Glutamate dehydrogenase (GDH) regulates bioenergetics and redox homeostasis in human glioma

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ABSTRACT

The mitochondrial enzyme, glutamate dehydrogenase (GDH), is commonly upregulated in human cancers. Whether and how GDH contributes to the development of glioma remains unknown. Here we report that GDH is a mitochondrial regulator for alpha-ketoglutarate (α-KG) in glioma cells. GDH expression and activity are up-regulated in human glioma cell lines and tissues, and high level of GDH predicts poor outcome. We demonstrate that GDH is an oncogenic factor *in vitro* and in *vivo* by using loss-of-function and gain-of-function strategies. GDH is important for bioenergetics (glutamine uptake, ATP production) and redox homeostasis (mitochondria ROS) in glioma cells by controlling the intracellular level of its product α-KG. Finally, we show that inhibiting GDH by overexpression of Sirt4 or by small molecule inhibitors EGCG and R162 leads to attenuated cancer cell proliferation and tumor growth *in vitro* and *in vivo*. These findings shed a light that GDH is a new potential target for treatment of human glioma.

INTRODUCTION

In central neuron system, 80% of tumors are malignant gliomas, which are essentially incurable. The overall five-year survival rate of glioma remains less than 5% and is even worse for elderly patients, despite decades of concerted effort and advances in surgery, radiation, and chemotherapy [1]. This dismal clinical outcome makes glioma an urgent subject of cancer research, and identification of new therapeutic targets is critically important.

Impaired cellular metabolism is the defining characteristic of nearly all cancers regardless of cellular or tissue origin. Quiescent cells invest large amounts of energy in the maintenance of functional and morphological integrity against extrinsic and intrinsic insults, including oxidative stress [2]. In contrast, proliferating cells take up abundant nutrients, including glucose and glutamine, and shunt their metabolites into anabolic pathways. The signals that promote cell proliferation direct the reprogramming of metabolic activities, which pushes quiescent cells into proliferative states [3].

Glutaminolysis is a mitochondrial pathway that involves the initial deamination of glutamine by glutaminase, yielding glutamate and ammonia. Glutamate is then converted to alpha-ketoglutarate (α-KG), a TCA cycle intermediate, to produce both ATP and anabolic carbons for the synthesis of amino acids, nucleotides, and lipids [4]. The conversion of glutamate to α-KG is catalyzed by either glutamate dehydrogenase (GDH) or other transaminases, including glutamate pyruvate transaminase 2 (GPT2), and glutamate oxaloacetate transaminase 2 (GOT2), which convert α-keto acids into their corresponding amino acids in mitochondria [5]. Fluxes of these enzymes are commonly elevated in human cancers [2, 6]. Although elevated glutaminolysis has been theoretically justified in various tumors [6-10], it remains unknown that whether modification of these enzyme(s) could determine the development of glioma.

Here in this work, we demonstrate GDH as a regulator for intracellular α-KG in mitochondria and GDH expression and activity are elevated in glioma. High level of GDH predicts poor survival. We show that

GDH promotes glioma cell growth *in vitro* and *in vivo* using loss-of-function and gain-of-function strategies. GDH promotes cellular bioenergetics and regulates redox homeostasis in glioma cells. Finally, either genetic or pharmacologic inhibition of GHD inhibits glioma development *in vitro* and *in vivo*.

MATERIALS AND METHODS

Human patients

One hundred and nine cases of glioma patients with full case history between January 2005 and December 2009 were selected and included in the present study (Table 1). Fresh glioma tissues were obtained, and the fresh tissues were stored at -80°C before use. All human tissue samples of normal brain and glioma were obtained from the *Department of Neurosurgery, Renji Hospital (Shanghai, China)*. All samples were classified according to the fourth edition of the histological grades of tumors of the nervous system published by the WHO in 2007 [11]. A written form of informed consent was obtained from all patients and donors. The study was approved by the *Clinical Research Ethics Committee of Renji Hospital*.

Cell culture

Human glioma cell lines T98G, U87MG, A172, U251 and CCF-STTG1 were purchased from the ATCC and cultured according to the guidelines recommended by the ATCC. All cells were maintained at 37 °C with 5% CO_2. The NHA cell line was purchased from the Lonza group and cultured with Clonetics medium and reagents. The other HA cell line was purchased from ScienCell Research Laboratories and cultured with astrocyte medium.

