children diagnosed with nonresectable advanced stage brain tumors (anaplastic astrocytoma stage IV and cerebellar astrocytoma stage III), both of which had undergone extensive radiation and chemotherapy. The goal of the study was to determine if dietary induced ketosis could decrease the availability of glucose to disrupt tumor metabolism while maintaining the nutritional status of the patients. Both children responded remarkably well to the KD, showing a reduction in glucose uptake and experiencing long-term tumor management.

In 2010, researchers in Italy published a case report on 65-year-old female patient with multicentric glioblastoma multiforme (GBM) that was treated with a restricted calorie ketogenic diet (RKD) during standard radiation and chemotherapy. The patient followed the 4:1 (ratio of fats:carbohydrate plus protein) ketogenic diet restricted to 600kcal/day which resulted in reduced levels of blood glucose and elevated levels of urinary ketones. After two months on the diet, the patient's body weight was reduced by about 20%, however most importantly; no observable brain tumor was detected using either Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) or magnetic resonance imaging (MRI). Ten weeks after stopping the diet, the tumor recurred and CPT11 (Irinotecan) and bevacizumab chemotherapy was initiated [68]. The patient succumbed to her disease less than 2 years after diagnosis. Nevertheless, this work demonstrated that the RKD could be tolerated in a brain tumor patient, at least for a short period of time, and it appeared to have some efficacy. In 2011, German researchers evaluated the restricted-calorie KD in 16 subjects with various types of advanced cancers who had exhausted all therapeutic options. This pilot trial showed that the KD did not cause any severe side effects or changes in cholesterol or blood lipids. Of the 16 subjects, 5 were able to complete the 3-month treatment period and none of these patients experienced further tumor progression while on the diet [69]. Two of the 11 remaining patients died early following the beginning of the trial, one was unable to tolerate the diet and

dropped out immediately, 2 patients dropped out for personal reasons, one was unable to continue the diet for more than a month and 3 had disease progression within less than 2 months of starting the diet and one dropped out to resume chemotherapy. On the whole, this pilot study demonstrated that the KD could be tolerated in some patients with advanced disease and it appeared to be beneficial in 5 of the 16 subjects; however, the overall results were hard to interpret based on the variety and severity of disease in the enrolled patients.

More recently, a number of prospective clinical trials have been initiated. A study in Germany is evaluating the efficacy of a calorie-restricted ketogenic diet and transient fasting during re-irradiation for patients with recurrent GBM (ClinicalTrials.gov, NCT01754350). Michigan State University is directing a similar trial evaluating a calorie-restricted KD for the management of recurrent GBM (NCT01535911). A third pilot study is evaluating the KD as adjunctive treatment in refractory/end-stage GBM (NCT01865162). In Pittsburgh, another study is evaluating the KD in a variety of advanced or metastatic cancers (NCT01716468). The goals for all of these studies are to obtain data on the safety, efficacy and tolerability of the KD as an adjunctive therapy for patients with GBM.

The only study using the KD as an up-front, concurrent therapy has recently been approved and is now open for enrollment at St. Joseph's Hospital and Medical Center and Barrow Neurological Institute in Phoenix, Arizona (NCT02046187). This trial for patients with primary GBM will evaluate the classic 4:1 ketogenic diet therapy during radiation treatment and concurrent temozolomide followed by the modified Atkins Diet (1:1 fat:carbohydrate plus protein) during temozolomide treatment.

While the KD holds promise as an anti-cancer therapy, clinical utilization is not without its challenges. More data is needed to define the optimum "therapeutic range" for blood glucose and ketone levels, and to determine if varying formulations of the KD would be more effective in different individuals. Further studies are also needed to determine the necessary duration and long term effects of the KD. A limited number of papers suggest that long term use of the UKD may in fact have deleterious effects including glucose intolerance in rats [70-72]. However, it should be noted that the composition of the diet is critical, and the ongoing support of a registered dietician well versed in its use can reduce the likelihood of adverse effects in humans. In addition, the medical community must be educated on the therapeutic value of metabolic alteration as an adjuvant therapy, even if it results in a small amount of healthy weight

loss, since the current dogma is to avoid weight loss in patients undergoing chemotherapy for fear of increased fatigue and further decline in overall patient health. In fact, it has been suggested that a high fat diet may even reduce cachexic weight loss, a source of reduced overall health in cancer patients [73]. As with any clinical decision, implementation of therapy must be guided by the assessment of the patient's individual situation, which should include nutritional status. Quality of life is also a concern as this type of nutritional therapy requires discipline, motivation and careful guidance by a registered dietician experienced in implementing the KD. Compliance can be made more difficult by the use of steroids (prescribed for peritumoral edema) that often increase hunger and raise blood glucose levels. Despite these caveats, the existing preclinical data suggesting anti-tumor efficacy and a synergistic effect with standard therapies provides a strong impetus to conduct controlled clinical trials, particularly those that will shed light on the interactions between the KD and other therapies.

Introduction to the Current Study

The current study aims to further explore the anti-tumor mechanisms underlying the KD, specifically in the context of tumor hypoxia and angiogenesis. Mediators of the hypoxic response hypoxia including HIF-1α, NF-κB, and carbonic anhydrase IX (CA IX) were analyzed along with tumor vasculature and key mediators in the pro-angiogenic pathways, including VEGF and VEGFR2. In addition, the influence of the KD on peritumoral edema was explored and expression of tight junction proteins and aquaporins were analyzed.

MATERIALS AND METHODS

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of St. Joseph's Hospital and Medical Center (protocol number 334 (A3510-01)). All surgery was performed under ketamine/xylazine anesthesia, and every effort was made to minimize suffering.

GL261 Mouse Model of Glioma

Bioluminescent GL261-luc2 cells were derived as previously described [44] and were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100µg/ml Geneticin® (G418, Invitrogen Corp, Carlsbad, CA) at 37°C with 5% CO_2. Cells were harvested by trypsinization, washed and resuspended at a concentration of 1-2 x 10^7 cells/ml in DMEM without FCS and implanted into 10 week old female C57BL/6–cBrd/cBrd/Cr (albino C57BL/6) mice (National Cancer Institute at Frederick Animal Production Program, Frederick, MD) at an average weight of 20 grams as described [43, 44, 74]. Mice were housed in groups of 5 in the animal care facility of St. Joseph's Hospital and Medical Center in rooms with controlled temperature and humidity under a 12-hour light-dark cycle according to the guidelines in the NIH Guide for Care and Use of Laboratory Animals.

Treatment and Animal Monitoring

Following surgery, animals were fed standard rodent chow for 3 days. Animals were then randomized to remain on standard rodent chow (SD) *ad libitum* or changed to KetoCal® (KC; a generous gift from Nutricia North America, Gaithersburg, MD) *ad libitum*. KC was obtained directly from the manufacturer and is a nutritionally complete diet providing a 4:1 ratio of fats to carbohydrates plus protein

(72% fat, 15% protein, and 3% carbohydrate). A paste was prepared by mixing KC with water (2:1). Animals in each cage received a cubic inch of the paste each day which was sufficient to provide *ad libitum* feeding.

Bioluminescence was analyzed to quantify tumor burden [43]. Animals received a subcutaneous (s.c.) injection of 150µg luciferin (Perkin Elmer, Waltham, MA) per kg body weight 15 min prior to *in vivo* imaging using an IVIS® Spectrum *in vivo* imaging system (Perkin Elmer). Tumor cells were detectable 3 days post implantation (the first day they were imaged) and quantitation was done using the system's Living Image® 4.3 software. Serum β-hydroxybutyrate levels were measured using a Precision Xtra® ketone monitoring system (Abbott Laboratories, Abbott Park, IL) and blood glucose levels were tested using Nova Max® Plus blood glucose monitoring system (Nova Biomedical, Waltham, MA). Animals were weighed every 3-4 days and were euthanized upon occurrence of visible symptoms of impending death such as hunched posture, reduced mobility and weight loss [44, 75].

In Vivo Imaging of Hypoxia

At 21 days post-implantation, animals were administered HypoxiSense680™ (2 nmol/100 µl; a generous gift from Perkin Elmer, Inc.) by intraperitoneal injection and imaged 24 hours later using the IVIS® Spectrum *in vivo* imaging system (675 ex/720 em; Perkin Elmer). Spectral unmixing was performed and quantitation was done using the system's Living Image® 4.3 software. Each tumor bearing animal was imaged prior to injection of the probe to quantitate signal generated by tissue autofluorescence. Healthy, non-tumor bearing mice were injected with the probe and imaged 24 hours later to quantitate non-specific binding of the probe.

Western Blotting

On Day 21 post implantation, GL261-luc2 tumors and contralateral non-tumor containing brain tissue were homogenized in ice-cold RIPA buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-Base (pH 8.0), protease inhibitor cocktail (Cell

Signaling Technology, Beverly, MA, USA) and PhosSTOP phosphate inhibitor tablets (Roche Applied Science, Indianapolis, IN, USA). Lysates were incubated on ice with agitation for 1.5 hours and then centrifuged at 12,000 RPM for 20 minutes. Protein concentrations were determined using the Pierce® BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Forty µg of total protein from each tissue sample was resolved by SDS-PAGE on 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA). Proteins were transferred to a nitrocellulose membrane for 1 hour in standard wet transfer buffer (25mM Tris, 192mM glycine, 20% methanol) and blocked in 5% BSA in Tris-buffered saline with Tween-20 (TBST) for 1 hour at room temperature. Blots were then incubated overnight at 4°C in primary antibodies against CA IX (1:1000; Proteintech, Chicago, IL), HIF-1α (1:500; Bioss, Woburn, MA), NF-κB (1:1000; Cell Signaling Technology), p-NF-κB (1:1000; Cell Signaling Technology), CD31 (1:5000; Novus Biologicals, Littleton, CO), VEGF (1:1000; Santa Cruz Biotechnology, Dallas, TX), VEGFR2 (1:5000; Novus Biologicals), ZO-1 (1:200; Biorbyt, Cambridge, UK), Occludin (1:40; Biorbyt), Aquaporin-1 (1:1000; Abcam, Cambridge, UK) and Aquaporin-4 (1:1000; Abcam). After washing with TBST, blots were incubated in horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature and bands were visualized using the Novex® ECL Chemiluminescent Substrate Reagent Kit (Life Technologies). Each membrane was stripped and reprobed for β-actin (1:6000; Abcam) as an internal loading control. Densitometry was used to determine the ratio of the target to β-actin (VisionWorks LS 7.1 software; UVP, Upland, CA).

