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pH, Lactate, and Hypoxia: Reciprocity in Regulating High-Affinity Monocarboxylate Transporter Expression in Glioblastoma[1]

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Abstract

Highly malignant brain tumors harbor the aberrant propensity for aerobic glycolysis, the excessive conversion of glucose to lactic acid even in the presence of ample tissue oxygen. Lactic acid is rapidly effluxed to the tumor microenvironment via a group of plasma-membrane transporters denoted monocarboxylate transporters (MCTs) to prevent "self-poisoning." One isoform, MCT2, has the highest affinity for lactate and thus should have the ability to respond to microenvironment conditions such as hypoxia, lactate, and pH to help maintain high glycolytic flux in the tumor. Yet, MCT2 is considered to not respond to hypoxia, which is counterintuitive. Its response to tumor lactate has not been reported. In this report, we experimentally identify the transcription initiation site/s for MCT2 in astrocytes (normal) and glioma (tumor). We then use a BACmid library to isolate a 4.2-kbp MCT2 promoter-exon I region and examine promoter response to glycolysis-mediated stimuli in glioma cells. Reporter analysis of nested-promoter constructs indicated response of MCT2 to hypoxia, pH, lactate, and glucose, the major physiological "players" that facilitate a tumor's growth and proliferation. Immunoblot analysis of native MCT2 expression under altered pH and hypoxia reflected the reporter data. The pH-mediated gene-regulation studies we describe are the first to record H^+-based reporter studies for any mammalian system and demonstrate the exquisite response of the MCT2 gene to minute changes in tumor pH. Identical promoter usage also provides the first evidence of astrocytes harnessing the same gene regulatory regions to facilitate astrocyte-neuron lactate shuttling, a metabolic feature of normal brain.

Neoplasia (2017) 19, 121–134

Abbreviations: GBM, glioblastoma multiforme; ACCA, α-cyano-4-hydroxycinnamic acid; PBS, Dulbecco's phosphate buffered saline; MCS, multicloning site; ds, double stranded

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Introduction

Malignant brain tumors are among the most lethal of all cancers. Glioblastoma multiforme (GBM), a highly aggressive brain tumor that arises from astrocytes (which form the bulwark of cells in the brain and play the primary metabolic support role in neuronal function), are the deadliest with mortality closely mirroring incidence [1]. In 2016 alone, it is estimated that 78,000 new cases of brain tumor will be diagnosed in the United States, whereas 17,000 deaths are projected to occur as a result (www.abta.org), both dismal statistics. A high rate of aerobic glycolysis (glucose metabolism) [2–4] and efflux of resultant lactic acid [5], and concomitant acidification of the tumor microenvironment are hallmarks of GBMs [6–8], which allow the tumor to poison the surrounding normal brain tissue, create dead zones, and spread diffusely within the brain [7,9]. Unfortunately, glioblastoma patients succumb to these diffusely spread tumors on average 15 months after initial diagnosis, as the tumors are refractory to complete surgical resection, chemotherapy, and radiotherapy.

Two modes for harnessing the aberrant metabolism of malignant tumors in attempts to destroy them have come to the forefront in preclinical and clinical studies. The first is metabolic targeting [8,10–14], where the aberrant metabolic pathways are disrupted or blocked to cause metabolic chaos within the tumor. The second is metabolic reengineering or reprogramming [15,16], where the aberrant metabolic pathways are forced to realign with those of normal tissues and thus cause tumor growth to be shut down, or for the tumor to become susceptible to disruption via abnormal buildup of reactive metabolites.

Crucial to maintenance of high glycolytic flux from tumor to the microenvironment is a family of plasma-membrane transporters (monocarboxylate transporters; MCTs) that are responsible for efflux of lactic acid. These help maintain an enhanced glycolytic flux within the tumor while preventing metabolic crisis, as such malignant tumors need to rapidly eliminate lactic acid to the tumor microenvironment. Although 14 isoforms of MCTs have been identified by *in silico* analysis of the human genome (known as the SLC16A membrane transporter family), only 4 (MCTs 1-4) are known to be involved in lactic acid transport in normal and tumor tissue [17–19]. We [10] and others [20–23] have previously demonstrated differential expression of MCT isoforms in brain tumors versus normal brain. *In vitro* and *in vivo* studies by us and others have used RNA interference and α-cyano-4-hydroxycinnamic acid (ACCA, CHC), a small-molecule inhibitor against MCTs, to target tumor expressed MCT isoforms and selectively interfere with lactate efflux by glioma [8,10,11,23]. These studies have provided proof that targeting these MCTs can cause an initial build-up of lactate and an altered intracellular pH, followed by collapse of the glycolytic metabolic flux, and can facilitate glioma cell death. Separate studies by us and others have also shown that inhibition of glycolysis by interfering with lactate efflux can enhance radiosensitivity of these frequently radioresistant tumors by up to 10-fold [11,24].

MCT isoforms 1 and 4 have received the most attention due to their relatively higher levels of expression in tumors. Isoform 2 harbors an affinity for lactate that is 5- and 38-fold higher than that of MCTs 1 and 4, respectively [17]. However, it has not been examined in similar detail due to its lower level of expression. Given its kinetic parameters, MCT2 should serve as a sensitive probe for tumor hypoxia, lactate, and perturbation of pH. With this in mind, we investigated the regulation of human MCT2 gene to glycolytic metabolites, oxygen tension, and pH. First, we used classical molecular biological methods to isolate the gene promoter. We experimentally identified the transcription initiation site/s via 5′-RACE (rapid amplification of cDNA ends) of both astrocyte and glioblastoma mRNA, results of which indicated identical initiation sites for both normal and tumor MCT2 gene. Then, the promoter-exon I region was mapped *in silico*, and a 4.2-kbp proximal promoter fragment was isolated from a 172-kbp BACmid (bacterial artificial chromosome) library. Nested deletion mutants of the promoter fused in frame to a firefly-luciferase reporter gene were constructed to identify response of the promoter to pH, lactate, glucose, and hypoxia in U-87MG and U-251MG, two established glioma cell lines, and TS667, a primary glioma "stem-like" cell line maintained as neurospheres. Results indicated that transcription of MCT2 is indeed highly sensitive to these key physiological and metabolic stimuli. The same changes were also observed at the translational level, although to a lesser extent. Methods developed by us for assay of pH-mediated promoter activation and the resulting data outcomes constitute the first report of studies to demonstrate pH-mediated transcription regulation via reporter-gene analysis in any mammalian cell system, including tumors.

Our findings demonstrate that MCT2 gene exquisitely responds to intracellular pH (pH_i) changes, in addition to robust responses to hypoxia and to glucose/lactate. Particularly, the studies can be extended to other high-affinity enzyme isoforms expressed along the glycolytic and other aberrantly expressed metabolic pathways in highly malignant tumors to identify the interplay between enzymes, transporters, metabolites, pH_i, and oxygen tension that facilitate, regulate, and maintain an enhanced glycolytic flux.

Materials and Methods

Cell Lines, Reagents, and Culture Media

Human glioma cell lines U-87MG (HTB14) and U-251MG (CRL-2612) were obtained from the American Type Culture Collection (Manassas, VA). Their authenticity were verified by short-tandem-repeat analysis. TS667 primary glioma neurosphere cell line [25,26] was a kind gift of Dr. Nicholas Szerlip of Memorial Sloan Kettering Institute, NY. Standard powdered Dulbecco's modified Eagle's medium (DMEM; #31600-083; low glucose, 5 mM), minimum essential medium (MEM; #41500-034), and Optimem-1 (#22600–050) were purchased from Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Life Technologies. U-87MG and U-251MG cells were maintained in standard DMEM supplemented with 10% FBS or in a modified MEM (mMEM) formulated for low-serum (2% v/v) glioma cell culture (Suppl. Table 1) [27] as specified under each described method. TS667 cells were maintained as spheroids in a serum-free "neurosphere" medium (Suppl. Table 2) [27]. Basal DMEM (#90-113-PB; glucose, glutamine, pyruvate, and phenol red free) was from Corning CellGro (Manassas, VA). Normal human astrocytes were obtained from Lonza (Walkerville, MD) and maintained up to five passages in astrocyte growth medium (a derivative medium formulation based on DMEM; Lonza) supplemented with EGF, b-FGF, 10% human serum supplement, and antibiotics per manufacturer's instructions. *Escherichia coli* strains SURE (recombination deficient) and SCS110 (methylation deficient) (Stratagene, Inc., Santa Clara, CA) were used to maintain the MCT2 promoter clones. The clones were propagated in LB medium [28] supplemented with ampicillin (50 μg/ml).

Reagent Preparations

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or from Life Technologies. Small-volume solution formulations were sterilized using 0.45-μm pore-sized PVDF syringe-filter units (#229745; Celltreat, Pepperell, MA). Molecular biology reagents were obtained from New England Bio Labs (NEB, Ipswich, MA) or Life Technologies. Plasmid DNA was prepared using Qiaprep Spin Miniprep kits (Qiagen, Valencia, CA). Reporter-gene assay reagents (Dual-Glo luciferase assay system; #E2920) were from Promega (Madison, WI).