Lentivirus packaging and infection

Sh-GDH, sh-GPT2, sh-GOT2, sh-GPx and control shRNA (sh-Ctrl) lentivirus particles were purchased from GenePharma. The shRNA sequences are shown in Supplementary Table S1. Lentivirus expressing human GDH or Sirt4 were generated by sub-cloning human GDH or Sirt4 cDNA to the pSLIK lentivirus expression system. For retroviral packaging, 293T cells were co-transfected with the retroviral particles. For transduction, cells were incubated with virus-containing supernatant in the presence of eight mg/ml polybrene. After 48 hours, infected cells were selected for 72 hours with puromycin (2 mg/ml) or hygromycin (200 mg/ml).

Intracellular metabolite measurements

The intracellular levels of α-KG was determined by using commercial kits (BioVision, # K677-100). Briefly, 2×10^6 cells were homogenized in PBS. The supernatant was collected, and proteins were removed by using 10 KD Amicon Ultra Centrifugal Filters (Millipore). The flow-through containing the metabolites was used for the measurement of α-KG following the manufacturer's instructions.

Western blot

Tissues and glioma cells were lysed with cell lysis buffer (Beyotime, #P0013) supplemented with protease inhibitor mixture (Biotool, # B14001). Western blot was performed as previously described[14]. The following antibodies were used: anti-GAPDH (Santa Cruz, # sc-365062), anti-GPT2 (Santa Cruz, # sc-398383), anti-GOT2 (Abcam, # ab90562), anti-GDH (Abcam, #ab34786), anti-Gpx (Santa Cruz, #sc-22145) and anti-Sirt4 (Santa Cruz, #sc-135053).

Antioxidant enzymatic activity assay

GDH enzyme activity assay was performed using GDH activity assay kit (BioVision, # K729-100). 20 mg of total cell lysates was added to the reaction mixture containing 50 mM triethanolamine (pH 8.0), 100 mM ammonium acetate, 100 mM NADPH, and 2.6 mM EDTA. The reaction was initiated by adding α-KG, and the activity was assessed by monitoring the oxidation of NADPH as a decrease in absorbance at 340 nm. GPx1 enzyme activities were determined by using commercially available kits from BioVision according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from cells or tissues with TRIzol and cDNA was synthesized from 1μg of RNA with One Step RT-PCR Kit (Clontech, # RR055A). q-PCR was performed with the SYBR Green (TAKARA, # RR820A) on an ABI-7500 RT-PCR system (Applied Biosystems). The primers were listed in Supplementary Table S2.

Cell proliferation assay

Cell proliferation was monitored by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (BioVision, # K299-1000) in according to the guidelines.

Cell colony formation assay

Glioma cells were suspended in 1.5 ml complete medium supplemented with 0.45% low melting point agarose (Invitrogen). The cells were placed in 35 mm tissue culture plates containing 1.5 ml complete medium and agarose (0.75%) on the bottom layer. The plates were incubated at 37°C with 5% CO_2 for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

Table 1: Clinical parameters of glioma patients

Clinical parameters		N (%)
Age (years)	<50	25 (23)
	>=50	84 (77)
Sex	Male	61 (56)
	Female	48 (44)
PS	0	65 (60)
	1	34 (32)
	2	10 (8)
Surgery	Partial	38 (35)
	Sutotal resection	60 (55)
	Total resection	11 (10)
Hemisphere	Left	59 (54)
	Right	33 (30)
	Bilateral	17 (16)
Location	Temporal	35 (32)
	Parietal	24 (22)
	Occipital	11 (10)
	Frontal	25 (23)
	Deep	4 (3)

Xenograft studies

Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University. Nude mice (athymic nu/nu, female, 4–6 weeks old; Harlan) were subcutaneously injected with 1×10^7 U251 cells harboring empty vector on the left flank, and cells with stable knockdown of GDH (or overexpression of GDH or Sirt4) on the right flank, respectively. To evaluate the efficacy of EGCG and R162, the drugs were administered from a day after U251 cells injection by daily intraperitoneal injection of 50 mg/kg EGCG (Sigma, # E4143) or 30 mg/kg R162 (provided by Medicilon, Shanghai) for 35 days, respectively. Fifty percent of DMSO in PBS was as a diluent control. Tumor growth was recorded by measurement of two perpendicular diameters of the tumors, and tumor size was calculated using the formula $4\pi/3 \times(\text{width}/2)^2 \times (\text{length}/2)$. The tumors were harvested and weighed at the experimental endpoint.

