Glucose-6-phosphatase Is a Key Metabolic Regulator of Glioblastoma Invasion

Sara Abbadi, Julio J. Rodarte, Ameer Abutaleb, Emily Lavell, Chris L. Smith, William Ruff, Jennifer Schiller, Alessandro Olivi, Andre Levchenko, Hugo Guerrero-Cazares, and Alfredo Quinones-Hinojosa

Abstract

Glioblastoma (GBM) remains the most aggressive primary brain cancer in adults. Similar to other cancers, GBM cells undergo metabolic reprogramming to promote proliferation and survival. Glycolytic inhibition is widely used to target such reprogramming. However, the stability of glycolytic inhibition in GBM remains unclear especially in a hypoxic tumor microenvironment. In this study, it was determined that glucose-6-phosphatase (G6PC/G6Pase) expression is elevated in GBM when compared with normal brain. Human-derived brain tumor–initiating cells (BTIC) use this enzyme to counteract glycolytic inhibition induced by 2-deoxy-D-glucose (2DG) and sustain malignant progression. Downregulation of G6PC renders the majority of these cells unable to survive glycolytic inhibition, and promotes glycogen accumulation through the activation of glycogen synthase (GYS1) and inhibition of glycogen phosphorylase (PYGL). Moreover, BTICs that survive G6PC knockdown are less aggressive (reduced migration, invasion, proliferation, and increased astrocytic differentiation). Collectively, these findings establish G6PC as a key enzyme with promalignant functional consequences that has not been previously reported in GBM and identify it as a potential therapeutic target.

Implications: This study is the first to demonstrate a functional relationship between the critical gluconeogenic and glycogenolytic enzyme G6PC with the metabolic adaptations during GBM invasion.

Visual Overview: http://mcr.aacrjournals.org/content/12/11/1547/F1.large.jpg.

Mol Cancer Res; 12(11); 1547–59. ©2014 AACR.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain cancer in adults (1, 2). The diffuse infiltrative nature of GBMs makes their total surgical resection impossible, leading to poor prognosis and short survival (3). A growing body of evidence has demonstrated that cancer cells display cellular hierarchies with a subset of cells believed to be responsible for resistance to conventional cancer therapies and for promotion of tumor growth (4). In cancers originating in the brain, these cells are called brain tumor–initiating cells (BTIC), cancer stem cells, or tumor-propagating cells (5, 6).

The rapid expansion of GBMs and other solid tumors causes them to frequently outgrow their blood supply leading to oxygen deficiency and nutrient deprivation (7, 8). As a response, tumors undergo angiogenesis to increase their oxygen and nutrient supply (9). Antiangiogenic therapy in solid tumors shows beneficial effects associated with a reduction of the vasculature and delayed tumor progression, but ultimately increases tumor hypoxia, and induces a treatment-resistant phenotype (10). These findings, which are unique to cancer cells, are driven by a constant metabolic reprogramming that enhances their survival adaptation in response to hypoxia (11). One well-established example of this reprogramming is the Warburg effect (preference for aerobic glycolysis), which has been of particular interest in cancer cell metabolism (12). However, the enhanced glycolysis seen with the Warburg effect cannot be completely functional under hypoxic conditions wherein nutrient supply is insufficient. Thus, cancer cells may additionally activate other metabolic processes, such as glycogen mobilization, to provide intermediates for their enhanced, reprogrammed glycolytic pathway (13).

Metabolic reprogramming allows BTICs to survive and adapt to restricted nutrition conditions (14). Targeting such adaptation mechanism, which is common to most cancer
cells, could be a crucial therapeutic tool (15). Previously, we demonstrated that glycolytic inhibition of BTICs with the glucose analog 2-deoxy-D-glucose (2DG) promoted neuronal commitment and decreased cell proliferation rate, inducing a less malignant phenotype (16). However, some BTICs are able to survive glycolytic inhibition and recover their aggressive phenotype. The precise mechanism by which BTICs counteract glycolytic inhibition remains unknown.