5′-RACE to Identify the Transcription Initiation Site/s of MCT2 in Astrocytes and Glioma

Total RNA was purified from exponentially growing normal human astrocytes and U-87MG glioma cells (16×10^6 cells). In brief, monolayer cultures were rinsed with cold PBS and lysed in situ using RNA STAT-60 (1 ml per 10×10^6 cells; Tel-Test, Inc., Friendswood, TX). Total RNA purified using RNeasy Mini Kit RNase-Free DNase Set (Qiagen) as described before [29] and dissolved in 1 mM EDTA (pH 8.0). Two-microgram aliquots of RNA were reverse transcribed using a reverse-transcriptase template switching system (5′ RACE System, Ver. 2.0; Invitrogen, Carlsbad, CA) to obtain sequence information for the 5′-untranslated region. Three gene-specific primers were designed based on *in silico* predicted MCT2 mRNA sequence data (NCBI accession no. NM_004731) 245 bp (5′ GAA-TGC-ATA-GGA-AAA-TCC-AA 3′), 213 bp (5′ GCT-GCT-CCA-ACC-ACA-ATC-CAA 3′), and 185 bp (5′ CCT-CCA-TCT-GGA-GGT-GGA-TGC-A 3′) downstream from the predicted 5′ terminal of mRNA. The protocol listed in the 5′-RACE system was followed except for the buffer used for polymerase chain reaction (PCR) amplification [×10 buffer consisting of 670 mM Tris-HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$, 67 mM $MgCl_2$, and 100 mM β-ME] [29]. PCR parameters used were 94°C, 2′30″ for initial denaturation; then 35 cycles of 94°C (1′), 55°C (1′), and 72°C (2′); followed by a final "polishing" step of 72°C for 5″. The PCR-amplified products were purified using a QiaQuick PCR purification system (Qiagen) and sequenced to identify the transcription initiation sites.

Isolation of MCT2 Proximal Promoter Region from a Human Genomic BACmid Clone

We conducted a gene-library-clone search in archives of the Human Genome Project (NCBI) based on our 5′-RACE data. We located a 172-kbp BAC clone (pBACe3.6 clone #RP11-597J22 in *E. coli* DH10B; BACPAC Resources, Oakland, CA) with a 160.5-kbp genomic DNA insert that harbored the transcription initiation site/s identified by 5′-RACE of MCT2 mRNA. The genomic DNA straddled the chromosomal location 12q14.1 and was mapped *in silico* using NEBcutter (tools.neb.com; NEB) to identify suitable restriction enzymes to isolate a promoter-exon I fragment of interest. The BACmid clone was propagated at 30°C in LB medium supplemented with 30 μg/ml chloramphenicol [30]. BACmid DNA was isolated using a large plasmid isolation system (PhasePrep BAC DNA kit, Sigma) and digested first with EcoRI to drop a 17.8-kbp DNA fragment. This was agarose gel purified (QiaEx II gel extraction kit; Qiagen) and double digested with AflII and MluI (based on *in silico* restriction digest analysis) to narrow down the MCT2 promoter-exon I region. A 4.2-kbp DNA fragment that harbored the transcription initiation site/s (first exon) and the proximal promoter region of MCT2 was isolated.

Putative transcription factor binding sites on the 4.2-kbp promoter region were identified by two methods: 1) the MatInspector program (ver. 8.2, 2015) under the Genomatix Software Suite (genomatix.de) [31] and 2) the CHIP-seq (chromatin immunoprecipitation-massively parallel DNA sequencing) data available under UCSC Genome Browser (genome.ucsc.edu) [32].

Luciferase Reporter Gene Construction

The promoter-exon I region was cloned into luciferase reporter gene vector pGL2-Basic (#E1641; Promega, Madison, WI) in frame with the luciferase coding sequence using a series of cloning steps (Suppl. Method 1). The plasmids were maintained in *E. coli* SURE strain to minimize recombination events within the promoter. Plasmid integrity was verified by DNA sequence analysis.

Nested Deletion Mutant Construction

The 1-kbp nested deletion mutants of the 4.2 kbp promoter were constructed from the 5′ end. In brief, an AflII (Exonuclease III sensitive) site was engineered into the 5′ terminus during cloning of the promoter into the pGL2 reporter gene vector. The construct was double digested with KpnI (Exonuclease III resistant) and AflII followed by exposure to Exonuclease III at 34°C, and the digestions were quenched at 1-min intervals under conditions we have previously reported [28,33–35].

In Vitro Reporter Gene Assays

Plasmid DNA for transfections were generated in methylation-deficient *E. coli* strain SCS110 to minimize the effects of *E. coli* DNA methylation imprinting on MCT2 promoter activity. Plasmid DNA was transfected into glioma cell lines using Fugene-HD or Fugene-6 reagent (Roche Biochemicals, Indianapolis, IN). Fugene-HD was used at a Fugene-HD:DNA ratio of 5 μl:2 μg for U-87MG glioma cells, whereas a ratio of 3 μl:2 μg was used for U-251MG and TS667 cell lines. These optimum ratios were identified by transfecting each cell line with pRL-SV40 (#E2231, *Renilla* luciferase control vector; Promega) per manufacturer's (Roche) instructions. Fugene-6 was used at a Fugene-6:DNA ratio of 3 μl:1 μg for all three cell lines. In brief, nested deletion mutants of the promoter-firefly luciferase constructs were mixed with the pRL vector at a 10:1 (μg: μg) ratio for transfection with Fugene reagents for MCT2 promoter reporter gene assays. The pRL reporter was also used to normalize transfection efficiency across experiments. The specific volumes of Fugene, plasmid DNA, and medium in wells at transfection are provided in Suppl. Method 2. Note: All transfections and reporter gene assays were conducted in antibiotic-free medium.

Metabolite and MCT Inhibitor-Based Reporter Gene Assays

Because these reporter gene studies require maintenance of cells under metabolite-supplemented but serum-starved conditions, U-87MG cells used for the studies were grown in DMEM supplemented with 10% FBS prior to harvest. Twenty-four hours prior to transfection, cells were "trypsinized" with Accutase (#AT-104; Innovative Cell Technologies, San Diego, CA; Suppl. Method 2) according to manufacturer's instructions. Cells were plated in 24-well plates in 1.0 ml of basal DMEM (HCO_3^- buffered and supplemented with 2 mM glutamine, pH 7.4; glutamine provided as a minimal energy substrate) at 0.025×10^6 cells/well for Fugene-6 transfection or at 0.05×10^6 for Fugene-HD transfection, respectively. Twenty-four hours postplating, medium in each well was replaced with 0.5 ml of prewarmed basal DMEM. Aliquots of the

transfection mix (plasmid DNA and Fugene in serum-free basal DMEM; Suppl. Method 2) were added to the wells. Eight to 12 hours posttransfection, spent medium in each well was replaced with 1.0 ml basal DMEM supplemented with the metabolites, 5 mM pyruvate, 10 mM lactate, 25 mM glucose, or with 25 mM glucose containing 10 mM ACCA [8,11]. Forty-eight hours postexposure to the metabolites, luciferase activity of cells were measured. In brief, spent medium was aspirated, and 250 µl firefly luciferase assay reagent was added to each well and mixed on a horizontal shaker for 1 minute. Fifty-microliter aliquots were pipetted into 96-well flat-bottomed white-walled polystyrene plates (#655083, Greiner Bio-One F-bottom, chimney well, Greiner, Monroe, NC). The plates were stored for 10 minutes in dark at room temperature and analyzed in a plate reader (Tecan Infinite M200; Tecan, Research Triangle Park, NC). Luminescence readings were integrated for 1000 milliseconds. Then, 50-µl aliquots of the Stop-Glo buffer were added to each well, mixed, and stored as before, and luminescence of *Renilla* luciferase was read under the same instrument settings.

Hypoxia-Based Reporter Gene Assays

Because these reporter studies provide optimal response under low serum conditions but can be masked at higher (10% v/v) standard cell culture serum levels, U-87MG and U-251MG cells destined for the studies were grown in mMEM supplemented with 2% FBS (Suppl. Table 1) prior to harvest. Twenty-four hours prior to transfection, cells were "trypsinized" with Accutase and plated in 24-well plates in 1.0 ml of Optimem-I medium containing 4% (v/v) FBS at 0.025×10^6 cells/well for Fugene-6 or at 0.05×10^6 for Fugene-HD transfection. TS667 cells were maintained as spheroids in neurosphere medium (Suppl. Table 2) prior to harvest, then dispersed with Accutase and plated in 24-well plates in Optimem-I medium containing 4% (v/v) FBS for monolayer culture for transfection. Twenty-four hours later, medium in each well (for all cell lines) was replaced with 0.5 ml of prewarmed Optimem-I medium containing 4% (v/v) FBS, and aliquots of each of the transfection mix (DNA and Fugene in serum-free Optimem-1 medium; Suppl. Method 2) were added. Eight to 12 hours posttransfection, media in the 24-well plates were replaced with 1.0 ml Optimem-I containing 4% FBS (same medium used to plate cells). The plates were placed in a modular hypoxia chamber (MIC-101; Billups-Rothenberg, Inc., Del Mar, CA) equipped with a lab-assembled O_2 monitor [36] and a flow meter (SFM3001; 3-25 LPM adjustable flow rate; Billups-Rothenberg). The chamber was purged with a 5% CO_2, 95% N_2 gas mixture until the chamber O_2 level dropped to 0.5%. The chamber was placed in a 37°C incubator and repurged 1 hour later to readjust gas-phase O_2 down to 0.5%. Note: This second step is critical for long-term maintenance of hypoxia between 0.5% and 1.0% [36]. Chamber O_2% was monitored (maintained below 1%) for the next 48 hours, the plates were removed, and luminescence of the cells was assayed as described before.