Glutamine uptake measurement

The labeling and measurement of glutamine uptake were performed as described previously [10].

ATP and ADP measurement

To measure ATP and ADP amounts, the cells were lysed in somatic releasing buffer (Sigma, #FLSAR-1VL). Whenever possible, all experiments were conducted in the cold room and all reagents were at 4°C prior to use. The total ATP amounts were measured via luciferase activity (Sigma) with linear ranges determined for both the samples and standard controls. The ADP levels were measured by adding excess amounts of phosphoenolpyruvate (PEP) and pyruvate kinase (both from Sigma, #9067-77-0 and #9001-59-6). A detailed protocol was described previously [10].

Mitochondrial ROS measurement

Mitochondrial ROS level was determined by using a specific mitochondrial H_2O_2 probe, MitoPY1 (Sigma, #SML0734). 2×10^5 cells were incubated with 10 mM of MitoPY1 for 30 min at 37°C. Cells were washed and analyzed by flow cytometry (BD FACSCanto).

Statistical analysis

Statistical analysis and graphical presentation was performed using Prism 6 (GraphPad). Data shown are from one representative experiment of multiple independent

experiments and are given as mean ± SEM. Statistical differences between two groups were determined using Student's *t* test. The Kaplan-Meier method was used to estimate overall survival. Survival differences according to GDH expression were analyzed by the log-rank test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

GDH regulates α-KG production in glioma cells

To better understand the glutamine metabolism in human glioma, we first tested intracellular α-KG level in human normal astrocyte cells (NHA and HA) and glioma cell lines (T98G, U251, U87MG, A172 and CCF-STTG1). We found that intracellular α-KG level in glioma cells was higher than that in astrocytes (Figure 1A). We next tested whether conversion of glutamate to α-KG, a crucial step in glutaminolysis, contributes to higher α-KG levels in glioma cells. We first knocked down GDH and other two mitochondrial enzymes, GOT2 and GPT2 in U251 cells (Figure 1B). Interestingly, we found that GDH but not GOT2 or GPT2 was the enzyme responsible for the conversion of glutamate to α-KG (Figure 1C). Furthermore, we found that GDH activity, mRNA level and protein levels in glioma cells were higher than in astrocytes (Figure 1D-1F). These results suggest a crucial role of GDH in glutaminolysis in human glioma cells.

GDH is overexpressed in human glioma and predicts survival

We determined the mRNA levels of GDH in fresh human glioma tissues (n=34) and normal brain tissues (n=24), and found that GDH expression in glioma tissues was much higher than that in normal brain tissues (Figure 2A; Supplementary Figure S1). In addition, we also determined the enzymatic activity of GDH in fresh normal brain tissues (n=8) and glioma tissues (n=8). The activity of GDH was significantly higher in glioma tissues compared to normal brain tissues (Figure 2B). We then tested GDH mRNA levels of glioma tissues from 109 patients, and divided the patients to GDH low and high expression groups. Kaplan-Meier curve comparing was performed to analyze overall survival of glioma patients with the low (n=26) versus high (n=83) GDH expression. Strictly, GDH high level predicts poor overall survival of glioma patients (Figure 2C). Taken together, GDH expression and activity were up-regulated in human glioma tissues and high GDH expression level predicts poor survival.

GDH promotes glioma cell growth *in vitro* and *in vivo*

We next determined whether GDH regulates cellular behaviors of glioma cells. To this end, two (T98G and U251) of the five glioma cell lines were used as models for further study of GDH function in glioma proliferation and colony formation. To investigate the role of GDH in glioma development, we used lentivirus system to knockdown GDH in glioma cells. GDH knockdown significantly reduced the proliferation rate of U251 and T98G cells (Figure 3A; Supplementary Figure S2A). We next probed the contribution of GDH in the transformative properties of glioma cells. Glioma cells with stably GDH knockdown were subjected to colony formation assay. We found that U251 and T98G with stable GDH knockdown possessed reduced colony-forming activity (Figure 3B). In consistent with these findings, we found that lentivirus-mediated GDH overexpression promoted proliferation and colony formation in U251 cells (Figure 3C and 3D; Supplementary Figure S2B).