A key enzyme in the regulation of glucose homeostasis and the glycogenolytic pathway is the glucose-6–phosphatase complex, which is located at the membrane of the endoplasmic reticulum (17). Because it serves a physiologically important role in the glycogenolytic pathway, we hypothesized that glucose-6–phosphatase is required for BTIC survival, and that targeting it will commit BTICs to cell death.

In this work, we report a regain of promalignant characteristics of BTICs following their recovery from glycolytic inhibition. We then identify that this recovery capacity is abolished when the hepatic isoform of glucose-6-phosphatase (G6PC) is inhibited. Furthermore, knocking down G6PC is sufficient to decrease the migratory and proliferative capacity of BTICs. These findings seem to be related to the alterations in the glycogen metabolism of brain cancer cells.

Materials and Methods

Cell culture

All protocols in this study have been approved by the Johns Hopkins Hospital Institutional Review Board. Primary cultures of human fetal–derived astrocytes were obtained as described previously (18, 19). Adult GBM tissue samples were collected after written informed consent. Patient characteristics are described in Supplementary Table S1. Cell lines were tested and authenticated by the GRCF at Johns Hopkins. Control media consisted of DMEM F12 (+)glutamine (Life Technologies), BIT9500 10% (STEM-CELL Technologies) for a final glucose concentration of 25 mmol/L, and 20 ng/mL EGF and bFGF (basic fibroblast growth factor; Pepro Tech). 2DG-treated groups were cultured for 18 hours in low-glucose media consisting of DMEM no-glucose (+)glutamine (Life Technologies), with BIT9500 (10%), 20 ng/mL EGF and bFGF (Pepro Tech) and 2DG 25 mmol/L (Sigma-Aldrich). Recovery groups were obtained by replacing 2DG-supplemented media (after 18 hours) with control media for 72 hours. Of note, 0.1 mmol/L chlorogenic (CHL) acid (Sigma-Aldrich) was added to recovery media in the recovery+CHL groups. CP-91149 40 μmol/L (Selleckchem) was used as glycogen phosphorylase (PYGL) inhibitor when indicated. All treatments and experiments were carried out in a hypoxia incubator at an atmosphere of 5% carbon dioxide, 1.5% oxygen balanced with nitrogen, and 37°C.

Lentiviral transduction

Viruses were prepared in 293T cells. BTICs were transduced with equal titers of concentrated virus in control media supplemented with 1 μg/mL polybrene (Sigma) for 24 hours and were given additional 24 hour to recover before selection in 0.5 μg/mL puromycin (Sigma) for a minimum of 6 days.

Invasion assay

Boyden Transwell assays were used to compare invasive capacity of BTICs among treatment groups. A total of 5 × 10^4 cells in each treatment group were resuspended in corresponding media. Of note, 500 μL of cell suspension was seeded into the top well of a Boyden chamber (BD Biosciences; 8-μm pores); 700 μL of media were added to the lower chamber. After 24 hours incubation at 37°C, cells were fixed, stained, and counted under light microscopy (10 fields/insert). All treatment groups were done in triplicate; all experiments were repeated three times.

Neurosphere assay

Neurosphere culture was performed as previously described by our group (20). Briefly, 2.5 × 10^3 BTICs were plated in uncoated T25 flasks in a total volume of 5 mL of control media. Size and number of spheres were measured after 14 days of culture and analyzed.

Migration assay

Migration of BTICs was assessed using topographic nanopatterned substratum, which consists of parallel ridges 350-nm wide, 500-nm high, spaced 1.5-μm apart, fabricated onto glass coverslips as previously described by our group (21–23).

Tumor xenografts in nude mice

All animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. A total of 5 × 10^3 GBM1 BTICs were resuspended in 2 μL of media and injected intracranially into each of 25 male nude mice (Coordinates AP 1.34; ML 1.5; and DV 3.5) as previously described by our group (24).