Fluorescence-Based pH Assays

Ratiometric titrations of intracellular pH were carried out as described previously by us [10]. In brief, U-87MG glioma cells (3×10^6 cells) grown in DMEM (10% FBS supplemented) to confluency were "trypsinized" with Accutase, washed once in 30 ml of the same (fresh) medium, and resuspended in the same volume of PBS. SNARF-4F ester (S-23921; Molecular Probes, Eugene, OR) was added to a final concentration of 1.25 µM, and the cell suspension was incubated for 30 minutes at room temperature in the dark. The cells were washed in PBS (30 ml), aliquoted into 3-ml samples, and resuspended in the same volume of Bis-Tris-Propane/MES buffer (120 mM KCl, 30 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM Bis-Tris propane, 20 mM MES) with pH adjusted from 6.25 to 8.25 at 0.25-pH unit increments with 1.0 M HCl or 1.0 M KOH. A total of 12.5 µl of the K^+ ionophore nigericin (#N7143, Sigma; 1 mg/ml in ethanol) was added to each sample for pH equilibration between cells and buffer for 10 minutes. Fluorescence emission spectra were obtained in a Perkin Elmer LS55 spectrometer equipped with FL WinLab software. Λ_{max} values were determined using the built-in scan application (590 nm and 650 nm, respectively). Then, time-driven ratiometric values (R) were obtained using the built-in TimeDrive application. The average of R values was tabulated to generate the ratiometric titration curve.

pH-Based Reporter Gene Assays

First, we determined the minimum KCl and nigericin concentrations that could be used in cell culture to equilibrate pH_i with pH_e while minimizing cell death. In brief, a 24-well plate was precoated with poly-L-lysine (10 µg/ml in H_2O; Sigma #P1524) to prevent potential cell detachment due to KCl and nigericin, and U-87MG glioma cells were plated in Optimem-I medium supplemented with 4% FBS (0.025×10^6 cells/well). Twenty-four hours later, KCl and nigericin were added to wells in an X-Y grid pattern. Zero, 20, 40, 60, 80, and 100 mM of KCl were placed in columns 1 to 6, and 0.5, 1, 2, and 4 µg/ml of nigericin placed in rows A to D. Twenty-four hours later, cell survival was assayed by MTT (thiazolyl blue tetrazolium bromide, #M5655, 5 mg/ml in PBS; Sigma) dye reduction assays [37]. The following protocol for pH-based reporter gene assays was developed based on the collected optimal parameters.

U-87MG and U-251MG cells (maintained in mMEM, 2% FBS medium) or TS667 spheroid cultures (maintained in neurosphere medium; Suppl. Table 2) were processed as described previously for hypoxia-based assays and plated in 24-well plates in 1.0 ml Optimem-I medium supplemented with 4% FBS at 0.05×10^6 cells/well. Eight to 12 hours postplating, medium was replaced with fresh 0.5 ml Optimem-I supplemented with 4% FBS. The glioma cells were transfected with the 3.2-kbp or 2.5-kbp nested deletion mutant constructs and the pRL control vector using Fugene-HD using the same conditions described for hypoxia-based reporter assays. Four to 6 hours later, media in well columns 1, 3, and 5 (control wells) of plate were replaced with 1.0 ml of fresh prewarmed Optimem-I medium supplemented with 4% FBS, preadjusted to pH 7.3, 7.4, and 7.5 (pH 7.4 ± 0.1), respectively (Suppl. Method 3). Media in columns 2, 4, and 6 (test) were replaced with equivalent, pH preadjusted media but supplemented with 5 mM KCl and 0.5 µg/ml nigericin. Twenty-four hours postexposure to pH-adjusted medium, the plates were analyzed for MCT2 promoter activation by luciferase assay as described previously for hypoxia-based assays.

Change in pH_i under above conditions was verified by fluorometric analysis of aliquots of U-87MG cells. Here, the cells were exposed to SNARF-4F in culture media, washed in PBS, and adjusted to pH 7.4 or 6.5 by adding 20 mM KCl and 0.5 µg/ml of nigericin immediately prior to fluorometric analysis as described before.

Culture of Glioma Cells to Evaluate Native MCT2 Expression under Hypoxia or Altered pH

For hypoxia-based studies, U-87MG, U-251MG, and TS667 cells were subcultured exactly as described under reporter gene assays

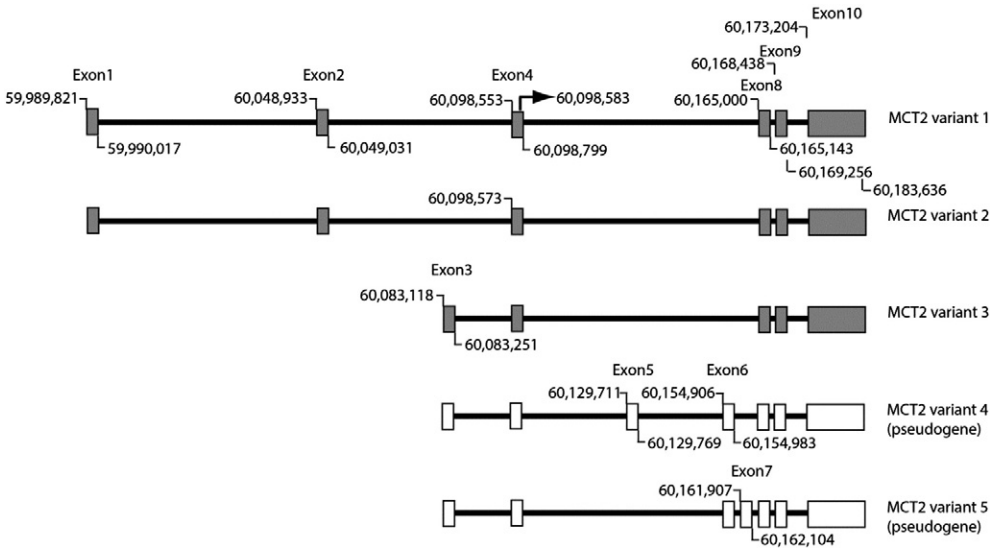

Figure 1. Schematic outline of the exon-intron structure and putative promoter region of predicted human MCT2 (SLC16A7) genomic variants. The map predictions were obtained from *Homo sapiens* chromosome 12, GRCh37.p10 primary assembly (ncbi.nlm.nih.gov; gi|224,589,803|ref.|NC_000012.11|; NM_001270623.1). Variants 1 to 3 (shaded) code for identical functional MCT2 protein. Variants 4 and 5 are nonfunctional pseudogenes (blank) with two alternate internal exons; the positioning of alternate start codons results in nonsense-mediated mRNA decay. Variants 1 and 2 are identical except for extra 20 bp in exon 4 prior to the ATG start site.

except for the final step. Here, the cells were maintained in Optimem-I medium containing 4% FBS for 16 hours, under either normoxia or hypoxia, prior to harvest of cells for membrane protein isolation (see below).

For pH-based studies, the cells were subcultured as described before and then maintained in Optimem-I medium containing 4% FBS supplemented with 5 mM KCl and 0.5 μg/ml of nigericin. One set of cell cultures was preadjusted to pH 7.3, whereas the other was maintained at pH 7.5. The cells were harvested for membrane protein isolation 16 hours post-pH exposure (see below). For comparison purposes, U-87MG and U-251MG spheroid cultures were generated in neurosphere medium as described by us previously [27].

Preparation of Membrane Fractions and Western Blotting

In brief, a protocol for subcellular fractionation of mammalian cells [38] was modified for isolation of MCT2 protein from membrane fractions of U-87MG, U-251MG, and TS667 glioma cells. First, glioma cells were suspended in a saline-HEPES buffer containing 250 μg/ml of digitonin and protease inhibitor cocktail to deplete the cells of cytosolic proteins. Then, the cells were resuspended in a saline-HEPES buffer containing 1% (v/v) Igepal CA-630 (Nonidet P-40 substitute) and protease inhibitor cocktail to extract the membrane fractions (Suppl. Method 4), which were stored at −80°C until use. Western blotting, protein quantitation, and densitometry for MCT2 expression were carried out as described in Suppl. Method 5.

Statistical Analysis

Statistical analyses were carried out using SigmaPlot 11.0 program (Systat Software, CA). Unless otherwise indicated, the results are reported as mean ± SD. *P* values were calculated using the Mann-Whitney rank sum test and considered statistically significant when *P* value was < .05.