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed paraffin-embedded tissue sections. Following deparaffinization, antigen retrieval was done in sodium citrate buffer (10mM; pH 6.0) at 98°C for 25 min and slides were then rinsed with cool dH_2O. Sections were blocked with 1% bovine serum albumin in TBST for 1 hour at room temperature and incubated in Image-iT® FX signal enhancer (Life Technologies, Carlsbad, CA) for 30 minutes at room temperature. The sections were then incubated in antibody against CD31 (1:2500, Novus Biologicals, Littleton, CO) primary antibody overnight at 4°C in a humidified chamber. Following washes in PBS, the sections were incubated with AlexaFluor® 488 goat

anti-rabbit IgG secondary antibody (1:1500, Life Technologies) for 1 hour at room temperature. To reduce lipofucsin induced autofluorescence, sections were incubated in 1 mM $CuSO_4$ diluted in 50 nM ammonium acetate buffer (pH 5) for 1 hour at room temperature. Sections were then rinsed in dH_2O and counterstained with VectaShield™ mounting media containing DAPI (Vector Laboratories, Burlingame, CA) prior to imaging.

Immunostaining was imaged using a Zeiss LSM 710 microscope (Carl Zeiss International, Gottingen, Germany) and Zen software (Zeiss). Total CD31 staining was determined by averaging the pixel density in 5 random, 200x fields within the same tumor for each animal using Image J software (NIH, Bethesda, MD). The percentage of CD31 positive staining per analyzed area of the tumor was determined by normalized pixel threshold analysis. All values were normalized to the standard diet (SD) values and represented as a fold change.

Gene Expression Analysis

Tumor samples were dissected from non-tumor containing brain and total cellular RNA was isolated using the TRIzol® LS Reagent (Life Technologies) and conditions specified by the manufacturer. RNA was further purified using an RNeasy® Mini Kit (Qiagen, Valencia, CA), DNased using TURBO™ DNase (Ambion®, Life Technologies) and the quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The Mouse Cancer RT² Profiler™ PCR Array was performed as a fee-for-service by Qiagen. Fold changes in gene expression were calculated for tumors from animals fed a standard rodent diet versus KetoCal®.

Measurement of Peritumoral Edema

On Day 14 following tumor implantation, MR images were acquired on a Bruker Biospec 7.0T small animal MR scanner (Bruker Medizintechnik, Karlsruhe, Germany) with 72mm transmit coil and a surface receive coil. A multiple slice 2D T2-weighted RARE sequence was acquired as reference image (29 slices, 0.1mmx0.1mm, thickness 0.5 mm, TR= 4000 ms, effective TE = 60 ms, RARE factor=8) to

locate the slice with maximum size of tumor. Then a multi-echo T2 relaxometry sequence was used for T2 mapping, in which a series of T2-weighted images were obtained at 28 different echo times, starting from 10.57ms with 10.57ms increments, with in-plane resolution of 0.078mmx0.078mm, slice thickness 1.0 mm, matrix size = 192x192, field of view = 15mmx15 mm, repetition time = 3000ms. The T2 map was derived by single-exponential fitting of the data.

Statistical Methods

Statistical analyses were performed using GraphPad Prism® v 5.04 (GraphPad Software, San Diego, CA). All values are represented as the mean ± SD and significance was determined using the Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Animals fed KC show an increase β-hydroxybutyrate levels and a reduction in blood glucose

Animals fed KC had a statistically significant increase in blood βHB levels (Figure 3A) and decrease in blood glucose (Figure 3B) both 7 and 14 days post-implantation. Body weight remained stable throughout the course of the experiment (Figure 3C) and on day 21 post-implantation, all animals were euthanized to obtain tissue for *ex vivo* analysis. These findings are consistent with our previous results [44].

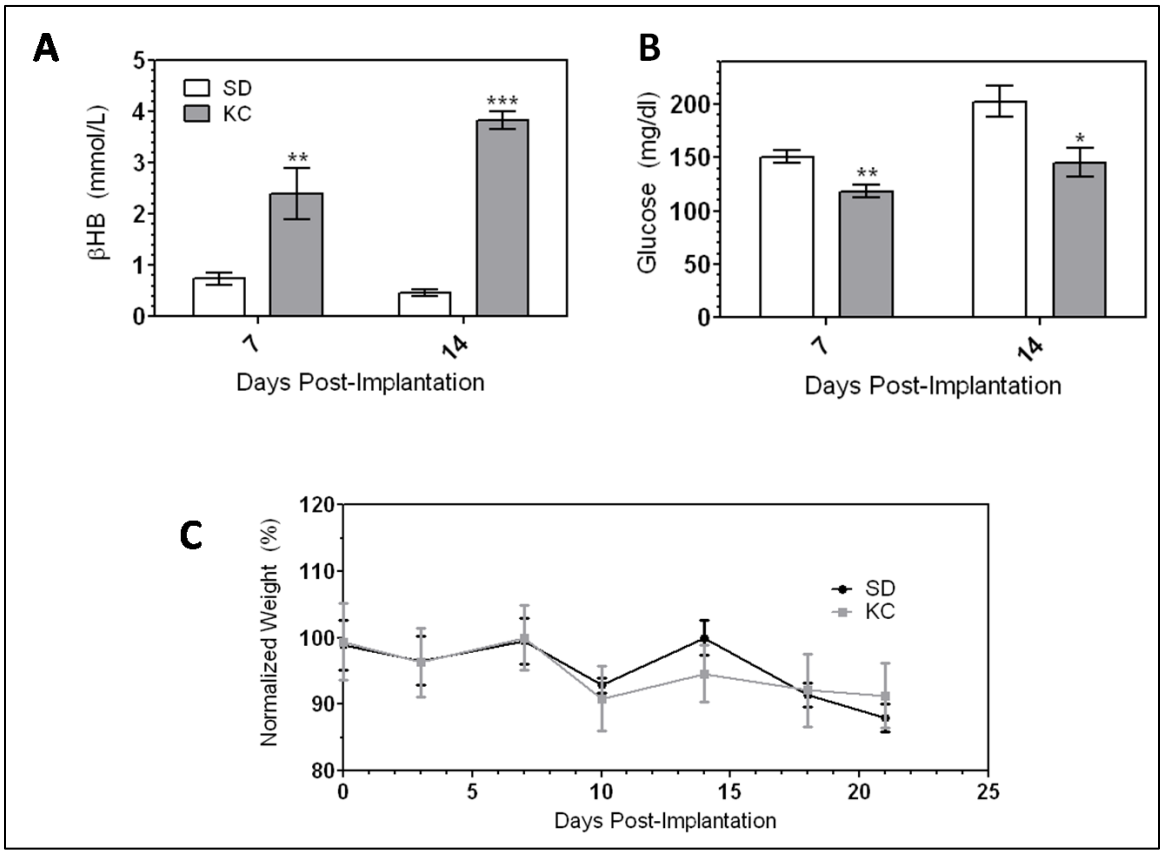

Figure 3: Animal weight, βHB and Glucose Measurements. Animal ketone and glucose measurements show (A) higher βHB blood levels and (B) lower glucose in animals treated with KetoCal®. (C) Weight measurements were taken every 3 days. Graph shows animals weights normalized to the average starting weight of each group on day zero. (N=5; *p<0.05; **p<0.01; ***p<0.001).

KC reduces *in vivo* expression of the hypoxia marker, carbonic anhydrase IX

The HypoxiSense 680™ fluorescent imaging agent detects expression of the hypoxia marker, carbonic anhydrase IX (CA IX) on the surface of tumor cells. Animals maintained on KC had a statistically significant reduction in HypoxiSense 680™ signal on Day 21 following implantation when compared to animals fed SD (Figures 4A and 4B). Tumor bearing animals were imaged prior to injection of the probe to quantitate signal generated by tissue autofluorescence (Figure 4B). Healthy, non-tumor bearing mice were injected with the probe and imaged 24 hours later to quantitate non-specific binding of the probe (Figure 4B). Bioluminescent tumor signal measured on the same day showed no significant difference in tumor signal between treatment groups (Figure 4C).

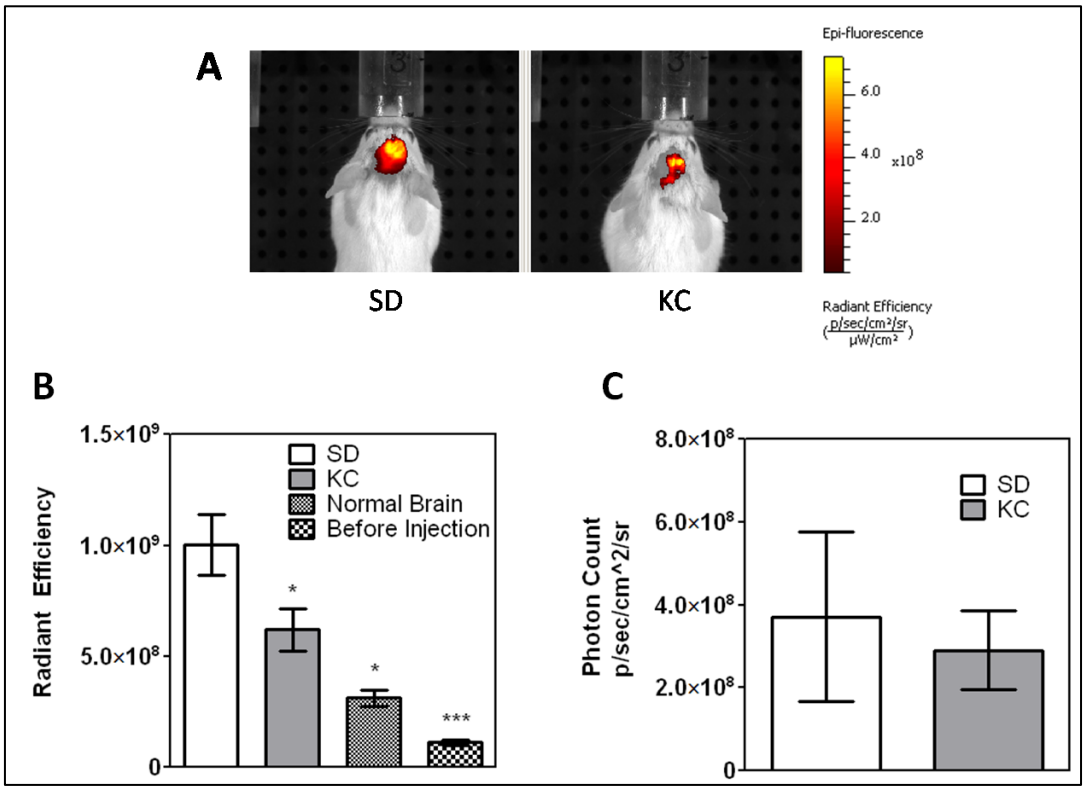

Figure 4: *In Vivo* Imaging of Hypoxia. (A) 21 days following tumor implantationHypoxiSense 680™ was used to analyze hypoxia *in vivo*. (B) Spectral unmixing was performed to quantitate fluorescent signal from tumor bearing mice on each diet (N=5; *p<0.05). Each tumor-bearing animal was imaged just prior to injection to analyze tissue autofluorescence ("Before injection"; N=5; ***p<0.001) and healthy, non-tumor bearing mice were injected to analyze non-specific binding of the probe ("Normal Brain"; N=2; *p<0.05). (C) Tumor bioluminescence was measured on the same day and showed no significant difference in tumor size between SD and KC (N=5).