Results

BTICs escape glycolytic inhibition with an aggressive phenotype

We performed all experiments using BTICs derived from intraoperative GBM samples. The capacity of our BTICs to form spheres in suspension, differentiate upon growth factor withdrawal, and form orthotopic tumors in animal models was previously described (Supplementary Fig. S1 and Supplementary Table S1; refs. 20, 25, 26). To recapitulate the hypoxic tumor niche, all the experiments were performed under chronic hypoxia.

First, we evaluated the effects of the glycolytic inhibitor 2DG on primary BTICs and their recovery potential upon reintroduction of glucose to their media (Fig. 1A). We did this in effort to simulate the transitory exposure of the cells to the nutrient-restricted environment during cycles of intermittent tumor hypoxia in vivo. Targeting the glycolytic pathway by means of the glucose analog 2DG has proven to sensitize cancer cells to additional treatments (27). However, specific cellular
adaptations and functional consequences following glycolytic inhibition had not been recognized. To address this matter, we first evaluated BTIC proliferative capacity using 5-ethynyl-2-deoxyuridine (EdU) incorporation after 18 hours exposure to 2DG. Compared with controls, 2DG-treated cells showed a decrease in proliferation ($P < 0.001$). However, placing them back in control media for 72 hours allowed them to recover from glycolytic inhibition (recovery group; $P < 0.001$; Fig. 1B and Supplementary Fig. S2A).

We then evaluated the invasive capacity of BTICs using a Transwell migration assay. As expected, glycolytic inhibition significantly impaired BTIC invasion (Fig. 1C). Interestingly, in the recovery group, the cells became more invasive...
Abbadi et al. found that glucose-6-phosphate is dephosphorylated (29). To determine whether 2DG-6P is localized in the cell and accumulates, inhibiting glycolysis until it is both phosphorylated by hexokinase. Unlike glucose, the G6PC is upregulated in BTICs and governs their aggressiveness (22, 23). Addition of 2DG significantly decreased the cell speed ($P < 0.001$), whereas the recovery group cells showed an increase in their speed beyond that seen on both, control- and 2DG-treated groups ($P < 0.001$; Fig. 1E–H; Supplementary Movie S1).

2DG-treated BTICs demonstrated increased expression of p21 and p53 and decreased Cyclin D1. This alteration in the expression of cell-cycle proteins was accompanied by an increase in cell death, as evidenced by a rise in cleaved poly ADP-ribose polymerase (PARP) expression. The reverse expression profile was seen in the recovery group (Fig. 1I and Supplementary Fig. S2G).

Finally, we aimed to determine whether addition of 2DG affected the ability of BTICs to uptake glucose. We observed no significant changes after the addition of 2DG in three of our primary BTIC lines (GBM1, GBM2, and GBM3; Supplementary Fig. S2H). Altogether, these results suggest that glycolytic inhibition induced by 2DG not only has a transient effect on BTICs, but more importantly, it stimulates their aggressiveness in vitro.

**G6PC is upregulated in BTICs**

To investigate the mechanism underlying the recovery from 2DG, we evaluated potential enzymes that could be mediating this effect. During glycosylation, 2DG and glucose are both phosphorylated by hexokinase. Unlike glucose, the intermediate 2DG-6-phosphate cannot be further metabolized in the cell and accumulates, inhibiting glycolysis until it is dephosphorylated (29). To determine whether 2DG-6P dephosphorylation indeed mediated the recovery process, we examined the expression of several glucose phosphatases. We found that glucose-6-phosphatase isoform α (G6PC) was upregulated in human GBM samples. Through immunohistochemistry, we observed that G6PC had a stronger presence in the human GBM cortex (Fig. 2A–D). We then evaluated differential expression of G6PC between BTICs and noncancer cortex cells from adult and fetal human brain via Western blot analysis and qRT-PCR. The expression of G6PC was higher in the GBM1 sample than in adult brain ($P < 0.001$) or fetal samples (F1, F2, and F3; $P < 0.05$; compared with F3; Fig. 2E and Supplementary Fig. S3A). Next, we examined a panel of seven different primary derived BTICs. Each showed a consistent increase in G6PC when compared with the adult brain noncancer cortex (Fig. 2F). The expression of G6PC has never been reported in brain cancer. To determine the exclusivity of the isoform’s upregulation, we also evaluated the expression of glucose 6-phosphatase isoform β (G6PC3), which had been reported to be expressed in the brain (30) but found no significant difference in its expression between normal adult brain, fetal brain, and BTICs (data not shown).