Results

MCT2 Gene Transcription Is Initiated at Identical Genomic Sites in Both Astrocytes and Glioma

Predicted exon structure for human MCT2 gene lists five variants, of which three code for identical functional proteins whereas the remaining two are pseudogenes (Figure 1). The transcription initiation sites have remained as *in silico* predictions to date [39,40]. Thus, we chose to experimentally verify the initiation site/s via 5′-RACE of total RNA from both normal human astrocytes and the human glioblastoma cell line U-87MG cultured under physiological (5 mM) glucose levels (DMEM). The sequenced products indicated identical initiation for MCT2 transcript in both normal and tumor cells (Figure 2*A*). The mapped site was 82 to 109 bp proximal from the sites previously predicted via *in silico* analysis by other investigators (Figure 2*B*) [39,40]. A 4.2-kbp proximal promoter region for MCT2 was isolated and cloned into pGL2 reporter vector (Suppl. Figure 3). *In silico* analysis of the 4.2-kbp proximal promoter clone identified a TATA box immediately 5′ to the transcription initiation site and, most significantly, enclosed a hypoxia response element [HIF-1; gcccctgaCGTGtcctc (−)] as the most proximal element to the transcription initiation site, which previously had been listed as part of the mRNA sequence [39]. Because hypoxia response is modulated by HIF-1/ARNT (aryl hydrocarbon receptor nuclear translocator) heterodimerization and translocation, significantly, a motif for ARNT [ttccgtgaAGTGatttg (+)] was also identified 545 bp upstream of the HIF-1 site. Multiple other key cis-acting elements primary to driving tumor metabolism and proliferation were identified on the 4.2-kbp promoter clone: cAMP response elements [221(−), 4041(−)], a carbohydrate response element [1894(+)], multiple C/EBP and octamer (OCT) binding sites, and a pair of PPARγ binding sites [1269(+), 1362(−)]. A p53 binding site [2385(+)] and multiple p53 half-sites were also located on the 4.2-kbp proximal promoter clone. The ~1-kbp nested deletion

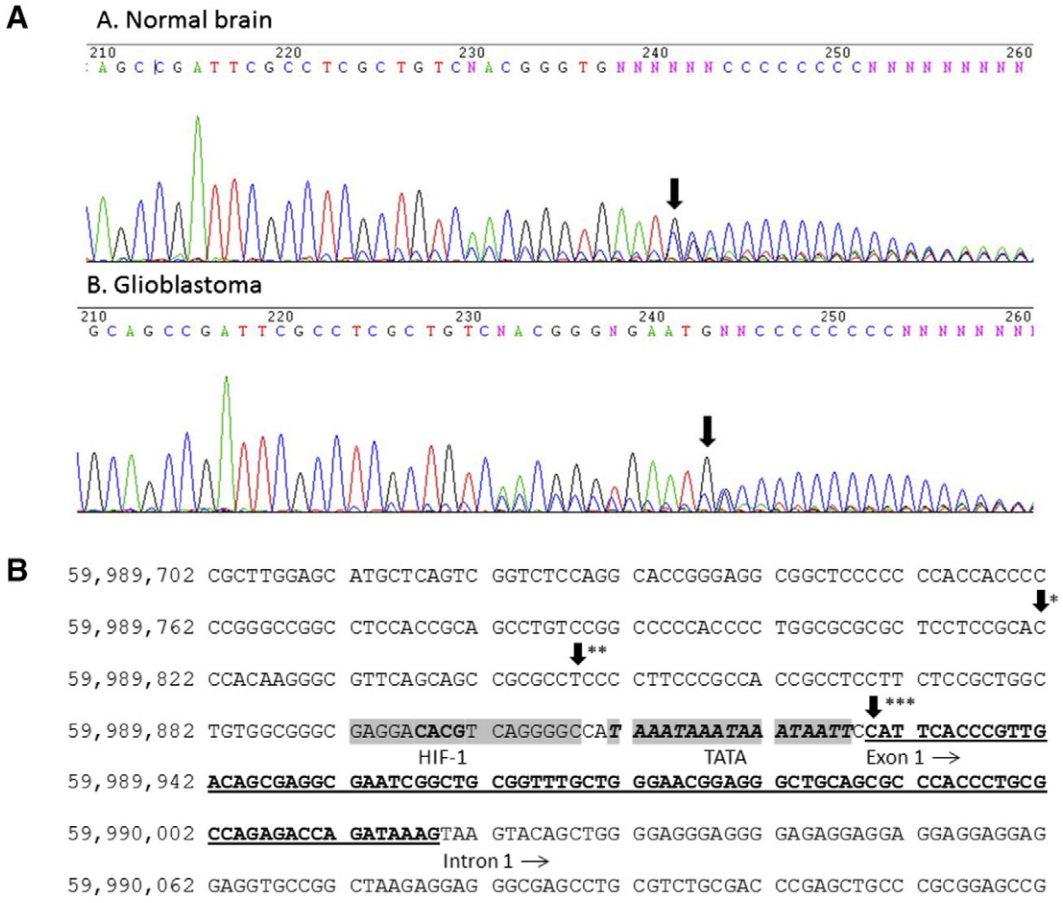

Figure 2. (A) Transcription initiation site of MCT2 is identical in both astrocytes (normal) and glioblastoma (tumor): 5′-RACE analysis of primary transcripts isolated from both astrocytes and U87-MG glioma cells indicates identical start sites (arrow). The complementary DNA sequence of the putative proximal promoter-exon I region is indicated above the sequence readout. (B) Identity of the experimentally verified transcription initiation site and comparison to site(s) previously identified by *in silico* analysis: (*) by *in silico* analysis (NCBI *H. sapiens* chr. 12, GRCh37.p10 Primary Assembly data); (**) by *in silico* analysis (RT-PCR–based cloning and isolation of putative promoter [39]; (***) by 5′-RACE (this publication).

mutants constructs of the clone (Figure 3) were then analyzed to delineate the regions within the proximal promoter that are most responsive to key physiological stimuli, i.e., metabolites of the glycolytic cascade, pH, and oxygen tension, to identify the relative contribution on MCT2 expression.

The MCT2 Promoter Is Highly Responsive to Glycolytic Metabolites

As a first step in identifying the proximal promoter regions that are most responsive to glycolytic metabolites, we transfected U-87MG glioma cells with nested deletion mutants of the MCT2 promoter-reporter vector. The 1.1-kbp and longer promoter-reporter constructs displayed ≥four-fold response to 25 mM glucose (×5 physiological glucose concentration), which represents enhanced glycolysis (Figure 4). When exposed to a 25 mM glucose, 10 mM ACCA mixture (conditions which we have previously demonstrated to simulate lactate buildup in cells [11]), 2.5-kbp and longer promoter constructs showed ≥three-fold response (Figure 4). In both cases, the highest fold activations were seen with the 3.2-kbp and 2.5-kbp promoter constructs. Of note is that the assay of the full-length (4.2 kbp) promoter with pyruvate (5 mM) and lactate (10 mM) showed responses of similar magnitude to glucose (25 mM) (Suppl. Figure 4*A*). Increase of glutamine concentration in medium from 0.5 mM (physiological concentration) to 2 mM (×4 physiological concentration; present in most cell culture media) did not cause further activation of the promoter (above and beyond that with glucose, pyruvate, or lactate), whereas even higher glutamine concentrations (5 mM) suppressed promoter activity (Suppl. Figure 4*B*).

The MCT2 Promoter Acutely Responds to Changes in Intracellular pH

Intracellular pH is an integral component of stimuli that should influence mammalian gene transcription. To date, this aspect has not been examined via reporter gene assays primarily because standard *in vitro* pH-based methods drastically affect cell culture viability. We addressed this issue by developing conditions under which cell viability was maintained while pH_i was altered via the use of a standard K^+ ionophore to equilibrate pH_e with pH_i (Suppl. Figure 5*A*). Changes to pH_i were verified using the fluorophore SNARF-4F as described in methods (Figure 5; Suppl. Figure 5*B*). Promoter-reporter activity was tested with the 2.4-kbp and 3.2-kbp nested deletion mutant clones that demonstrated the maximal (five- to six-fold higher) response to glucose and glucose + ACCA (increased lactate flux) (Figure 4) and to lower pH (6.5) (Suppl. Figure 5*C*). We observed exquisite responses of the MCT2 proximal promoter to pH