KC Reduces Expression of Proteins Involved with the Hypoxic Response

Western blot analysis of tumor lysates was performed to examine the effects of KC on the expression of proteins important in the hypoxic response. Both CA IX and HIF-1α levels were significantly reduced in the tumors from animals fed KC when compared to those fed SD (Figure 5; N=6; *p<0.05; **p<0.01). Further, there was no difference in total NF-κB levels; however, there was a significant reduction in expression of the phosphorylated form, suggesting a reduction in the activation of NF-κB (Figure 5).

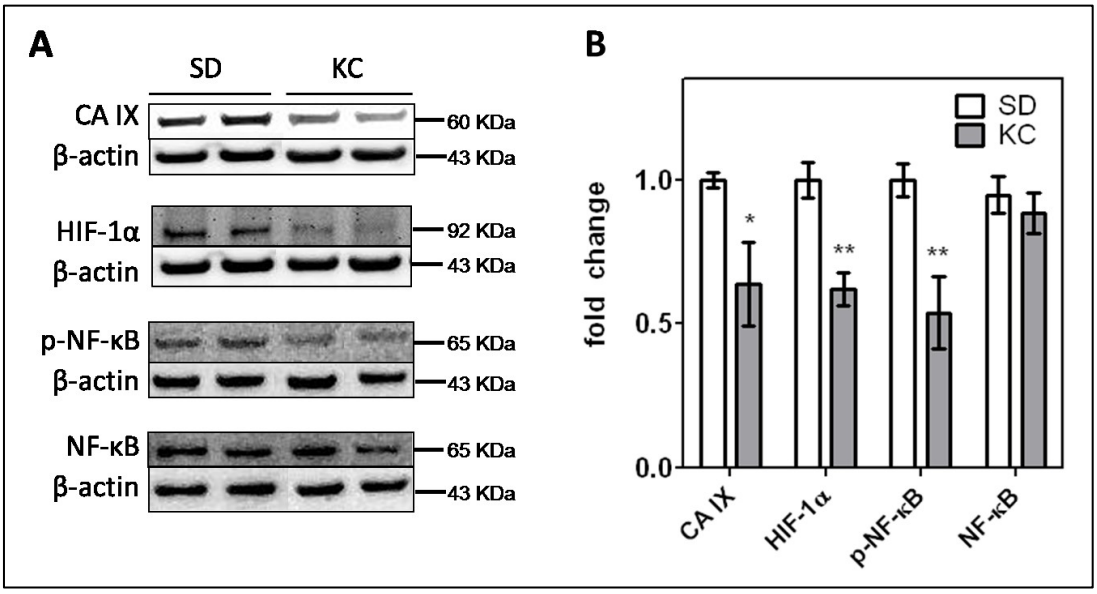

Figure 5 Western Blot Analysis of CA IX, HIF-1α, phospho-NF-κB, and total NF-κB. (A) Western blot analysis of CAIX, HIF-1α, phospho-NF-κB and total NF-κB protein expression was performed on whole lysates of tumor tissue and β-actin was used as a loading control. (B) Expression was quantified and represented as a fold change from SD (N=6; *p<0.05; **p<0.01).

KC Reduces Tumor Microvasculature

Western blot analysis was performed on whole lysate of GL261-luc2 tumors to examine the expression of the angiogenic marker, CD31 (Figure 6A). The results show a statistically significant 2-fold decrease in expression of CD31 in animals fed KC when compared to SD fed animals (Figure 6B).

These results were confirmed using immunohistochemical staining of paraffin-embedded tissue sections. We showed a reduction in blood vessels in tumors from animals fed the KC when compared to

those fed SD (Figure 6C). Quantitation of staining demonstrated a statistically significant decrease in the percentage of CD31 positive areas within the tumors of animals fed KC (Figure 6D).

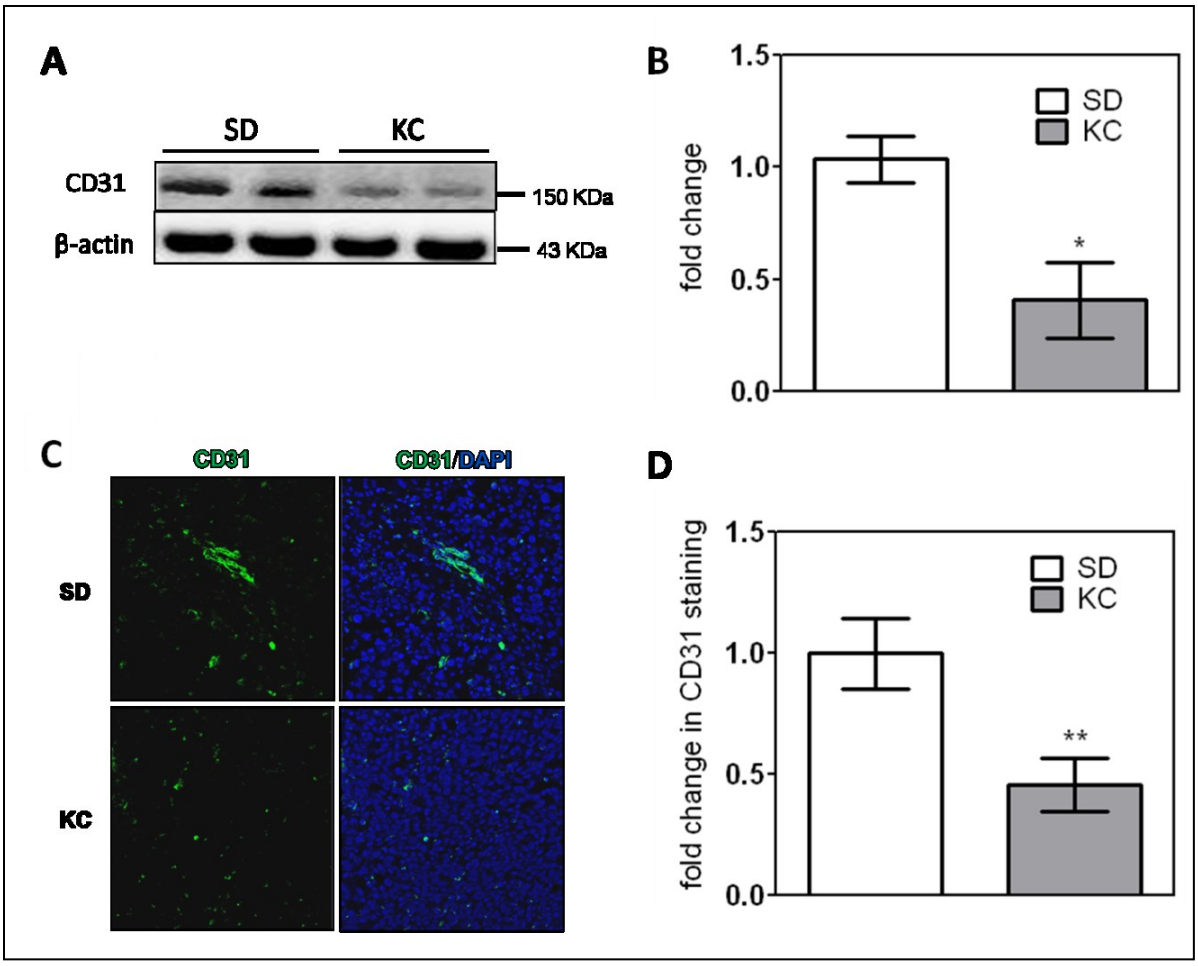

Figure 6: CD31 Expression. (A) Western blot analysis of total CD31 protein expression was performed on the whole lysates of tumor tissue and β-actin was used as a loading control. (B) Expression was quantified and represented as a fold change from SD (N=5; *p<0.05). (C) Immunostaining for CD31 was performed on formalin-fixed, paraffin-embedded tissue. Representative images from each tissue type are shown. (D) Quantification of CD31 staining was performed on 2 independent mouse brain tumors from each group. Data calculated as the average pixel density in 5 random, 200x fields within the same tumor and represented as a fold change from SD (**p<0.01).

KC alters expression of genes involved in angiogenesis and vascular modeling

Western blot analysis of tumor tissue was performed to examine the expression of the key regulators of tumor angiogenesis, VEGF and VEGFR2. Tumors from animals fed a KC showed no

difference in total VEGF expression but a significant reduction in VEGFR2 expression was found (Figure 7A and 7B).

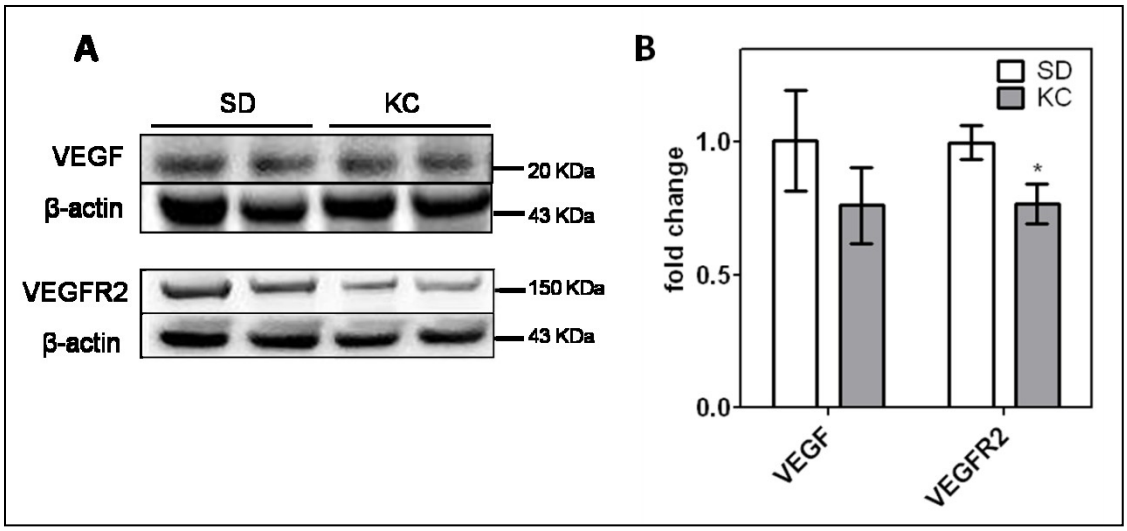

Figure 7: Western Blot Analysis of VEGF and VEGFR2. (A) Western blot analysis of total VEGF and VEGFR2 protein expression was performed on the whole lysates of tumor tissue and β-actin was used as a loading control. (B) Expression was quantified and represented as a fold change from SD (N= 6 for SD; N=5 for KC; *p<0.05).