We next evaluated the effects of glycolytic inhibition via 2DG on the expression of G6PC in BTICs. Glycolytic inhibition induced an almost 2-fold increase in expression of G6PC at both protein and mRNA levels ($P < 0.01$; Fig. 2G and H and Supplementary Fig. S3B and S3C). Culturing the cells in low-glucose media for 18 hours did not cause a significant change in the expression level of this isoform ($P = 0.095$; Supplementary Fig. S3B). However, in the recovery group a decrease in the expression of G6PC was observed ($P < 0.01$; Fig. 2G and H and Supplementary Fig. S3C and S3F). Collectively these results demonstrate that G6PC is more highly expressed in BTICs than in noncancer cortex. The fact that the expression of G6PC is further increased by 2DG suggests it has an important role in the recovery mechanism under investigation.

**G6PC is required to recover (i.e., survive) from glycolytic inhibition**

To further determine the involvement of G6PC in the recovery of BTICs from glycolytic inhibition, we performed a lentiviral-mediated knockdown of its gene (shG6PC; Fig. 3A), and tested several key malignant cancer characteristics. The shG6PC in BTICs induced an increase in p21, p53, and cleaved PARP expression and a decrease in Cyclin D1 (Fig. 3B). These effects were similar to those observed after induction of glycolytic inhibition with 2DG (Fig. 3B). We further investigated the protein level of two major transcription factors, hypoxia-inducible factor 1α (HIF1α) and signal transducer and activator of transcription 3 (STAT3), known for causing enhancement in cancer cell survival, migration, and cellular metabolism (31, 32). We found that 2DG reduced the expression of these two proteins, however, recovered cells regained the expression of HIF1α and pSTAT3 (recovery group; Fig. 3C). Downregulation of both HIF1α and pSTAT3 expression was also observed in the recovery–shG6PC group (Fig. 3C).

In addition, we observed an increase in the activation of caspase-3 ($P < 0.001$) and a decrease in cell proliferation ($P < 0.001$) in the control–shG6PC cells when compared with the control–EV cells (Fig. 3D and E and Supplementary Fig. S3D). Next, we investigated two of the most critical malignant properties of GBM, invasion and migration. shG6PC strongly decreased the ability of the cell to invade ($P < 0.001$) and migrate ($P < 0.001$) when compared with EV cells (Fig. 3F and G; Supplementary Fig. S3E and Supplementary Fig. S4, and Supplementary Movie S2).

To evaluate whether the effects observed after knocking down G6PC could be mimicked chemically, we tested the effects of CHL, an inhibitor of glucose-6-phosphate translocase, which transports glucose-6-phosphate from the cytoplasm into the lumen of the endoplasmic reticulum (33). At the transcription level, we found that recovery of
CHL-treated BTICs displayed increased transcription levels of G6PC when compared with those of the control \((P < 0.01)\), suggesting a rescue mechanism induced in the recovery group to CHL (Supplementary Fig. S3F). Interestingly, recovered CHL-treated cells displayed a significant reduction in cell invasion when compared with nontreated recovered cells \((P < 0.05; \text{Fig. } 3H)\). CHL treatment also decreased cell migration in recovered cells \((P < 0.05; \text{Fig. } 3I\) and Supplementary Fig. S3G). To determine whether these findings were specific to BTICs, we evaluated the invasive capacity of other human cancer cell lines, upon G6PC knockdown. We tested human breast adenocarcinoma (MDA-MB-231), human melanoma (A375), and human pancreatic carcinoma (Panc-1) cell lines. Although the effects of shG6PC were similar in the pancreatic cells (a decrease in their invasive capacity), this result was not observed in the cells of breast or melanoma origin when compared with brain tumors (Supplementary Fig. S3H). Collectively, these results demonstrate that not only does shG6PC prevent escape from glycolytic inhibition in BTICs, but also induces a decrease in migration, invasion, and cell viability in vitro.
G6PC regulates astrocytic differentiation in BTICs