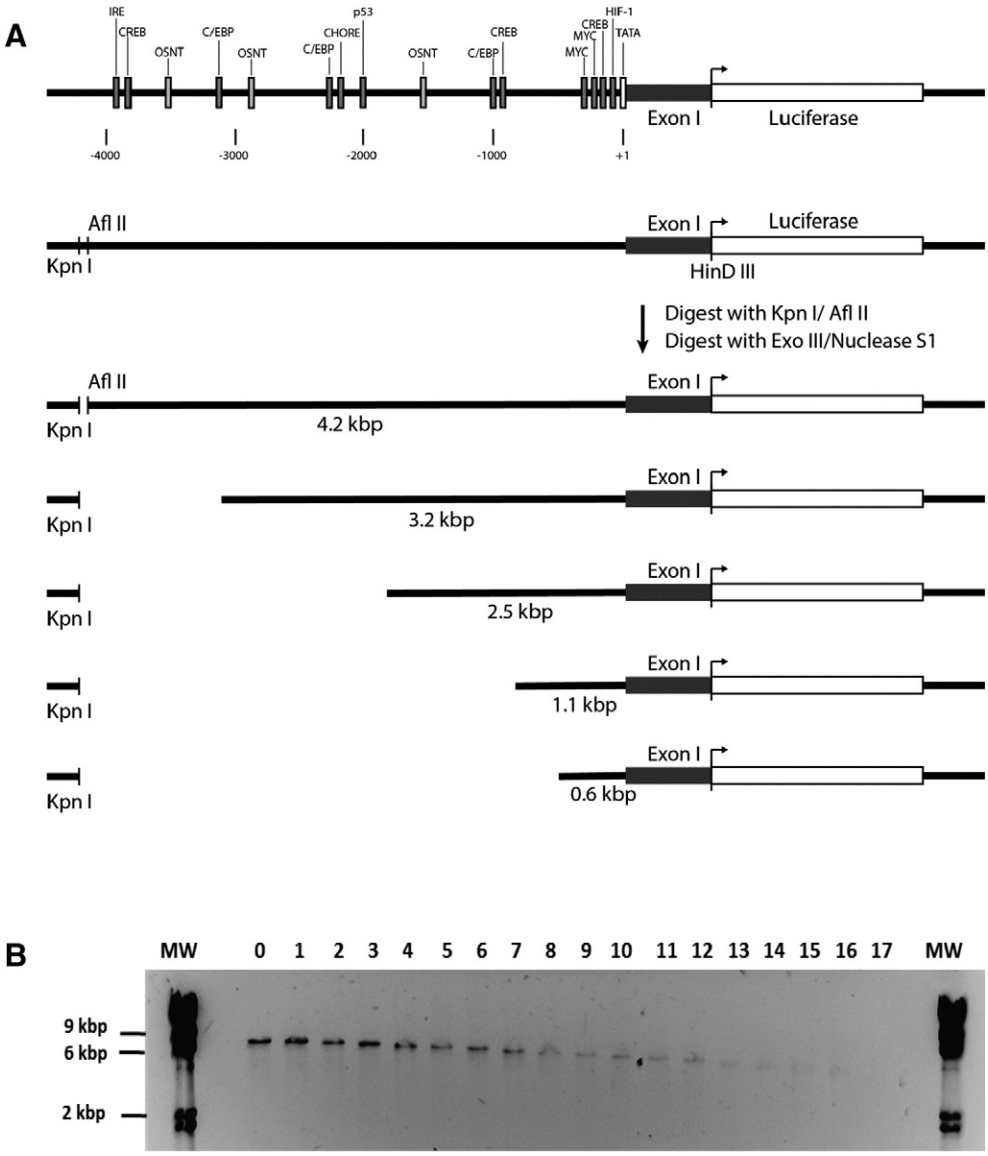

Figure 3. Construction of nested deletion mutants of MCT2 promoter-luciferase reporter gene constructs: (A) The 4.2-kbp promoter-exon I fragment was cloned into pGL2-firefly luciferase reporter gene vector at indicated restriction sites, followed by digestion with exonuclease III to generate a series of 5′ nested deletion mutants to identify the promoter regions most responsive to pH, hypoxia, glucose, and lactate. (B) Agarose gel electrophoresis pattern of sequentially generated nested deletion mutants (λ-HinD III markers were used as reference standards). Putative transcription factor binding sites on the 4.2-kbp promoter region identified by the MatInspector program (Genomatix Software Suite) are listed above the nested deletion mutant map.

in U-87MG and U-251MG glioma cells when pH_i was lowered from 7.5 to 7.3 in 0.1 pH units (Figure 6 and Suppl. Figure 6). The promoter responded with increased levels of activation (approximately 1.5-, 2-, and 2.5-fold for U-87MG and 2-, 2.2-, and 3.5-fold for U-251MG, respectively) when intracellular pH was lowered by 0.1 pH units (Figure 6, *A* and *B*). Promoter activity in the glioma "stem" cell line TS667 "dropped" in each of the pH sets (−nigericin/−KCl versus +nigericin/+KCl). Still, an increase in response of promoter to lower pH_i (1.0-, 1.1-, and 2.0-fold, respectively) could be observed when the +nigericin/+KCl cohort is considered independently (Figure 6*C*; 7.4+, 7.3+, and 7.2+).

We also exposed U-87MG, U-251MG, and TS667 cells to pH 7.3 or 7.5 (in the presence of KCl and nigericin) and observed that native MCT2 protein expression patterns recapitulate reporter data (Figure 7). The established cell lines U-87MG and U-251MG showed 1.2- and 1.3-fold enhancements at low pH, whereas TS667 cells showed a 1.1-fold enhancement (Figure 7*C*). Spheroid cultures of each cell line (maintained in neurosphere medium, pH 7.4) showed robust MCT2 expression (Figure 7*A*). However, when normalized to total protein profile (Figure 7*B*), these levels were on par with monolayer cultures (Figure 7*C*). The exception was U-251MG spheroids, which showed high net MCT2 expression.

Hypoxia Is a Primary Driver of MCT2 Promoter Activation

Hypoxia is a key stimulus for increased glycolytic metabolism in tumors [41,42]. Thus, reported nonresponse of MCT2 gene to hypoxia [39] has remained an anomaly. We examined the reaction of all MCT2 promoter nested deletion mutants to hypoxia, where the

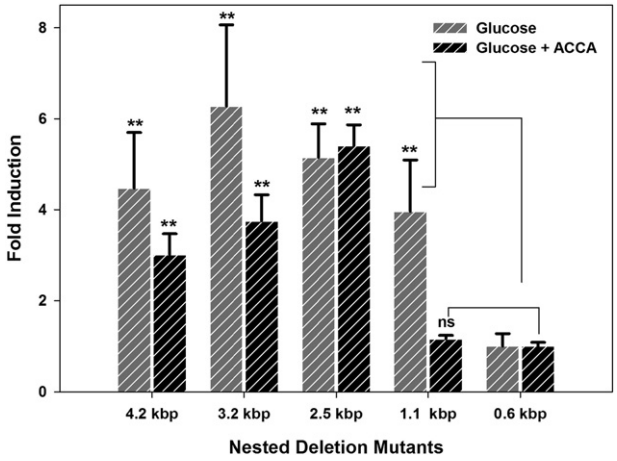

Figure 4. The MCT2 promoter is responsive to enhanced glycolytic flux and to intracellular lactate: U-87MG glioma cells transfected with nested deletion mutant-reporter constructs were exposed to 1) 25 mM glucose for 72 hours to mimic enhanced glycolytic flux through the tumors, and 2) to 25 mM glucose and 10 mM ACCA to inhibit lactate efflux and cause buildup of intracellular lactate. Firefly luciferase reporter activity was normalized against *Renilla* luciferase (see Methods). The promoter was highly responsive to both glucose and lactate with the most activity retained with the 3.2- to 2.5-kbp proximal promoter region. Data are presented as mean ± SD ($n = 4$ for each plot). (**$P < .05$, Mann-Whitney rank sum test; ns, not significant).

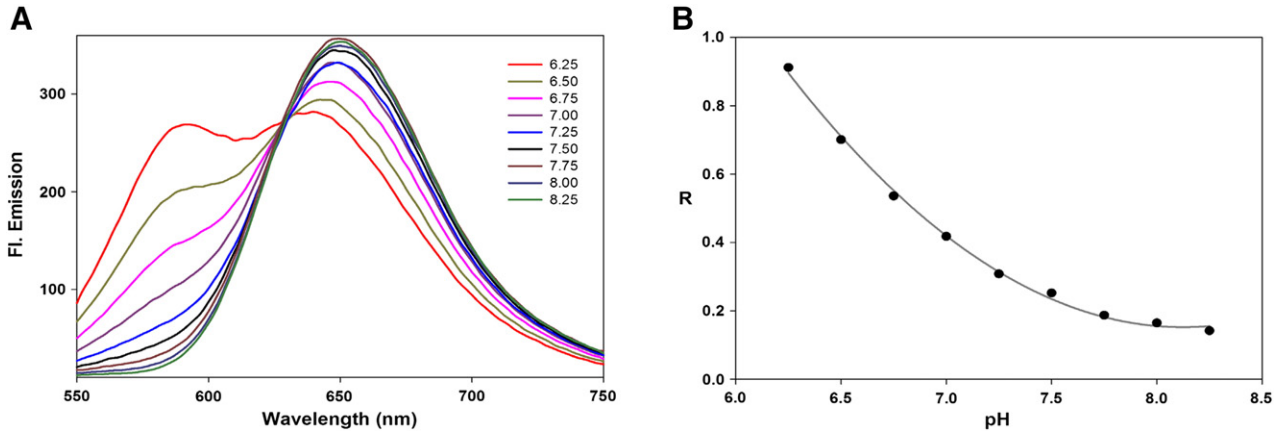

Figure 5. SNARF-4F–based fluorometric titration of pH_i: (A) U-87MG glioma cells were resuspended in Bis-Tris-Propane/MES buffer (see Methods) and pH adjusted from 6.25 to 8.25 at 0.25-pH unit increments. Nigericin was added to each sample for pH equilibration between cells and buffer. Fluorescence emission spectra were obtained between 550 nm and 750 nm. (B) Time-driven ratiometric values (*R*) were obtained at λ_{max} values (590 nm and 650 nm). Average of *R* values was plotted to generate the ratiometric pH titration curve.

transfected U-87MG, U-251MG, and TS667 cells were exposed to ≤1% O_2 for 48 hours. The assayed promoter activity was significant and reproducible. We saw a much higher response (two- to four-fold) in the established U-87MG and U-251MG glioma cells (Figure 8, *A* and *B*). The proximal-most deletion mutant (0.6 kbp) showed a hypoxia response of similar magnitude to deletion mutants of the rest of the promoter (Figure 8, *A* and *B*), which would be expected with a functional HIF-1 response element that is proximal to the transcription initiation site. In contrast, response of TS667 cells was more muted for most of the nested deletion mutant constructs (Figure 8*C*).