KC Reduces Expression of Genes Involved with Angiogenesis

Tumor RNA was analyzed from animals fed KC and animals fed from SD using a mouse Cancer RT² Profiler™ PCR Array (Qiagen). The expression of a number of genes involved in various angiogenenic processes were found to be altered by KC (Figure 8). Of those genes, *Vegfb*, *Plau*, *Timp1*, *Tek*, and *Itgb1* were expressed significantly lower in the tumors from animals fed a KC when compared to tumors from animals fed SD (Figure 8).

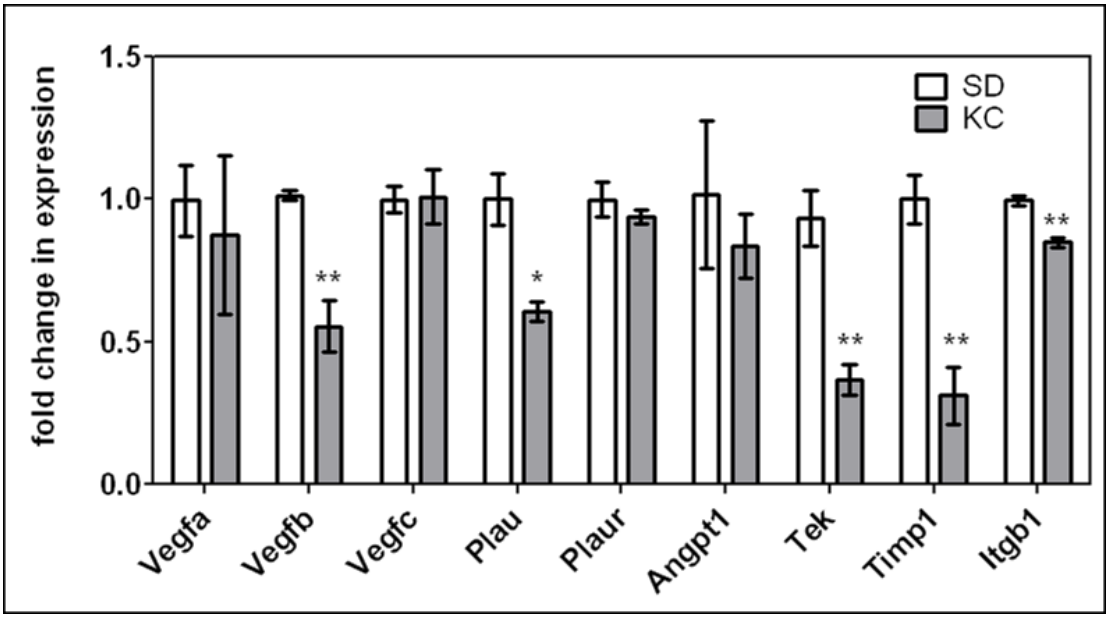

Figure 8: Gene Expression Analysis: The expression of genes involved in angiogenesis was analyzed. Data represented as the fold change difference in expression from SD tumors (N=3; *p<0.05; **p<0.01).

KC Reduces Expression and Activation of MMP-2

Western blot analysis was performed on tumor tissue to determine the expression levels of both the pro-form and the proteolytically processed activated form of MMP-2. Tumors from animals maintained on KC showed a significant reduction in both the pro- (72 KDa) and activated (65KDa) form of MMP-2 (Figure 9A and 9B).

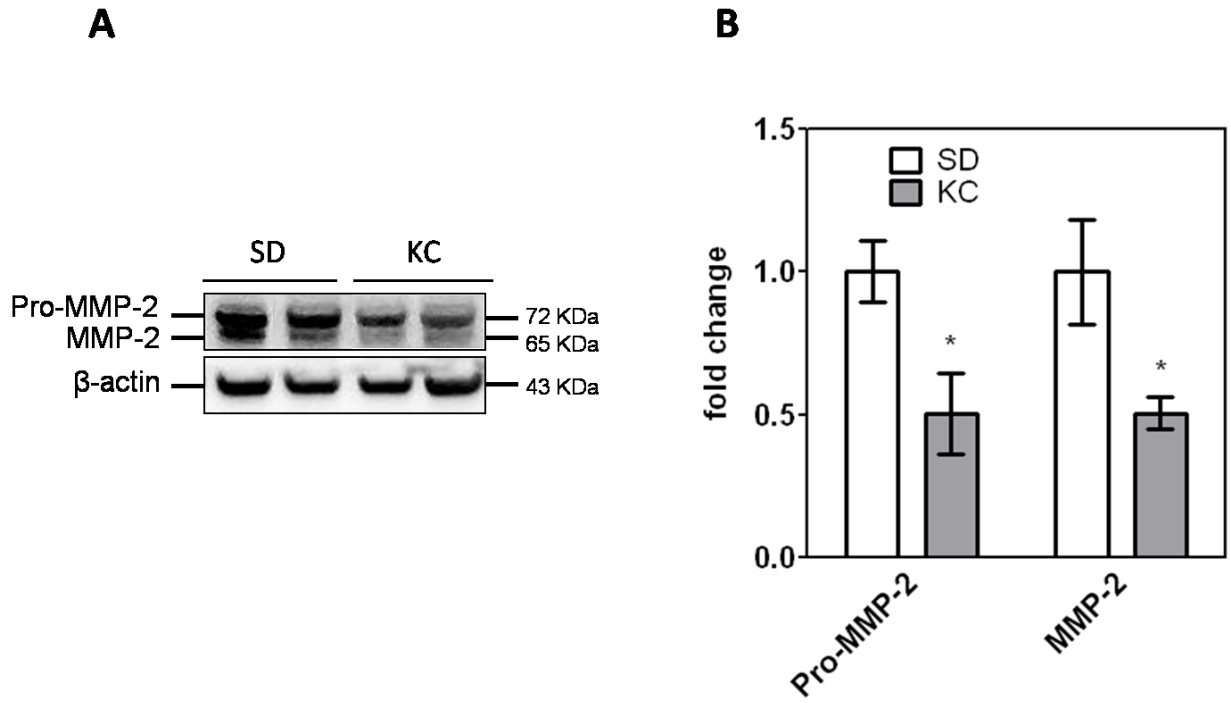

Figure 9: Western Blot Analysis of Pro- and Activated- MMP-2. (A) Western blot analysis of the pro- and activated- form of MMP-2 protein expression was performed on the whole lysates of tumor tissue and β-actin was used as a loading control. (B) Expression was quantified and represented as a fold change from SD (N=6 for SD; N=5 for KC; *p<0.05).

KC Reduces Peritumoral Edema

Peritumoral edema was measured by MRI on day 14 following implantation. Results show a statistically significant 2-fold decrease in peritumoral edema in animals fed KC when compared to those fed SD (Figure 10A). To demonstrate that the reduction in edema with the KC was not purely a function tumor size, bioluminescent tumor signals from the same day were analyzed and there was no statistical difference in signal between groups (Figure 10B). These results recapitulate previous results seen in a separate experiment (unpublished data) evaluating a higher fat formula of the ketogenic diet (6:1 fat to protein/carbohydrate; Bio-Serv F3666 KD, Frenchtown, NJ). The animals maintained on the 6:1 KD demonstrated elevated blood ketones, reduced blood glucose and a statistically significant reduction in peritumoral edema (N=6; *p<0.05; unpublished data).

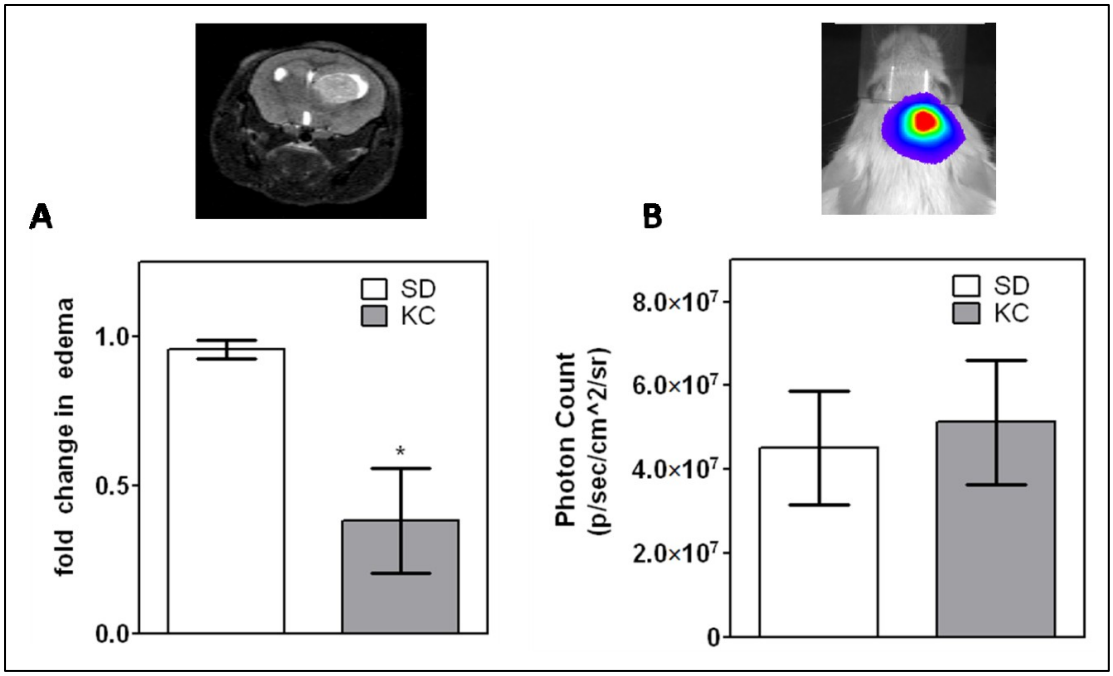

Figure 10: Peritumoral Edema Measurements. (A) 14 days following tumor implantation, edema was measured by MRI, showing a 2-fold decrease in peritumoral edema in animals fed KC when compared to those fed the SD (N=3; *=p<0.05). (B) Tumor bioluminescence was measured on the same day and showed no significant difference in tumor size between animals fed SD or KC.