We previously observed that glycolytic inhibition induced the commitment of glioma cells to a neuronal lineage (16). Building upon this work, we were interested in evaluating the morphology and expression of differentiation markers in our experimental groups. Expression of the progenitor cell marker nestin, the astrocytic marker GFAP, and the neuronal marker Tuj1 was examined before and after 2DG exposure. In GBM1, we found a significant decrease in the number of nestin-positive cells in the 2DG group ($P < 0.05$; Supplementary Fig. S5A and S5B). Consistent with our previous results (16), we observed no significant increase in the GFAP-expressing cells ($P = 0.8248$; Supplementary Fig. S5A and S5C); however, 2DG did induce an increase in Tuj1 compared with controls ($P < 0.05$; Supplementary Fig. S5A and S5D). These results suggest that glycolytic inhibition with 2DG induces differentiation toward a neuronal lineage, shown as an increase in Tuj1.

Microscopic observation of BTICs after shG6PC revealed major alterations in their morphology. Unlike the flat polygonal morphology of the control cells, the shape of shG6PC cells was similar to that of mature astrocytes (Fig. 4A). To further explore this finding, we examined the expression of Tuj1 and GFAP after shG6PC. Immunocytochemical staining confirmed a significant decrease in the expression of Tuj1 after shG6PC in both control and recovery groups ($P < 0.001$). However, a concomitant upregulation of GFAP expression was observed in the control–shG6PC group ($P < 0.001$) and was higher in the recovery group ($P < 0.01$; Fig. 4B and C). These findings
were consistent with the results obtained after the addition of CHL (Supplementary Fig. S5). No significant changes were observed in nestin expression after knocking down G6PC or adding CHL (data not shown). Altogether these findings indicate that shG6PC can induce differentiation of BTICs into an astrocytic lineage. This led us to further investigate the possible role that G6PC might play on the stem-like properties of BTICs. This subpopulation of tumor cells is believed to play a major role in tumor recurrence (34).

**G6PC is required for maintenance of stem-like properties in BTICs**

Neurosphere formation assays are currently the standard in vitro method for identifying the presence of stem cells derived from both tumor and nontumor tissues (35–37). Therefore, we determined the role of G6PC in a sphere formation assay. Two weeks after lentiviral transduction, we evaluated the sphere-forming capacity of control, 2DG, and recovery groups. shG6PC significantly decreased sphere formation (P < 0.001) and size (control P < 0.01, recovery P < 0.001) in both control and recovery groups (Fig. 4D–F).

Next, we sought to investigate the expression of CD133, a membrane marker used to enrich for stem cells and expressed by both neural stem cells and brain cancer stem cells (38, 39). We found that the addition of 2DG strongly decreased the expression of CD133 in control–EV cells (data not shown). Furthermore, culturing of the EV 2DG cells in control media for 72 hours (recovery) allowed them to rescue (or

Figure 4. shG6PC of BTICs induces astrocytic differentiation and decreases stemness. A to C, immunocytochemistry staining comparing the expression of Tuj1 and GFAP in GBM1. D to F, neurosphere assay comparing sphere size and number in GBM1. G, flow cytometric analysis of the expression of CD133 in GBM1. H, representative Western blot analysis of total and pAKT(Ser473) in GBM1. Data, mean ± SEM (n = 3) from three independent experiments; *, P < 0.05; **, P < 0.01; ***, P < 0.001; scale bars, 100 μm.
induce) expression of CD133 (Fig. 4G). However, shG6PC dramatically decreased the expression of CD133 in both control and recovery cells ($P < 0.05$; Fig. 4G).