Native MCT2 protein expression under hypoxia also replicated reporter assay data in a pattern similar to our observations for pH_i. MCT2 expression under hypoxia for U-87MG and U-251MG cultures was quite robust (Figure 9), whereas response of TS667 cells to hypoxia was more mild (Figure 9*C*). Spheroid cultures of all three cell lines were also assayed alongside for comparison.

Discussion

Glioblastoma, in step with most other highly malignant tumors, maintains an abnormal metabolic capacity that effluxes lactic acid to the tumor microenvironment to both destroy and remodel the surrounding healthy brain tissue. This facilitates its highly infiltrative nature. Thus, whereas the tumor itself maintains a more "neutral" pH than surrounding normal tissue, it acidifies and poisons the tumor microenvironment via a series of facilitated organic anion/H^+ and monovalent cation/H^+ transporters. Key among them are the MCTs that help support the rapid elimination of lactic acid to the tumor microenvironment.

In this report, we examined transcriptional regulation of MCT2 [40], the isoform with the highest affinity for lactate [43]. Based on its kinetics, MCT2 expression should be a sentinel indicator of response of the tumor to physiological changes in glycolytic metabolic flux, hypoxia, and pH to help facilitate survival and function of the tumor, and maintain maximal metabolite flux across the tumor/

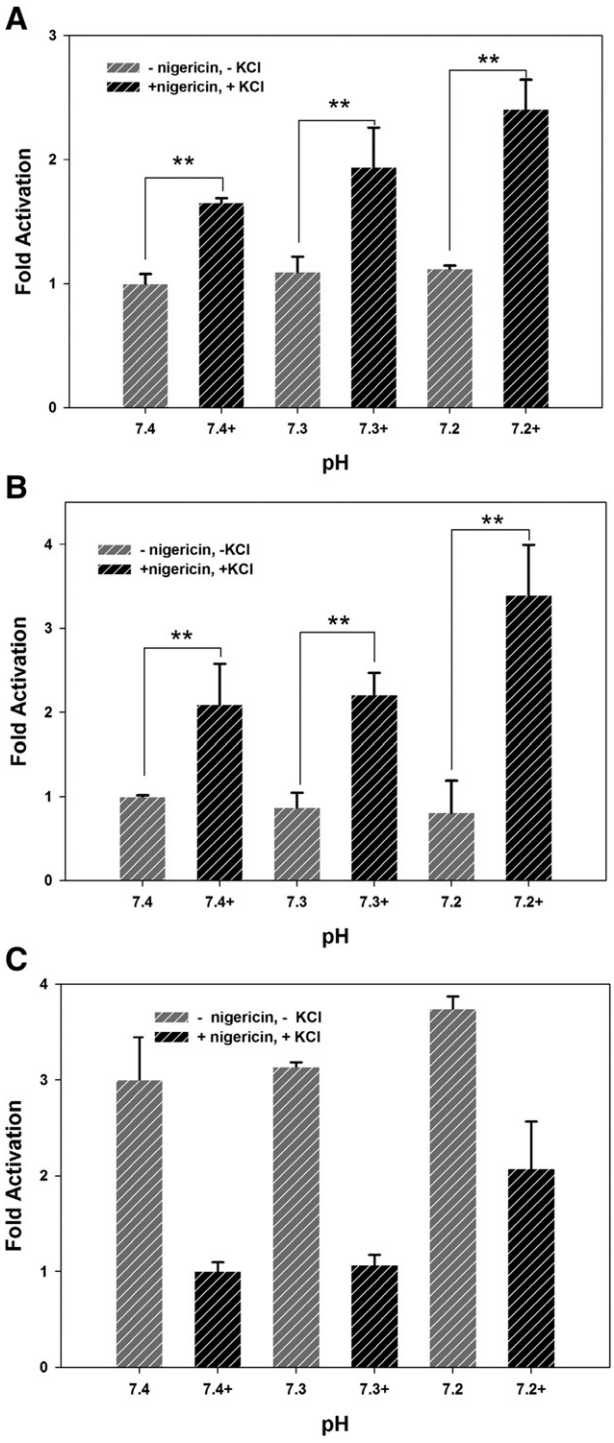

Figure 6. The MCT2 promoter is highly sensitive to minute changes in pH_i: (A) U-87MG, (B) U-251MG, and (C) HS667 glioma cells transfected with the 2.5-kbp MCT2 promoter-reporter construct were exposed to cell culture medium with 0.1-unit deviations from physiological pH in the presence of the ionophore nigericin and KCl for 24 hours. Luciferase reporter activity was normalized against *Renilla* luciferase and plotted. pH_i changes were verified by SNARF-4F–based fluorescence spectrometry (see Methods and Supplementary Figure 5*B* for supporting information). Data are presented as mean ± SD ($n = 4$). (**$P < .05$, Mann-Whitney rank sum test; ns, not significant).

microenvironment interphase. Alternate MCT2 promoters have been predicted based on listed mRNA profiles in the human genomic databases [39]. Murine MCT2 promoter profiling also points to alternate promoter use [44], which may be tissue and species specific. MCT2 is reported to be crucial in shuttling lactate between neurons and astrocytes in healthy brain tissue [45]. Our findings indicate that, in humans, MCT2 is transcribed and regulated by identical promoter regions in both astrocytes and glioblastoma, revealing that the MCT2 transcriptional unit is preserved during malignant transformation of astrocytes, during which glycolysis becomes enhanced as the tumor becomes more aggressive with increased production of lactate [4]. Thus, although methylation patterns may play a role in epigenetic control of MCT2 transcription between astrocytes and glioma, our data show that the signaling cascades, which facilitate the neuron-astrocyte lactate shuttle in healthy brain, are usurped but maintained during malignant transformation to help promote tumor dissemination within the brain parenchyma. In fact, DNA methylation profiling array data have indicated differential methylation patterns at the MCT2 locus in the case of prostate cancer, indicating epigenetic influence in MCT2 expression between normal and tumor tissues [46].

Glucose, pyruvate, and lactate are the respective initial and final metabolites of the glycolytic cascade. Glucose is a primary driver of tumor proliferation and is known to regulate most of the genes of the glycolytic cascade studied to date in tumors [33,47]. The effects are mediated via a carbohydrate response element [48,49], which we identified on the MCT2 proximal promoter also. The robust upregulation of the MCT2 transcription by glucose, pyruvate, and lactate we observed supports our finding that MCT2 is a tightly regulated TATA-element–driven gene as opposed to a generic "housekeeping" gene.

We observed a more vigorous MCT2 promoter response to glucose alone, although this could also be due to pyruvate and lactate derived from the intracellular glucose-to-lactate flux in the glioma cells. If so, this should be reflected in the glucose + ACCA treatments also, where an even higher promoter response would be expected due to greater lactate buildup upon MCT inhibition. Despite the expectations, promoter response was similar (2.5-kbp construct) or lower with all other nested deletion mutants). The outcome was most likely the result of glycolytic pathway beginning to undergo collapse (after an initial buildup of lactate upon inhibition of MCTs with ACCA), an observation we have made in prior magnetic resonance spectroscopy–based studies [11].

Lactate-derived transcriptional upregulation of MCT1, another MCT isoform, has also been reported in skeletal muscle–derived cells [50]. Furthermore, *in silico* screening of the proximal promoter-exon I region with CHIP-seq data from the ENCODE database [32] identified numerous cis-elements including MYC, CEBPB, and FOS/JUN (Suppl. Figure 10) that play a fundamental role in maintaining the tumor glycolytic phenotype [51].

Our data on activation of MCT2 gene by exogenous physiological levels of pyruvate and lactate (Suppl. Figure 4*A*) agree with a transporter that senses build-up of either metabolite, as the status of metabolic flux is altered from primarily oxidative phosphorylation (mitochondrial respiration) to supplementation with enhanced aerobic glycolysis during malignant transformation [52–54]. Healthy tissues will harbor lower intracellular pyruvate levels due to lower glycolytic flux and efficient uptake of pyruvate by mitochondria. However, pyruvate may be a factor for MCT2 activation in neuronal tissues, in which pyruvate is also derived from lactate shuttled from adjacent astrocytes [45,55]. Furthermore, both lactate and pyruvate are reported to regulate HIF-1 expression independent of hypoxia in highly glycolytic tumors by stabilizing HIF-1 [56]. Thus, both these