KC Influences Expression of ZO-1 and Aquaporin-4

Western blot analysis of tumor tissue was performed to analyze the expression of proteins involved with peritumoral edema and glioma progression including the tight junction proteins, ZO-1 and occludin, as well as the water channeling proteins, AQPN-1, AQPN-4. Results show that tumors from animals fed a KC had a significant increase in the expression of ZO-1 but no difference in occludin expression (Figure 11A and 11B). Animals maintained on KC also showed a reduction in expression of AQPN-4 expression but not AQPN-1 (Figure 11A and 11B).

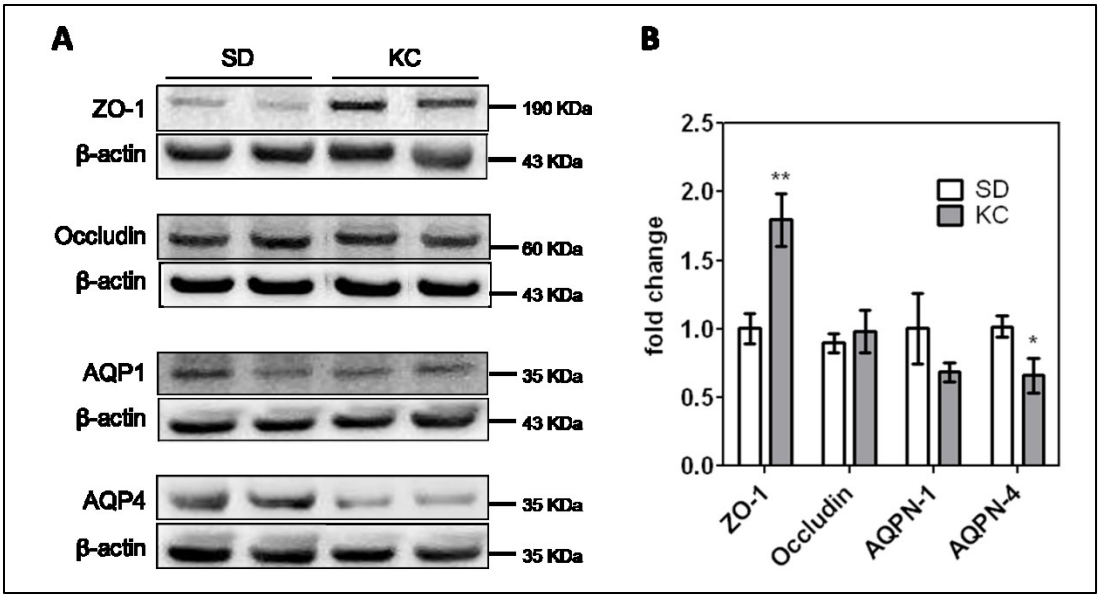

Figure 11: Western Blot Analysis of ZO-1, Occludin, Aquaporin-1 and Aquaporin-4. (A) Western blot analysis of zona occludens-1 (ZO-1), occludin, aquaporin-1 (AQP1) and aquaporin-4 (AQP4) protein expression was performed on the whole lysates of tumor tissue and samples were normalized to β-actin. (B) Quantification of expression was performed and data is represented as a fold change from SD (N=6 for SD; N=5 for KC; *p<0.05; **p<0.01).

DISCUSSION

Early attempts to use metabolism as a therapeutic target often focused on caloric restriction (CR). In 1914, Payton Rous was the first to suggest that restricted food intake reduced tumor growth by reducing the tumor blood supply [76]. More recently it has been suggested that CR reduces growth and angiogenic biomarker expression in prostate cancer and breast cancer [77-80]. The combination of caloric restriction and the ketogenic diet (restricted ketogenic diet; RKD) has also been studied as a cancer therapy. The anti-proliferative and anti-angiogenic effects of CR and the RKD have been substantiated in a number of studies using experimental mouse and human brain tumor models [14, 32, 46, 48, 81, 82]. Evidence also suggests that CR and the RKD alters inflammatory pathways, normalizes vasculature and may reduce peritumoral edema. In a mouse astrocytoma model, CR reduced expression of pro-inflammatory markers, cyclooxygenase-2 (COX-2), nuclear factor kappa B (NF-κB) and macrophage inflammatory protein (MIP-2) [83]. Seyfried and colleagues recently showed that CR promoted vessel maturation by preventing vascular VEGF signaling in the CT-2A mouse astrocytoma model [47]. Further, a recent study in the U87 human glioma model showed that CR normalized a variety of factors involved in tumor vessel instability and leakiness, including VEGF, and showed a reduction of peritumoral edema [46].

While CR and RKD can easily be administered in animal models of malignant tumors and there is anecdotal evidence and a few case reports of efficacy in humans, there has been resistance in the medical community to use CR for cancer patients. An alternative strategy is the unrestricted KD which has a long safety record for the treatment of pediatric epilepsy and may be easier for patients to maintain [84]. Thus, this approach may meet with less resistance from the clinical community.

In the present study, a bioluminescent mouse model of malignant glioma was used to demonstrate for the first time that an unrestricted KD can (1) reduce expression of the tumor hypoxia marker, CA IX; (2) alter the transcriptional response to hypoxia by reducing HIF-1α expression and decreasing activation of NF-κB; (3) reduce expression of VEGFR2 and decrease tumor microvasculature; (4) reduce peritumoral edema; (5) alter the expression of proteins that modify the tumor microenvironment during hypoxia.

Hypoxia is one of the fundamental biological phenomena found in a variety of solid tumors. Hypoxia-inducible factor-1 (HIF-1), which is controlled by rapid stabilization of the HIF1α subunit, is a pivotal transcriptional factor in the cellular response to hypoxia. The current study found a reduction in expression of HIF-1α and CA IX in tumors from animals maintained on KC. HIF-1 is a potent activator of angiogenesis and invasion through its upregulation of target genes critical for these functions including vascular endothelial growth factors (VEGF), VEGF receptors (VEGFR) and matrix metalloproteinases (MMP) [85]. Carbonic anhydrase IX (CA IX) expression is also controlled by HIF-1α and is thus considered a marker for hypoxia. Overexpression of CA IX is common in malignant glioma and correlates with poor survival in patients [86, 87]. Animals maintained on KC also showed a significant reduction in activation of nuclear factor kappa B (NF-κB) in their tumors, which is also a key regulator of the transcriptional response to hypoxia [88]. NF-κB is linked to various signal transduction pathways and to transcriptional activation events that mediate inflammation, cell proliferation, cell migration, and angiogenesis [89-91].

The hypothesis that tumor growth and progression can be limited by targeting angiogenesis has been demonstrated extensively in both preclinical and clinical studies [92]. Vascular endothelial growth factor (VEGF) pathways are the main focus of anti-angiogenic therapies, which include bevacizumab, the monoclonal antibody targeting VEGFA [93]. The current study demonstrated that KC altered the VEGF pathway at the receptor level, showing significantly reduced protein expression of VEGFR2, which is the main receptor responsible for modulating tumor angiogenesis [94]. Recent studies have shown that selective inhibition of VEGFR2 tyrosine kinase induces a radiologic response, normalizes tumor vasculature, and reduces edema while increasing patient quality of life [95-97]. Further, it has been suggested that a VEGFR2 blockade creates a "normalization window" where vessel structure is normalized and hypoxia is reduced, enhancing radiation therapy in human glioblastoma xenograft models [98]. Total VEGF at the protein level was unaltered by the KD however gene expression analysis showed a significant decrease in the expression of *Vegfb*. Although the role of VEGFB in tumor angiogenesis is not well understood, it has been suggested that it may be important for angiogenic processes in a context dependent manner [99-101]. Evidence suggests that decreased VEGFB expression is associated with improved survival and may be an indicator of response to anti-angiogenic therapies in ovarian cancer patients [102, 103]. Further analysis of gene expression showed tumors in mice fed KC also had significantly

lower expression of plasminogen activator urokinase (*Plau*) and angiopoeitin-1 receptor (*Tek*). Both are thought to play a central role in tumor invasion, metastasis and angiogenesis [104-106]. PLAU inhibitors are being actively explored as anticancer strategies in glioblastoma [107-109]. Downregulation of the uPA pathway was shown to inhibit TEK signaling and led to a reduction in angiogenesis in glioblastoma cells [110]. The angiogenic properties of TEK were substantiated in both a rat glioma model [111] and human GBM xenografts [112]. TEK was also shown to be a key molecular regulator of pathological vascularization characteristic of malignant astrocytomas [113]. We also found that β1 integrin (*Itgb1*) and tissue inhibitor of metalloproteinase 1 (*Timp1*) expression was significantly reduced when animals were maintained on KC. β1 integrin inhibitors are being explored as a treatment strategy for various cancers [114-116] and have been shown to potentiate antiangiogenic therapy in bevacizumab resistant glioblastoma [117]. In addition, β1 integrin has also been implicated in tumorigenesis, therapy resistance, invasion and metastasis [118]. Low expression levels of TIMP-1 have been associated with longer survival times in GBM patients [119, 120]. Recently TIMP-1 upregulation has also been shown to be involved in mechanisms of developed resistance to anti-VEGF treatment [121].

Currently, bevacizumab is the only FDA-approved molecular drug targeting angiogenesis in GBM; however, this drug often results in adverse effects and only a limited improvement in survival [122]. In addition, it has been shown that bevacizumab treatment can lead to increased regions of hypoxia, enhanced matrix metalloproteinase-2 (MMP-2) function, and a more invasive, treatment-resistant glioma phenotype [123, 124]. MMPs are enzymes that remodel the extracellular matrix and alter surface protein expression. Increased expression and activation of MMP-2, which is linked to HIF-1α expression [125], can lead to cellular invasion [126] and increased BBB permeability [127]. A study using a human xenograft model showed that silencing pro-MMP-2 resulted in decreased expression of VEGFR2 and enhanced radiosensitivity [128]. Protein analysis of tumors from animals fed KC showed reduced expression of both pro- and activated-MMP-2, suggesting that KC may reduce the invasive potential of cells within the tumor. If metabolic therapy could be used to provide a less toxic way to limit angiogenesis it may mimic the beneficial effects of bevacizumab while limiting the pro-invasive selection seen with anti-angiogenic agents.