Finally, we evaluated the expression of Akt, a well-characterized downstream key effector of the PI3K, because Akt is commonly upregulated in human cancers (40) and is known to play an important role in the regulation of proliferation and cell survival (41). Moreover, it has been suggested that CD133 plays an important role in the activation of the Akt pathway in glioma stem cells (42). We, thus, investigated whether the decrease in CD133, after shG6PC, was associated with a decrease in the activation of Akt. Through immunoblot analysis, we found that there was indeed a significant decrease in pAkt (Ser473) in both control and recovery groups after shG6PC (Fig. 4H). Together, our results suggest that G6PC is required for sphere formation and plays a positive role in the activation of the cytoprotective Akt pathway—possibly through CD133.

**G6PC knockdown promotes glycogen accumulation**

Recent studies have suggested the critical role of glycogen in promoting cancer cell survival (43, 44). Moreover, inhibiting glycogen breakdown induces apoptosis and early cell senescence in cancer cells (43, 44). Given the physiologic importance of G6PC in the glycogenolytic pathway, we proceeded to investigate glycogen metabolism after shG6PC.

Glucose-6-phosphate is an allosteric activator of glycogen synthase (GS) that regulates glycogen synthesis (45, 46). We hypothesized that shG6PC would lead to glycogen over-accumulation and decrease the malignant phenotype of our BTICs. To test this hypothesis, we first examined the intracellular glycogen levels of our groups. We found that shG6PC promoted glycogen accumulation in all groups (Fig. 5A). We then examined the expression of GS and PYGL key enzymes that regulate synthesis and degradation of glycogen, respectively (47, 48). Previous studies have found that hypoxia induced an increase in the expression of the muscle isofrom of glycogen synthase (GYS1), and the liver isofrom of PYGL in GBM cells (43). Therefore, we decided to investigate the expression of these two isoforms in our BTICs.

At the protein level, there was a decrease in inactive, phosphorylated GYS1 (serine 641) in all groups after shG6PC when compared with the EV groups (Fig. 5B and Supplementary Fig. S6A). GYS1 mRNA was found to be significantly higher in the shG6PC–control group when compared with the EV–control group ($P < 0.001$; Supplementary Fig. S6C). These results were in accordance with the increase in GYS1 activity (Supplementary Fig. S6A). Moreover, in shG6PC groups, the PYGL protein level was found to be significantly decreased when compared with the EV groups (Fig. 5B and Supplementary Fig. S6B). However, at the mRNA level PYGL was found to be decreased only in the 2DG and recovery shG6PC groups ($P < 0.01$; Supplementary Fig. S6D). These findings imply that shG6PC cells exhibit an uncontrolled increase in glycogen synthesis whereas EVs exhibit a preference for glycogen degradation.

Before its entry into the glycolytic or pentose phosphate pathways, glycogen is converted to glucose–1–phosphate by PYGL (49). Using the glycogen phosphorylase inhibitor (GPI) CP-91149, we determined the consequences of preventing the conversion of glycogen to glucose–1–phosphate on cell invasion and proliferation (Fig. 5C). The presence of GPI decreased the ability of BTICs to invade in control, 2DG, and recovery EV groups ($P < 0.001$; Fig. 5D–G), indicating that the conversion of endogenous glycogen to glucose–1–phosphate plays a critical role in GBM cell invasion. Strikingly, the addition of GPI to shG6PC cells, further decreased cell invasion in all conditions (control $P < 0.001$, 2DG $P < 0.05$, recovery $P < 0.001$; Fig. 5D–G). Next, we sought to investigate the effect of GPI on cell proliferation. In EV cells, GP inhibition induced a decrease in cell proliferation in both control and recovery groups (control $P < 0.001$, recovery $P < 0.01$; Fig. 5H and J). In shG6PC cells there was a decrease in cell proliferation exclusively in the recovery group (data not shown). These data support the hypothesis that G6PC is required for cells to fully eliminate 2DG and recover their proliferative capacity. Incubation of cells with GPI and 2DG did not demonstrate any additional effect on cell proliferation (Fig. 5I), presumably due to the profound cell proliferation inhibition obtained with 2DG.