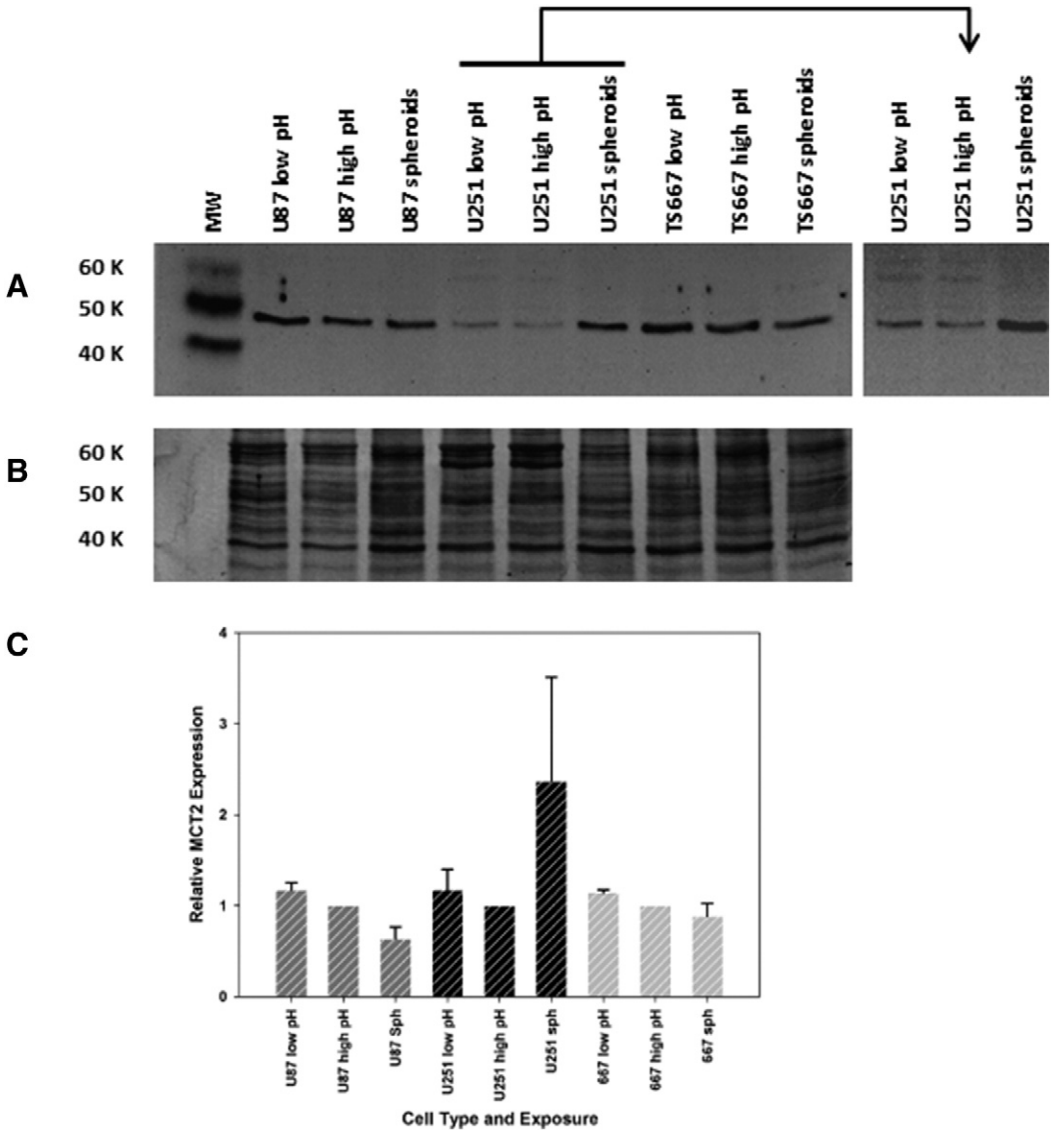

Figure 7. Native MCT2 protein expression responds to minute changes in pH_i: U-87MG, U-251MG, and TS667 monolayer cultures ($n = 2$) were exposed to pH 7.5 (high pH_i) or pH 7.3 (low pH_i) facilitated by ionophore nigericin and KCl for 16 hours. Western blot analysis of membrane protein fractions (A) for MCT2 and normalization to protein profile (B) indicated enhanced expression of MCT2 protein at lower pH (C). Membrane proteins isolated from spheroids of each cell line were used for comparison purposes. Panel to the right of (A): Expression pattern of MCT2 in U-251MG at lower camera exposure settings to visualize changes. (MCT2 expression at high $pH_i = 1$; Western blot and Coomassie stain of same membrane shown).

end products of glycolysis appear to play a multifaceted role in regulating MCT genes either via direct metabolite effects or indirectly by affecting trans-acting factors.

Furthermore, glioblastomas are suggested to have an inclination for increased uptake of glutamine to maintain their aberrant metabolism [57]. We queried whether glutamine supplementation (2 to 5 mM; frequently found in cell-culture medium and used in most published glutamine-based studies) could influence the MCT2 promoter activity. Incapability of excess glutamine to enhance MCT2 promoter activity beyond that in 5 mM glucose +0.5 mM glutamine (physiological levels of both substrates; Suppl. Figure 4*B*) suggests a potential metabolic demarcation between the glycolytic cascade and glutamine uptake and metabolism by these brain tumor cells. Our observations also agree with recent studies that suggest that these highly malignant tumors do not harbor a higher avidity for glutamine over glucose as a metabolic fuel [58,59].

Highly aggressive gliomas are known to be subjected to severe hypoxia ($pO_2 \approx 0.1\%$), whereas glioma of lesser grades can be exposed to moderate levels ($pO_2 \approx 2.5$ to 10%) [60,61]. Of the MCT 1, 2, and 4 isoforms that are expressed in malignant tumors including glioma, the isoforms 1 and 4 are known to respond to hypoxia [23,39,62]. MCT2, despite its highest affinity for lactate transport, has remained an outlier and is assumed to be nonresponsive [39]. Conflicting MCT2 mRNA or protein expression data have also been reported in literature, with indications that hypoxia response of MCT2 may be contextual or cell type based [63,64].

Our observation of pronounced activation of MCT2 promoter under hypoxia lends support to our hypothesis that this high-affinity lactate transporter should respond to lower oxygen tension in the tumor microenvironment. The data bolster our identification of an HIF-1 element as being the most proximal cis-element on the MCT2 promoter. The more robust response of MCT2 promoter to hypoxia

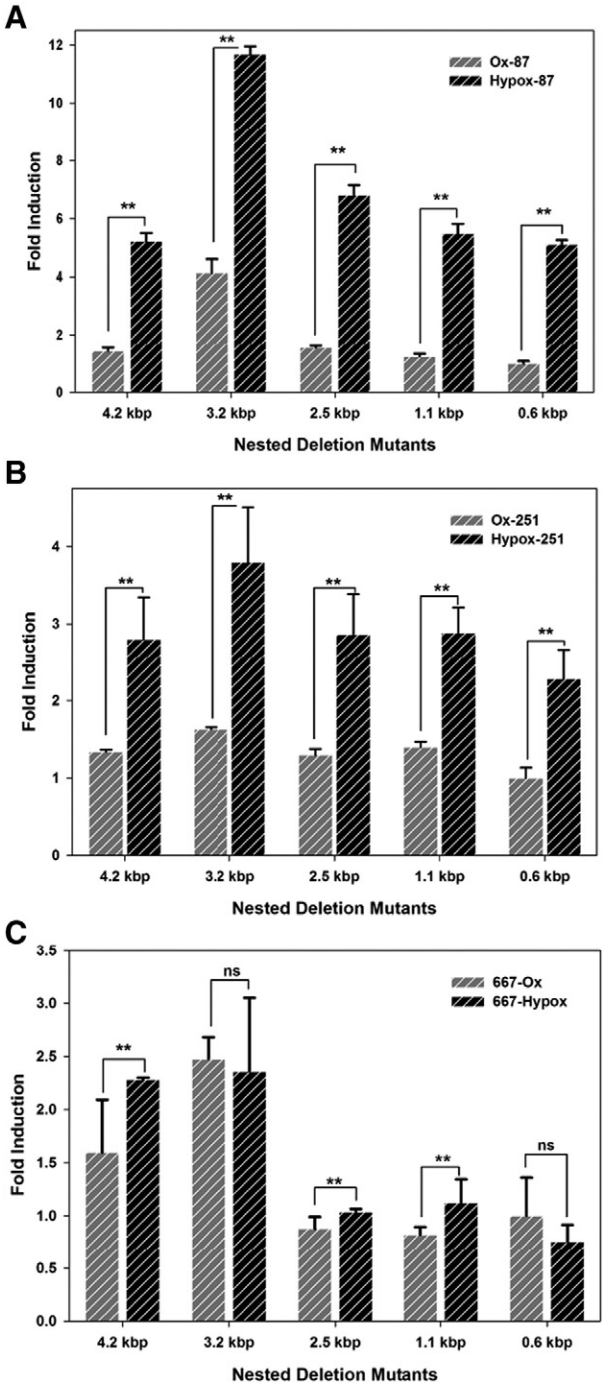

Figure 8. The MCT2 promoter demonstrates a robust response to changes in oxygen tension: (A) U-87MG, (B) U251MG, and (C) TS667 glioma cells transfected with reporter gene constructs were exposed to normoxia (~17% O_2) or hypoxia (≤1% O_2) for 48 hours. Luciferase reporter analysis of promoter activity indicated that MCT2 promoter was responsive to low oxygen conditions. (**$P < .05$, Mann-Whitney rank sum test; ns, not significant).

in U-87MG and U-251MG established glioma cells lines, and a lesser one in the TS667 glioma "stem" cell line, can be interpreted when we analyze the native MCT2 expression in tumor spheroids of each cell line. To expound, spheroids are known to maintain a hypoxic core under routine (normoxic) culture [65], which likely mimics conditions in solid tumors. The strong native MCT2 protein expression observed in spheroids of each glioma cell line (including TS667 neurospheres) by Western blot analysis can provide an answer. Native MCT2 protein expression in neurosphere-derived TS667 cells even after 48 hours of exposure to oxia under monolayer culture was still strong. Thus, the weaker response of MCT2 promoter in TS667 during reporter studies is likely due to intracellular negative feedback signaling loops because native MCT2 was already being highly expressed in the cells. This aspect of potential feedback cues needs to be investigated in future experiments. In contrast, both U-87MG and U-251MG glioma cells were routinely maintained as monolayer cultures and thus could robustly respond to hypoxia when challenged.