An additional reason angiogenesis presents a clinical challenge is that tumor vasculature, in contrast to healthy vessels, is often immature, highly permeable, structurally and functionally abnormal [129, 130]. This can impair delivery of therapeutic agents, alter the tumor microenvironment, drive tumor cell extravasation, allow for the rapid influx of inflammatory cells, and lead to peritumoral edema [131]. A byproduct of this inflammation is the buildup of fluid around the tumor, or peritumoral edema, which is a frequent cause of morbidity and mortality in patients with gliomas. Dexamethasone is the current treatment of choice for peritumoral inflammation and edema, yet it comes with adverse side effects such as hyperglycemia, cardiovascular effects, osteoporosis, weight gain, insomnia, infection and cognitive effects which ultimately reduce the quality of life for patients [132, 133]. The current study found a reduction in peritumoral edema in animals maintained on KC when compared to those fed SD. Leaky vasculature and disruption of the blood brain barrier caused by tumors is thought to occur in part because of defects in interendothelial tight junctions. Zona occludens-1 (ZO-1) and occludin are critical for maintaining the stability and functions of the tight junctions and their loss is associated with increased blood brain barrier permeability [134, 135]. ZO-1 expression was significantly increased in the tumors from animals fed KC when compared to SD; however, there was no difference in expression of occludin. Further, both irradiation and hypoxic conditions have been implicated in the breakdown of the blood brain barrier (BBB) via down-regulation of ZO-1 expression in gliomas [136, 137]. Our results suggest that KC may help mitigate peritumoral edema and BBB-breakdown by preserving tight junction proteins such as ZO-1.

Brain edema and BBB permeability are also propagated by HIF-1α expression which can increase expression of MMPs and aquaporin-4 (AQP4) [138]. Several groups have proposed the involvement of aquaporins in the pathophysiology of brain edema [139-141]. Aquaporins are the principle pathway for water movement across most cellular membranes. Of these, AQP1 and AQP4 proteins are found highly expressed in the most malignant gliomas [142, 143]. The current study found a reduction in expression of AQP4 but not AQP1 in the tumors from animals fed a KD. In addition to its well-known function in brain edema, AQP4 has also been recently reported to play a role in cell migration, invasion and survival [144-146]. When observed together, decreased AQP4 and MMP2 expression corresponded to decreased invasive ability of glioma cells [147].

CONCLUSION

The anti-tumor mechanisms through which the ketogenic diet, caloric restriction (and intermittent fasting) and other potential metabolic therapies act are not completely understood; however, the animal model data strongly suggest that metabolic alteration may be a highly effective therapy as well as a potent adjuvant to the current standard of care for malignant brain tumors. The KD and/or CR are the only therapeutic approaches that simultaneously target multiple hallmarks of cancer such as energy metabolism, hypoxia, inflammation, angiogenesis and invasion. This suggests a number of avenues for further research such as: (i) Can we mimic the effects of some current chemotherapies using metabolic alteration; (ii) will the use of the KD provide an effective way to reduce the confounding effects of tumor heterogeneity by targeting the abnormal metabolic processes underlying brain tumors and many other cancers; and (iii) can the use of standard therapies be augmented by altering the cancer's intrinsically aberrant cellular metabolism? These and other questions can only be answered using carefully constructed clinical trials that include metabolic alteration. Our increased understanding of metabolism as both a driving hallmark of cancer and an important therapeutic strategy provides important insight and suggest the KD may be an effective way to enhance the way brain tumors are treated.

REFERENCES

(1) Bloch O, Han SJ, Cha S et al. Impact of extent of resection for recurrent glioblastoma on overall survival: clinical article. *J Neurosurg* 2012;117(6):1032-1038.

(2) Anton K, Baehring JM, Mayer T. Glioblastoma multiforme: overview of current treatment and future perspectives. *Hematol Oncol Clin North Am* 2012;26(4):825-853.

(3) Weller M, Stupp R, Hegi M, Wick W. Individualized targeted therapy for glioblastoma: fact or fiction? *Cancer J* 2012;18(1):40-44.

(4) The Cancer Genome Atlas Research Group. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008;455(7216):1061-1068.

(5) Brennan C. Genomic profiles of glioma. *Curr Neurol Neurosci Rep* 2011;11(3):291-297.

(6) Brennan C, Momota H, Hambardzumyan D et al. Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS ONE* 2009;4(11):e7752.

(7) Lee Y, Scheck AC, Cloughesy TF et al. Gene expression analysis of glioblastomas identifies the major molecular basis for the prognostic benefit of younger age. *BMC Med Genomics* 2008;1:52.:52.

(8) Verhaak RG, Hoadley KA, Purdom E et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010;17(1):98-110.

(9) Masui K, Cloughesy TF, Mischel PS. Review: molecular pathology in adult high-grade gliomas: from molecular diagnostics to target therapies. *Neuropathol Appl Neurobiol* 2012;38(3):271-291.

(10) Warburg O. On the origin of cancer cells. *Science* 1956;123(3191):309-314.

(11) Warburg O, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE BODY. *J Gen Physiol* 1927;8(6):519-530.

(12) Ward PS, Thompson CB. Metabolic reprogramming: A cancer hallmark even Warburg did not anticipate. *Cancer Cell* 2012;21:297-308.

(13) Mischel PS. HOT models in flux: mitochondrial glucose oxidation fuels glioblastoma growth. *Cell Metab* 2012;15(6):789-790.

(14) Seyfried TN, Kiebish MA, Marsh J, Shelton LM, Huysentruyt LC, Mukherjee P. Metabolic management of brain cancer. *Biochim Biophys Acta* 2011;1807(6):577-594.

(15) Wolf A, Agnihotri S, Guha A. Targeting metabolic remodeling in glioblastoma multiforme. *Oncotarget* 2010;1(7):552-562.

(16) Nijsten MW, van Dam GM. Hypothesis: using the Warburg effect against cancer by reducing glucose and providing lactate. *Med Hypotheses* 2009;73(1):48-51.

(17) Marie SK, Shinjo SM. Metabolism and brain cancer. *Clinics (Sao Paulo)* 2011;66 Suppl 1:33-43.:33-43.

(18) Puzio-Kuter AM. The Role of p53 in Metabolic Regulation. *Genes Cancer* 2011;2(4):385-391.

(19) Robey RB, Hay N. Is Akt the "Warburg kinase"?-Akt-energy metabolism interactions and oncogenesis. *Semin Cancer Biol* 2009;19(1):25-31.

(20) Yang C, Sudderth J, Dang T, Bachoo RG, McDonald JG, DeBerardinis RJ. Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res* 2009;69(20):7986-7993.

(21) Semenza GL. Regulation of cancer cell metabolism by hypoxia-inducible factor 1. *Semin Cancer Biol* 2009;19(1):12-16.

(22) Yeung SJ, Pan J, Lee MH. Roles of p53, MYC and HIF-1 in regulating glycolysis - the seventh hallmark of cancer. *Cell Mol Life Sci* 2008;65(24):3981-3999.

(23) Kim DY, Rho JM. The ketogenic diet and epilepsy. *Curr Opin Clin Nutr Metab Care* 2008;11(2):113-120.

(24) Cross JH. New research with diets and epilepsy. *J Child Neurol* 2013;28(8):970-974.

(25) Morris AAM. Cerebral ketone body metabolism. *Journal of Inherited Metabolic Diseases* 2005;28:109-121.

(26) Cahill GF, Jr., Veech RL. Ketoacids? Good medicine? *Trans Am Clin Climatol Assoc* 2003;114:149-161.

(27) Vanitallie TB, Nufert TH. Ketones: metabolism's ugly duckling. *Nutr Rev* 2003;61(10):327-341.

(28) Veech RL, Chance B, Kashiwaya Y, Lardy HA, Cahill GF, Jr. Ketone bodies, potential therapeutic uses. *IUBMB Life* 2001;51(4):241-247.

(29) Stafstrom CE, Rho JM. The ketogenic diet as a treatment paradigm for diverse neurological disorders. *Front Pharmacol* 2012;3:59. Epub@2012 Apr 9.:59.

(30) Maalouf M, Rho JM, Mattson MP. The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. *Brain Res Rev* 2009;59(2):293-315.

(31) Maurer GD, Brucker DP, Bahr O et al. Differential utilization of ketone bodies by neurons and glioma cell lines: a rationale for ketogenic diet as experimental glioma therapy. *BMC Cancer* 2011;11:315:315.

(32) Zhou W, Mukherjee P, Kiebish MA, Markis WT, Mantis JG, Seyfried TN. The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutr Metab (Lond)* 2007;4:5:5.

(33) Seyfried TN, Mukherjee P. Targeting energy metabolism in brain cancer: review and hypothesis. *Nutrition and Metabolism* 2005;2:30-38.

(34) Tisdale MJ, Brennan RA. Loss of acetoacetate coenzyme A transferase activity in tumours of peripheral tissues. *Br J Cancer* 1983;47(2):293-297.

(35) Fredericks M, Ramsey RB. 3-Oxo acid coenzyme A transferase activity in brain and tumors of the nervous system. *J Neurochem* 1978;31(6):1529-1531.

(36) Seyfried TN. *Cancer as a metabolic disease: on the origin, management and prevention of cancer*. Hoboken, NJ: John Wiley and Sons, Inc.; 2012.

(37) Magee BA, Potezny N, Rofe AM, Conyers RA. The inhibition of malignant cell growth by ketone bodies. *Aust J Exp Biol Med Sci* 1979;57(5):529-539.

(38) Skinner R, Trujillo A, Ma X, Beierle EA. Ketone bodies inhibit the viability of human neuroblastoma cells. *J Pediatr Surg* 2009;44(1):212-216.

(39) Scheck AC, Abdelwahab MG, Fenton K, Stafford P. The ketogenic diet for the treatment of glioma: Insights from genetic profiling. *Epilepsy Research* 2012;100:327-337.

(40) Maalouf M, Sullivan PG, Davis L, Kim DY, Rho JM. Ketones inhibit mitochondrial production of reactive oxygen species production following glutamate excitotoxicity by increasing NADH oxidation. *Neuroscience* 2007;145(1):256-264.

(41) Fruehauf JP, Meyskens FL, Jr. Reactive oxygen species: a breath of life or death? *Clin Cancer Res* 2007;13(3):789-794.

(42) Weinberg F, Chandel NS. Reactive oxygen species-dependent signaling regulates cancer. *Cell Mol Life Sci* 2009;66(23):3663-3673.

(43) Stafford P, Abdelwahab MG, Kim DY, Preul MC, Rho JM, Scheck AC. The ketogenic diet reverses gene expression patterns and reduces reactive oxygen species levels when used as an adjuvant therapy for glioma. *Nutr Metab (Lond)* 2010;7:74.