Uncontrolled glycogen accumulation has been shown to lead to cell death and to have deleterious consequences in cancer cells. These results provide a potential mechanism accounting for the phenotype observed in the shG6PC cells, i.e., through an accumulation of glycogen.

**G6PC mediates invasiveness of brain cancer in vivo**

To test the *in vivo* roles of G6PC during cancer development, GBM1 with shG6PC or EV were implanted intracranially into athymic nude mice, as described previously (Fig. 6A; refs. 23, 24). We evaluated the proliferation and invasive capacity of BTICs through the corpus callosum 7 weeks after transplantation (Supplementary Fig. S7A). Ki67 staining was performed to evaluate the proliferation index. We found that the total number of Ki67–positive cells was significantly lower after knocking down G6PC in both control and recovery ($P < 0.001$; Fig. 6B and Supplementary Fig. S7B). However, after determining the proliferation index, we found no significant difference between groups (Supplementary Fig. S7C). This may be due to the increase in cell death as demonstrated by the *in vitro* activation of caspase-3 after shG6PC (Fig. 3D). More importantly, cell counts across the corpus callosum revealed that shG6PC resulted in a profound reduction in the invasiveness of BTICs in both control and recovery groups ($P < 0.001$; Fig. 6C–E and Supplementary Fig. S7D). These findings strongly suggest that G6PC could be playing an important role *in vivo* in regulating cell invasion, one of the biggest determinants in the recurrence and progression of GBM.

**Discussion**

Our results are the first to demonstrate a relationship between G6PC, glycolytic inhibition, and BTICs...
aggressiveness via glycogen metabolism. In this study, we provide evidence that BTICs are able to find alternative survival pathways to escape glycolytic inhibition and acquire a more aggressive phenotype in doing so. We further propose that the ability of BTICs to counteract glycolytic inhibition occurs, at least partly, via a G6PC-dependent mechanism potentially through intracellular glycogen degradation. Our hypothesis is supported by the observation that shG6PC prevented the recovery from glycolytic inhibition and resulted in a decreased aggressive phenotype and increased glycogen accumulation.

We observed that BTICs recovered from glycolytic inhibition and were more invasive, migratory, and proliferative compared with control cells. We believe that this increase in invasion, migration, and proliferation is analogous to the cellular responses that occur during the cyclic hypoxia phenomenon in which growth of the tumor increases once it recovers from hypoxia/nutrient deprivation and is consistent with previous studies that also reported an increase in tumorigenesis and selection for cancer-initiating cells following hypoxia and or nutrient deprivation (14, 50).

We used 2DG as a model of glycolytic inhibition. 2DG is a glucose analog that is phosphorylated by hexokinase to inhibit glycolysis (51). Knowing that a phosphatase was required to clear the cell from its presence (29), we tested the expression of glucose-6–phosphatase isofrom α (G6PC) and the ubiquitously expressed glucose 6 phosphatase isofrom β (G6PC3). We found upregulation of G6PC and no significant increase of G6PC3 after the addition of 2DG.

Figure 5. shG6PC induces glycogen accumulation through activation of GYS1 and inhibition of PYGL. A, glycogen quantification in EV or shG6PC GBM1. B, representative Western blot analysis of pGYS1(Ser641), total-GYS1, and PYGL in EV or shG6PC GBM1. C, experimental design illustrating the time points in which PYGL inhibitor CP-91149 (GPi) and 2DG were added to the groups depicted in D to J. D, representative images of cell invasion through the Boyden chamber from control, 2DG and recovery GBM1 treated with GPi or DMSO. E to G, comparison of the invasive capacity of EV or shG6PC in the presence or absence of GPi. H to J, cell viability and proliferation evaluated using alamarBlue in EV or shG6PC GBM1, in the presence or absence of GPi. Data, mean ± SEM (n = 3) from three independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001.
G6PC is a phosphatase that is predominantly expressed in the liver, kidney, and β cells of the pancreatic islets (17). No studies have looked specifically at the expression of G6PC in BTICs. We tested the expression of G6PC in several primary derived glioma BTICs and on tissue samples and found it to be highly upregulated.