Another hallmark of malignant tumors including glioblastoma that reflect the elevated glycolytic flux is the presence of a reversed pH gradient between the tumor and the microenvironment [66–69]. Whereas the pH_i of normal tissues is ~7.2 with pH_e being ~7.4 (the physiological pH), malignant tumors harbor a pH_i of ~7.4 with pH_e of ~6.8 [70]. Acid–base regulation in tumor cells is facilitated by an assemblage of plasma-membrane transporters including MCTs, Na^+/H^+ exchangers, HCO_3^- transporters and associated anion exchangers, vacuolar H^+-ATPases, and carbonic anhydrases [71,72]. Regulatory factors that recruit cytoplasmic signaling proteins to the membrane receptors to facilitate and promote glioma dissemination have also been reported [73]. An important facet in this milieu is the series of intracellular (pH_i) and plasma-membrane–bound (pH_e) acid–base sensor proteins that have been reported in mammalian systems. These include specific G-protein–coupled receptors, ion channels, receptor and nonreceptor tyrosine kinases, and a soluble form of adenylyl cyclase [71,74]. Although pH_i has to be a requisite component among metabolic stimuli that influence tumor gene promoters, to date, this aspect has not been investigated in any mammalian system via reporter gene analysis. Ionophores coupled to extracellular buffer systems are a prerequisite to modulating pH_i via pH-sensitive fluorescent, conditions which also drastically affect cell viability. We addressed this issue and developed conditions that maintain cell viability while facilitating reporter gene studies.

Our investigations demonstrated that the MCT2 promoter is highly sensitive to changes in pH_i, with proportionate net activation at progressively lower pH_i. The trend is pronounced in U-87MG and U-251MG glioma cells lines, whereas it is perceptible in the TS667 "stem" cell line when the ionophore/K^+-exposed cell cohort (7.4+/7.3+/7.2+) is separately examined. The drop in reporter readouts within each "pH pair" of TS667 cells (7.4/7.4+ etc.) is likely due to ionophore/K^+-exposed cells still showing some degree of sensitivity, which, in turn, can affect the reporter gene readout. The control TS667 cohort (7.4/7.3/7.2; naive to ionophore/K^+) did not show net activation at progressively lower pH_i, in line with that observed in the same cohorts for U-87MG and U-251MG glioma cells.

Native MCT2 protein was also overexpressed in response to low pH_i in all three glioma cell lines. In fact, net native MCT2 protein expression was higher in U-87MG and TS667 gliomas in comparison to the levels in spheroid cultures, an interesting outcome given that spheroid cultures are capable of robust MCT2 expression. It is possible that lower pH_i supersedes hypoxia in upregulating MCT2 expression or that it can stabilize the transcript or the protein. This observation also needs to be investigated further. The much lower raw native MCT2 expression in U-251MG monolayer cultures despite a robust response during promoter-reporter studies may indicate a sensitivity of these cells to ionophore/K^+. Nonetheless, upregulation of native MCT2 protein expression in response to low pH_i could still be observed in these cells.

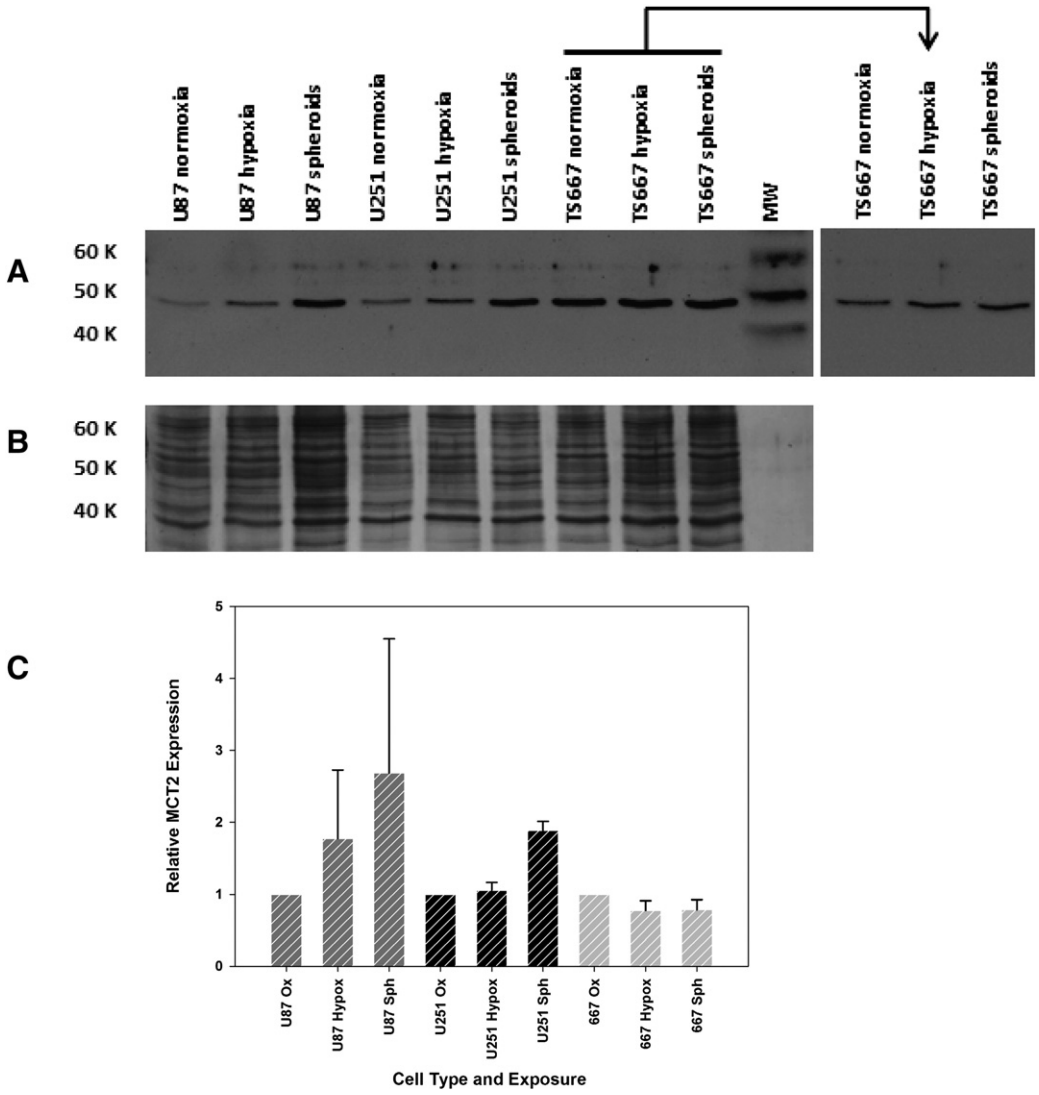

Figure 9. MCT2 protein is overexpressed upon exposure of glioma cells to hypoxia: U-87MG, U-251MG, and TS667 monolayer cultures ($n = 2$) were exposed to ≤1% O_2 (hypoxia) or normoxia (~17% O_2) for 16 hours. Western blot analysis of membrane fractions for MCT2 (A) was normalized to protein profile (B). The data indicated enhanced expression of MCT2 protein under hypoxia. Membrane protein fractions from TS667 spheroids and from spheroids generated from U-87MG and U-251MG were used for comparison purposes. Panel to the right of (A): Expression pattern of MCT2 in TS667 monolayer cultures at lower camera exposure settings to visualize changes. (MCT2 expression under normoxia = 1; Western blot and Coomassie stain of same membrane shown).

Overall, our study outcomes demonstrated the highly sensitive response of the MCT2 promoter to minute changes in pH_i. The MCT2 promoter showed increased activation with progressive lowering of pH_i, expected if a gene is to respond to even slight changes in pH_i due to increased tumor glycolytic metabolism, for downstream modulation of lactate efflux. H^+ plays a major role in aberrant tumor metabolism, tumor invasion, and dissemination [69]. Thus, our studies demonstrate for the first time how a key metabolic gene that facilitates tumor glycolysis responds to pH_i, with an exquisite response to cellular H^+, necessary to compensate for altered metabolic flux. Our studies were further supported by the protein data, thus linking the promoter activity to transporter expression.

In conclusion, recognition of response of this high-affinity lactate transporter isoform's transcription to hypoxia, pH_i, substrate, and products of the glycolytic cascade amalgamates how these key metabolic stimuli complement and integrate to further the malignancy of glioblastoma. Furthermore, reporter methods developed during the studies can now be extended to investigate and identify the trans-activating factors or signaling cascades that transduce changes in pH_i to metabolic genes to promote aggressiveness of these tumors. Identification and silencing of such factors can present unique metabolic strategies to target or reprogram metabolism of these tumors via forcing the pH_i to return to "normal" levels.

Disclosure Statement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.12.011.

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