(44) Abdelwahab MG, Fenton KE, Preul MC et al. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. *PLoS ONE* 2012;7(5):e36197.

(45) Woolf EC, Stafford P, Abdelwahab MG, Fenton KE, Preul MC, Scheck AC. The ketogenic diet potentiates radiation therapy in a mouse model of glioma: effects on inflammatory pathways and reactive oxygen species. Cancer Research 73[8 Supplement 1], 4441. 4-13-2013.

(46) Jiang YS, Wang FR. Caloric restriction reduces edema and prolongs survival in a mouse glioma model. *J Neurooncol* 2013;114(1):25-32.

(47) Urits I, Mukherjee P, Meidenbauer J, Seyfried TN. Dietary restriction promotes vessel maturation in a mouse astrocytoma. *J Oncol* 2012;2012:264039.

(48) Mukherjee P, Abate LE, Seyfried TN. Antiangiogenic and proapoptotic effects of dietary restriction on experimental mouse and human brain tumors. *Clin Cancer Res* 2004;10(16):5622-5629.

(49) Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. *Nat Rev Cancer* 2011;11(10):726-734.

(50) Venneti S, Thompson CB. Metabolic modulation of epigenetics in gliomas. *Brain Pathol* 2013;23(2):217-221.

(51) Yun J, Johnson JL, Hanigan CL, Locasale JW. Interactions between epigenetics and metabolism in cancers. *Front Oncol* 2012;2:163.

(52) Qureshi IA, Mehler MF. Developing epigenetic diagnostics and therapeutics for brain disorders. *Trends Mol Med* 2013;19(12):732-741.

(53) Azad N, Zahnow CA, Rudin CM, Baylin SB. The future of epigenetic therapy in solid tumours--lessons from the past. *Nat Rev Clin Oncol* 2013;10(5):256-266.

(54) Shimazu T, Hirschey MD, Newman J et al. Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* 2013;339(6116):211-214.

(55) Kobow K, Kaspi A, Harikrishnan KN et al. Deep sequencing reveals increased DNA methylation in chronic rat epilepsy. *Acta Neuropathol* 2013;126(5):741-756.

(56) Lee C, Raffaghello L, Brandhorst S et al. Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. *Sci Transl Med* 2012;4(124):124ra27.

(57) Scheck AC, Abdelwahab MG, Stafford P et al. Mechanistic studies of the ketogenic diet as an adjuvant therapy for malignant gliomas. Cancer Research 70[8 Supplement], 638. 2011.

(58) Allen BG, Bhatia SK, Buatti JM et al. Ketogenic Diets Enhance Oxidative Stress and Radio-Chemo-Therapy Responses in Lung Cancer Xenografts. *Clin Cancer Res* 2013;19(14):3905-3913.

(59) Raffaghello L, Lee C, Safdie FM et al. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc Natl Acad Sci U S A* 2008;105(24):8215-8220.

(60) Lee C, Safdie FM, Raffaghello L et al. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. *Cancer Res* 2010;70(4):1564-1572.

(61) Seyfried TN, Marsh J, Shelton LM, Huysentruyt LC, Mukherjee P. Is the restricted ketogenic diet a viable alternative to the standard of care for managing malignant brain cancer? *Epilepsy Res* 2012;100(3):310-326.

(62) Safdie F, Brandhorst S, Wei M et al. Fasting enhances the response of glioma to chemo- and radiotherapy. *PLoS ONE* 2012;7(9):e44603.

(63) Marsh J, Mukherjee P, Seyfried TN. Drug/diet synergy for managing malignant astrocytoma in mice: 2-deoxy-D-glucose and the restricted ketogenic diet. *Nutrition and Metabolism* 2008;5:33.

(64) Aldea M, Tomuleasa C, Petrushev B et al. Antidiabetic pharmacology: a link between metabolic syndrome and neuro-oncology? *J BUON* 2011;16(3):409-413.

(65) Kohshi K, Beppu T, Tanaka K et al. Potential roles of hyperbaric oxygenation in the treatments of brain tumors. *Undersea Hyperb Med* 2013;40(4):351-362.

(66) Poff AM, Ari C, Seyfried TN, D'Agostino DP. The ketogenic diet and hyperbaric oxygen therapy prolong survival in mice with systemic metastatic cancer. *PLoS ONE* 2013;8(6):e65522.

(67) Nebeling LC, Miraldi F, Shurin SB, Lerner E. Effects of a ketogenic diet on tumor metabolism and nutritional status in pediatric oncology patients: two case reports. *J Am Coll Nutr* 1995;14(2):202-208.

(68) Zuccoli G, Marcello N, Pisanello A et al. Metabolic management of glioblastoma multiforme using standard therapy together with a restricted ketogenic diet: Case Report. *Nutrition and Metabolism* 2010;7:33-53.

(69) Schmidt M, Pfetzer N, Schwab M, Strauss I, Kammerer U. Effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer: A pilot trial. *Nutr Metab (Lond)* 2011;8(1):54.

(70) Bielohuby M, Sisley S, Sandoval D et al. Impaired glucose tolerance in rats fed low-carbohydrate, high-fat diets. *Am J Physiol Endocrinol Metab* 2013;305(9):E1059-E1070.

(71) Ellenbroek JH, van DL, Tons HA et al. Long-term ketogenic diet causes glucose intolerance and reduced beta and alpha cell mass but no weight loss in mice. *Am J Physiol Endocrinol Metab* 2014.

(72) Coppola G, Natale F, Torino A et al. The impact of the ketogenic diet on arterial morphology and endothelial function in children and young adults with epilepsy: A case-control study. *Seizure* 2013.

(73) Tisdale MJ, Brennan RA, Fearon KC. Reduction of weight loss and tumour size in a cachexia model by a high fat diet. *Br J Cancer* 1987;56(1):39-43.

(74) Abdelwahab MG, Sankar T, Preul MC, Scheck AC. Intracranial Implantation with Subsequent *In Vivo* Bioluminescent Imaging of Murine Gliomas. *JOVE* 2011;57:e3403.

(75) Jouanneau E, Poujol D, Gulia S et al. Dendritic cells are essential for priming but inefficient for boosting antitumour immune response in an orthotopic murine glioma model. *Cancer Immunol Immunother* 2006;55(3):254-267.

(76) Rous P. THE INFLUENCE OF DIET ON TRANSPLANTED AND SPONTANEOUS MOUSE TUMORS. *J Exp Med* 1914;20(5):433-451.

(77) Mukherjee P, Sotnikov AV, Mangian HJ, Zhou JR, Visek WJ, Clinton SK. Energy intake and prostate tumor growth, angiogenesis, and vascular endothelial growth factor expression. *J Natl Cancer Inst* 1999;91(6):512-523.

(78) De Lorenzo MS, Baljinnyam E, Vatner DE, Abarzua P, Vatner SF, Rabson AB. Caloric restriction reduces growth of mammary tumors and metastases. *Carcinogenesis* 2011;32(9):1381-1387.

(79) Phoenix KN, Vumbaca F, Fox MM, Evans R, Claffey KP. Dietary energy availability affects primary and metastatic breast cancer and metformin efficacy. *Breast Cancer Res Treat* 2010;123(2):333-344.

(80) Thompson HJ, McGinley JN, Spoelstra NS, Jiang W, Zhu Z, Wolfe P. Effect of dietary energy restriction on vascular density during mammary carcinogenesis. *Cancer Res* 2004;64(16):5643-5650.

(81) Mukherjee P, El-Abbadi MM, Kasperzyk JL, Ranes MK, Seyfried TN. Dietary restriction reduces angiogenesis and growth in an orthotopic mouse brain tumour model. *Br J Cancer* 2002;86(10):1615-1621.

(82) Shelton LM, Huysentruyt LC, Mukherjee P, Seyfried TN. Calorie restriction as an anti-invasive therapy for malignant brain cancer in the VM mouse. *ASN Neuro* 2010;2(3):e00038.

(83) Mulrooney TJ, Marsh J, Urits I, Seyfried TN, Mukherjee P. Influence of caloric restriction on constitutive expression of NF-kappaB in an experimental mouse astrocytoma. *PLoS ONE* 2011;6(3):e18085.

(84) Kossoff EH, Wang HS. Dietary therapies for epilepsy. *Biomed J* 2013;36(1):2-8.

(85) Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ, Van Meir EG. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro Oncol* 2005;7(2):134-153.

(86) Proescholdt MA, Mayer C, Kubitza M et al. Expression of hypoxia-inducible carbonic anhydrases in brain tumors. *Neuro Oncol* 2005;7(4):465-475.

(87) Proescholdt MA, Merrill MJ, Stoerr EM, Lohmeier A, Pohl F, Brawanski A. Function of carbonic anhydrase IX in glioblastoma multiforme. *Neuro Oncol* 2012;14(11):1357-1366.

(88) Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S. Mechanism of hypoxia-induced NF-kappaB. *Mol Cell Biol* 2010;30(20):4901-4921.

(89) Nogueira L, Ruiz-Ontanon P, Vazquez-Barquero A, Moris F, Fernandez-Luna JL. The NFkappaB pathway: a therapeutic target in glioblastoma. *Oncotarget* 2011;2(8):646-653.

(90) Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 2006;25(51):6680-6684.

(91) Jiang L, Song L, Wu J et al. Bmi-1 promotes glioma angiogenesis by activating NF-kappaB signaling. *PLoS ONE* 2013;8(1):e55527.

(92) Jain RK. Lessons from multidisciplinary translational trials on anti-angiogenic therapy of cancer. *Nat Rev Cancer* 2008;8(4):309-316.

(93) Reardon DA, Herndon JE, Peters K et al. Outcome after bevacizumab clinical trial therapy among recurrent grade III malignant glioma patients. *J Neurooncol* 2012;107(1):213-221.

(94) Navis AC, Bourgonje A, Wesseling P et al. Effects of dual targeting of tumor cells and stroma in human glioblastoma xenografts with a tyrosine kinase inhibitor against c-MET and VEGFR2. *PLoS ONE* 2013;8(3):e58262.

(95) Friedman HS, Prados MD, Wen PY et al. Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. *J Clin Oncol* 2009;27(28):4733-4740.

(96) Jain RK, di TE, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT. Angiogenesis in brain tumours. *Nat Rev Neurosci* 2007;8(8):610-622.

(97) Verhoeff JJ, van TO, Claes A et al. Concerns about anti-angiogenic treatment in patients with glioblastoma multiforme. *BMC Cancer* 2009;9:444.