Next, we examined the effects of GDH on tumor growth using subcutaneous xenograft model of U251 cells in mice. We found that U251 cell growth was markedly reduced by GDH knockdown (Figure 4A-4C). In contrast, GDH stable overexpression facilitated U251 cell growth *in vivo* (Figure 4D and 4E). These findings demonstrate that GDH is an oncogenic factor in human glioma.

GDH regulates bioenergetics in glioma cells

To decipher the role of GDH in bioenergetics of cancer cells, we performed a set of metabolic assays using glioma cells U251 and T98G with GDH knockdown. We tested whether GDH itself directly regulates cellular glutamine uptake. The stable knockdown of GDH resulted in the repression of glutamine uptake in U251 and T98G cells (Supplementary Figure S3A), whereas GDH overexpression promoted glutamine uptake (Supplementary Figure S3B). We found that attenuation of GDH in cancer cells decreased the intracellular ATP levels compared with control cells harboring an empty vector (Supplementary Figure S3C). In contrast, the intracellular ATP/ADP ratio was significantly up-regulated by GDH overexpression in human glioma cells (Supplementary Figure S3D).

GDH is a mitochondrial enzyme critical for conversion of glutamate to α-KG, which enters TCA cycle and contributes to ATP generation. We therefore tested whether α-KG supplement could rescue the effect of GDH knockdown on ATP level and cell proliferation in glioma cells. The results showed that supplement of cell-permeable methyl-α-KG significantly rescued the attenuated intracellular ATP/ADP ratio and cellular proliferation ability in U251 and T98G cells (Supplementary Figure S3E and S3F). These findings indicate that GDH regulates bioenergetics in glioma cells.

GDH regulates ROS homeostasis in glioma cells

We also interested in whether other mechanism contributed to the function of GDH in human glioma

cells. A current report showed that GDH is a regulator for mitochondrial redox homeostasis by promoting the activity of glutathione peroxidase 1 (GPx1) [6]. We determined the effect of GDH on mitochondrial ROS level in glioma cells. The results showed that GDH knockdown increased mitochondrial H_2O_2 level in U251 cells, whereas GDH overexpression reduced mitochondrial H_2O_2 level (Figure 5A and 5B). When treatment with the mitochondrial antioxidant, MitoQ, the effects of GDH knockdown on mitochondrial ROS was blocked (Figure 5C). In addition, MitoQ also rescued the effects of GDH knockdown on cell growth of U251 cells (Figure 5D). We found that