We found that BTICs use G6PC to counteract glycolytic inhibition and depend on it even in the absence of glycolytic inhibition. Evidence for this was first suggested by the upregulation of G6PC expression upon 2DG treatment and further corroborated by the observation that inhibiting G6PC in BTICs stops their ability to recover from glycolytic inhibition. Remarkably, the sole knockdown of G6PC was able to decrease the aggressive phenotype of BTICs, potentially through the downregulation of the CD133/AKT axis and an increase in glycogen accumulation, which has been previously shown to induce cell death in cancer cells if it is not properly metabolized (43, 44). Glycogen was recently identified as a marker of astrocytic differentiation (52). Interestingly, we also observed astrocytic differentiation along with glycogen accumulation in the shG6PC BTICs.

Collectively, these results demonstrate that not only does G6PC knockdown prevents the escape from glycolytic inhibition in BTICs, but it also induces a decrease in migration, invasion, and cell viability; a phenomenon that is worth exploring in other cancers.

In the liver, targeted deletion of G6PC induces glucose-6-phosphate and glycogen accumulation (53). A number of observations have suggested that cancer cells have increased levels of glycogen (54), and that hypoxia stimulates glycogen buildup for later use in several cancer cell lines (13). Furthermore, in U87 glioma cells, glycogen accumulation induces premature cell senescence (43). Therefore, it is possible that altered glycogen metabolism could be part of the survival mechanism adopted by BTICs upon glycolytic inhibition, and, thus, a potential therapeutic target.

We observed in our BTICs that shG6PC induces an increase in the activity of GYS1, a decrease in PYGL, and...
subsequent glycogen accumulation. Given that accumulation of glucose-6-phosphate activates GYS1 and inhibits PYGL (45, 46), we hypothesized that glycogen levels increased upon shG6PC possibly as a result of glucose-6-phosphate accumulation (Fig. 7). This suggests that the channeling of glucose through glycogen may possess additional physiologic functions that go beyond its role as an energy source in situations with increased glucose demand. Furthermore, we found that HIF1α and STAT3 to be significantly downregulated upon shG6PC. Interestingly, HIF1α and STAT3 are found to be, at least in part, involved in glycogen regulation (13, 55). For instance, the expression of the G6PC is also regulated by HIF1α in hepatocytes (56). The specific role of HIF1α and STAT3 in the response of BTICs to G6PC inhibition needs to be further investigated.

In summary, this work gives a novel insight on the expression and effects of G6PC in primary BTICs and contributes to our understanding of the metabolic adaptations occurring in cancer cells. The heightened expression of G6PC in GBMs when compared with normal brain can potentially serve as a novel therapeutic target that could render brain cancer cells less aggressive and more vulnerable to additional metabolic inhibitors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Abbadi, E. Lavell, A. Quinones-Hinojosa
Development of methodology: S. Abbadi, J.J. Rodarte, E. Lavell, A. Quinones-Hinojosa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Abbadi, J.J. Rodarte, C.L. Smith, A. Olivi, H. Guerrero-Cazares, A. Quinones-Hinojosa
Writing, review, and/or revision of the manuscript: S. Abbadi, J.J. Rodarte, E. Lavell, C.L. Smith, A. Olivi, H. Guerrero-Cazares, A. Quinones-Hinojosa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases, etc.): S. Abbadi, A. Levchenko, J.J. Rodarte, C.L. Smith, A. Olivi, H. Guerrero-Cazares, A. Quinones-Hinojosa
Study supervision: A. Levchenko, H. Guerrero-Cazares, A. Quinones-Hinojosa

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Molecular Cancer Research

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