(98) Winkler F, Kozin SV, Tong RT et al. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* 2004;6(6):553-563.

(99) Mould AW, Greco SA, Cahill MM et al. Transgenic overexpression of vascular endothelial growth factor-B isoforms by endothelial cells potentiates postnatal vessel growth in vivo and in vitro. *Circ Res* 2005;97(6):e60-e70.

(100) Olofsson A, Pajusola k, Kaipainen A et al. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 1996;93:2576-2581.

(101) Li X, Kumar A, Zhang F, Lee C, Tang Z. Complicated life, complicated VEGF-B. *Trends Mol Med* 2012;18(2):119-127.

(102) Dai W, Zeller C, Masrour N, Siddiqui N, Paul J, Brown R. Promoter CpG island methylation of genes in key cancer pathways associates with clinical outcome in high-grade serous ovarian cancer. *Clin Cancer Res* 2013;19(20):5788-5797.

(103) Vecchione A, Belletti B, Lovat F et al. A microRNA signature defines chemoresistance in ovarian cancer through modulation of angiogenesis. *Proc Natl Acad Sci U S A* 2013;110(24):9845-9850.

(104) Rao JS, Gujrati M, Chetty C. Tumor-associated soluble uPAR-directed endothelial cell motility and tumor angiogenesis. *Oncogenesis* 2013;2:e53.

(105) Al-Sha'er MA, Khanfar MA, Taha MO. Discovery of novel urokinase plasminogen activator (uPA) inhibitors using ligand-based modeling and virtual screening followed by in vitro analysis. *J Mol Model* 2014;20(1):2080.

(106) Liu D, Martin V, Fueyo J et al. Tie2/TEK modulates the interaction of glioma and brain tumor stem cells with endothelial cells and promotes an invasive phenotype. *Oncotarget* 2010;1(8):700-709.

(107) Raghu H, Nalla AK, Gondi CS, Gujrati M, Dinh DH, Rao JS. uPA and uPAR shRNA inhibit angiogenesis via enhanced secretion of SVEGFR1 independent of GM-CSF but dependent on TIMP-1 in endothelial and glioblastoma cells. *Mol Oncol* 2012;6(1):33-47.

(108) Kim CK, Hong SH, Joe YA, Shim BS, Lee SK, Hong YK. The recombinant kringle domain of urokinase plasminogen activator inhibits in vivo malignant glioma growth. *Cancer Sci* 2007;98(2):253-258.

(109) Gondi CS, Lakka SS, Dinh DH, Olivero WC, Gujrati M, Rao JS. Intraperitoneal injection of a hairpin RNA-expressing plasmid targeting urokinase-type plasminogen activator (uPA) receptor and uPA retards angiogenesis and inhibits intracranial tumor growth in nude mice. *Clin Cancer Res* 2007;13(14):4051-4060.

(110) Raghu H, Lakka SS, Gondi CS et al. Suppression of uPA and uPAR attenuates angiogenin mediated angiogenesis in endothelial and glioblastoma cell lines. *PLoS ONE* 2010;5(8):e12458.

(111) Machein MR, Knedla A, Knoth R, Wagner S, Neuschl E, Plate KH. Angiopoietin-1 promotes tumor angiogenesis in a rat glioma model. *Am J Pathol* 2004;165(5):1557-1570.

(112) Zadeh G, Koushan K, Pillo L, Shannon P, Guha A. Role of Ang1 and its interaction with VEGF-A in astrocytomas. *J Neuropathol Exp Neurol* 2004;63(9):978-989.

(113) Zadeh G, Reti R, Koushan K, Baoping Q, Shannon P, Guha A. Regulation of the pathological vasculature of malignant astrocytomas by angiopoietin-1. *Neoplasia* 2005;7(12):1081-1090.

(114) Park CC, Zhang H, Pallavicini M et al. Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. *Cancer Res* 2006;66(3):1526-1535.

(115) Wang H, Zhu Y, Zhao M et al. miRNA-29c suppresses lung cancer cell adhesion to extracellular matrix and metastasis by targeting integrin beta1 and matrix metalloproteinase2 (MMP2). *PLoS ONE* 2013;8(8):e70192.

(116) Wiktorska M, Sacewicz-Hofman I, Stasikowska-Kanicka O, Danilewicz M, Niewiarowska J. Distinct inhibitory efficiency of siRNAs and DNAzymes to beta1 integrin subunit in blocking tumor growth. *Acta Biochim Pol* 2013;60(1):77-82.

(117) Carbonell WS, DeLay M, Jahangiri A, Park CC, Aghi MK. beta1 integrin targeting potentiates antiangiogenic therapy and inhibits the growth of bevacizumab-resistant glioblastoma. *Cancer Res* 2013;73(10):3145-3154.

(118) Jahangiri A, Aghi MK, Carbonell WS. beta1 Integrin: Critical Path to Antiangiogenic Therapy Resistance and Beyond. *Cancer Res* 2014;74(1):3-7.

(119) Crocker M, Ashley S, Giddings I et al. Serum angiogenic profile of patients with glioblastoma identifies distinct tumor subtypes and shows that TIMP-1 is a prognostic factor. *Neuro Oncol* 2011;13(1):99-108.

(120) Aaberg-Jessen C, Christensen K, Offenberg H et al. Low expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in glioblastoma predicts longer patient survival. *J Neurooncol* 2009;95(1):117-128.

(121) Lucio-Eterovic AK, Piao Y, de Groot JF. Mediators of glioblastoma resistance and invasion during antivascular endothelial growth factor therapy. *Clin Cancer Res* 2009;15(14):4589-4599.

(122) Patel M, Vogelbaum MA, Barnett GH, Jalali R, Ahluwalia MS. Molecular targeted therapy in recurrent glioblastoma: current challenges and future directions. *Expert Opin Investig Drugs* 2012.

(123) Furuta T, Nakada M, Misaki K et al. Molecular analysis of a recurrent glioblastoma treated with bevacizumab. *Brain Tumor Pathol* 2014;31(1):32-39.

(124) Hu YL, DeLay M, Jahangiri A et al. Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. *Cancer Res* 2012;72(7):1773-1783.

(125) Fujiwara S, Nakagawa K, Harada H et al. Silencing hypoxia-inducible factor-1alpha inhibits cell migration and invasion under hypoxic environment in malignant gliomas. *Int J Oncol* 2007;30(4):793-802.

(126) Ding T, Ma Y, Li W et al. Role of aquaporin-4 in the regulation of migration and invasion of human glioma cells. *Int J Oncol* 2011;38(6):1521-1531.

(127) Ishihara H, Kubota H, Lindberg RL et al. Endothelial cell barrier impairment induced by glioblastomas and transforming growth factor beta2 involves matrix metalloproteinases and tight junction proteins. *J Neuropathol Exp Neurol* 2008;67(5):435-448.

(128) Badiga AV, Chetty C, Kesanakurti D et al. MMP-2 siRNA inhibits radiation-enhanced invasiveness in glioma cells. *PLoS ONE* 2011;6(6):e20614.

(129) De BK, Cauwenberghs S, Carmeliet P. Vessel abnormalization: another hallmark of cancer? Molecular mechanisms and therapeutic implications. *Curr Opin Genet Dev* 2011;21(1):73-79.

(130) Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307(5706):58-62.

(131) Azzi S, Hebda JK, Gavard J. Vascular permeability and drug delivery in cancers. *Front Oncol* 2013;3:211.

(132) de GJ, Reardon DA, Batchelor TT. Antiangiogenic therapy for glioblastoma. *Am Soc Clin Oncol Educ Book* 2013;2013:71-78.

(133) Rutz HP, Herr I. Glucocorticoid administration in antiemetic therapy: Is it safe? *Cancer* 2005;103(12):2656-2657.

(134) Ma J, Wang P, Liu Y, Zhao L, Li Z, Xue Y. Kruppel-like factor 4 regulates blood-tumor barrier permeability via ZO-1, occludin and claudin-5. *J Cell Physiol* 2014;229(7):916-926.

(135) Liu LB, Xue YX, Liu YH, Wang YB. Bradykinin increases blood-tumor barrier permeability by down-regulating the expression levels of ZO-1, occludin, and claudin-5 and rearranging actin cytoskeleton. *J Neurosci Res* 2008;86(5):1153-1168.

(136) Fan L, Liu Y, Ying H et al. Increasing of blood-tumor barrier permeability through paracellular pathway by low-frequency ultrasound irradiation in vitro. *J Mol Neurosci* 2011;43(3):541-548.

(137) Fischer S, Wobben M, Marti HH, Renz D, Schaper W. Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvasc Res* 2002;63(1):70-80.

(138) Higashida T, Kreipke CW, Rafols JA et al. The role of hypoxia-inducible factor-1alpha, aquaporin-4, and matrix metalloproteinase-9 in blood-brain barrier disruption and brain edema after traumatic brain injury. *J Neurosurg* 2011;114(1):92-101.

(139) Badaut J, Fukuda AM, Jullienne A, Petry KG. Aquaporin and brain diseases. *Biochim Biophys Acta* 2014;1840(5):1554-1565.

(140) Stokum JA, Kurland DB, Gerzanich V, Simard JM. Mechanisms of Astrocyte-Mediated Cerebral Edema. *Neurochem Res* 2014.

(141) Ribatti D, Ranieri G, Annese T, Nico B. Aquaporins in cancer. *Biochim Biophys Acta* 2014;1840(5):1550-1553.

(142) Yang L, Wang X, Zhen S, Zhang S, Kang D, Lin Z. Aquaporin-4 upregulated expression in glioma tissue is a reaction to glioma-associated edema induced by vascular endothelial growth factor. *Oncol Rep* 2012;28(5):1633-1638.

(143) Wang D, Owler BK. Expression of AQP1 and AQP4 in paediatric brain tumours. *J Clin Neurosci* 2011;18(1):122-127.

(144) Nico B, Ribatti D. Role of aquaporins in cell migration and edema formation in human brain tumors. *Exp Cell Res* 2011;317(17):2391-2396.

(145) Suero Molina EJ, Ardon H, Schroeteler J et al. Aquaporin-4 in glioma and metastatic tissues harboring 5-aminolevulinic acid-induced porphyrin fluorescence. *Clin Neurol Neurosurg* 2013;115(10):2075-2081.

(146) Ding T, Zhou Y, Sun K et al. Knockdown a water channel protein, aquaporin-4, induced glioblastoma cell apoptosis. *PLoS ONE* 2013;8(8):e66751.

(147) Ding T, Ma Y, Li W et al. Role of aquaporin-4 in the regulation of migration and invasion of human glioma cells. *Int J Oncol* 2011;38(6):1521-1531.