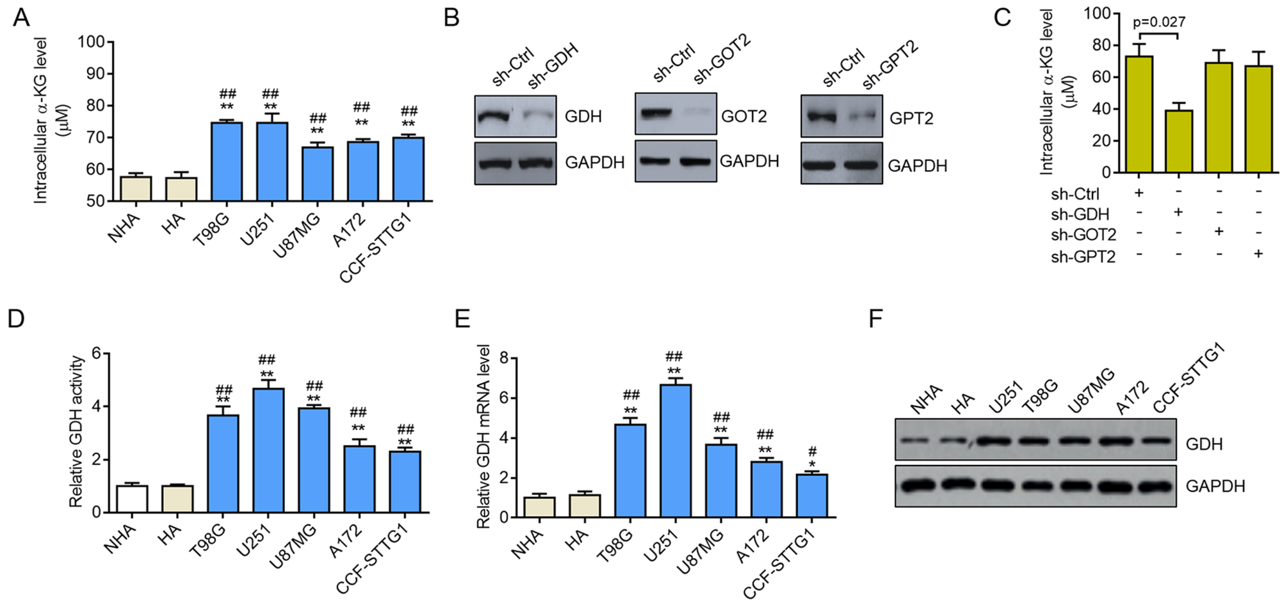

Figure 1: GDH regulates α-KG production in glioma cells. A. Intracellular α-KG level in human normal astrocyte cells (NHA and HA) and glioma cell lines (T98G, U251, U87MG, A172 and CCF-STTG1). **p<0.01 *vs.* NHA, ##p<0.01 *vs.* HA. **B.** Knockdown of GDH, other two mitochondrial enzymes, GOT2 and GPT2 with lentivirus-mediated shRNA in glioma U251 cell line. U251 cells were infected with indicated lentivirus for 24 hours and then protein levels were determined. **C.** Intracellular α-KG level of U251 cells with GDH, GOT2 and GPT2 knockdown. U251 cells were infected with indicated lentivirus for 24 hours and then intracellular α-KG levels were determined. **D.** GDH enzymatic activity was determined in normal glia cells and glioma cell lines. **p<0.01 *vs.* NHA, ##p<0.01 *vs.* HA. **E.** GDH mRNA levels were determined in normal glia cells and glioma cell lines. **p<0.01 *vs.* NHA, ##p<0.01 *vs.* HA. **F.** GDH protein levels were determined by western blot in normal glia cells and glioma cell lines.

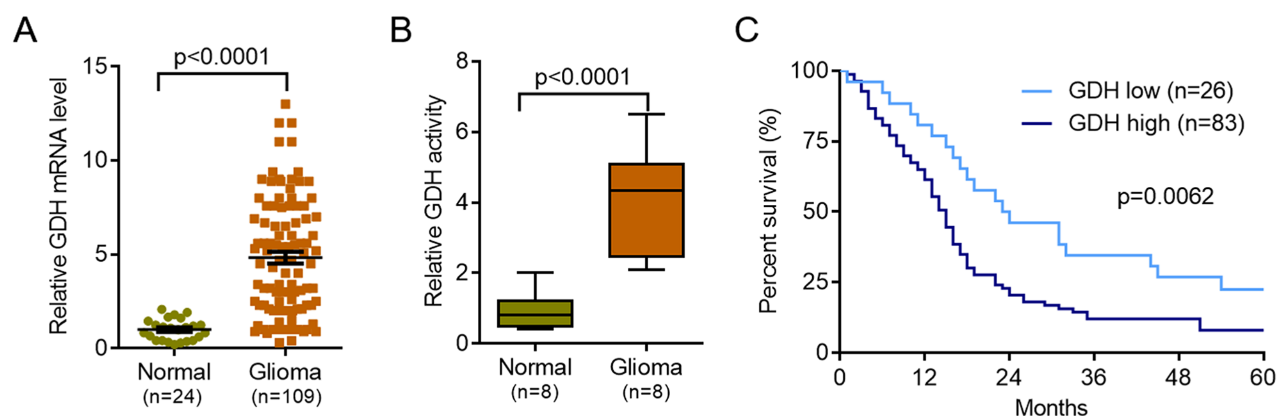

Figure 2: GDH is upregulated in human glioma and predicts overall survival. A. GDH mRNA levels were determined in fresh normal brain tissues and glioma tissues in human patients. N=24 in normal group and n=109 in glioma group. **B.** GDH enzymatic activity was determined in normal brain tissues and glioma tissues in human patients. N=8 per group. **C.** GDH level predicts survival of patients. Kaplan-Meier curve comparing overall and disease-free survival of glioma patients with the low versus high GDH expression using the q-PCR data. Those with GDH level higher than the median value were enrolled into the GDH high group (n=83) and the others into the GDH low group (n=26). We included this information in the Figure legends.

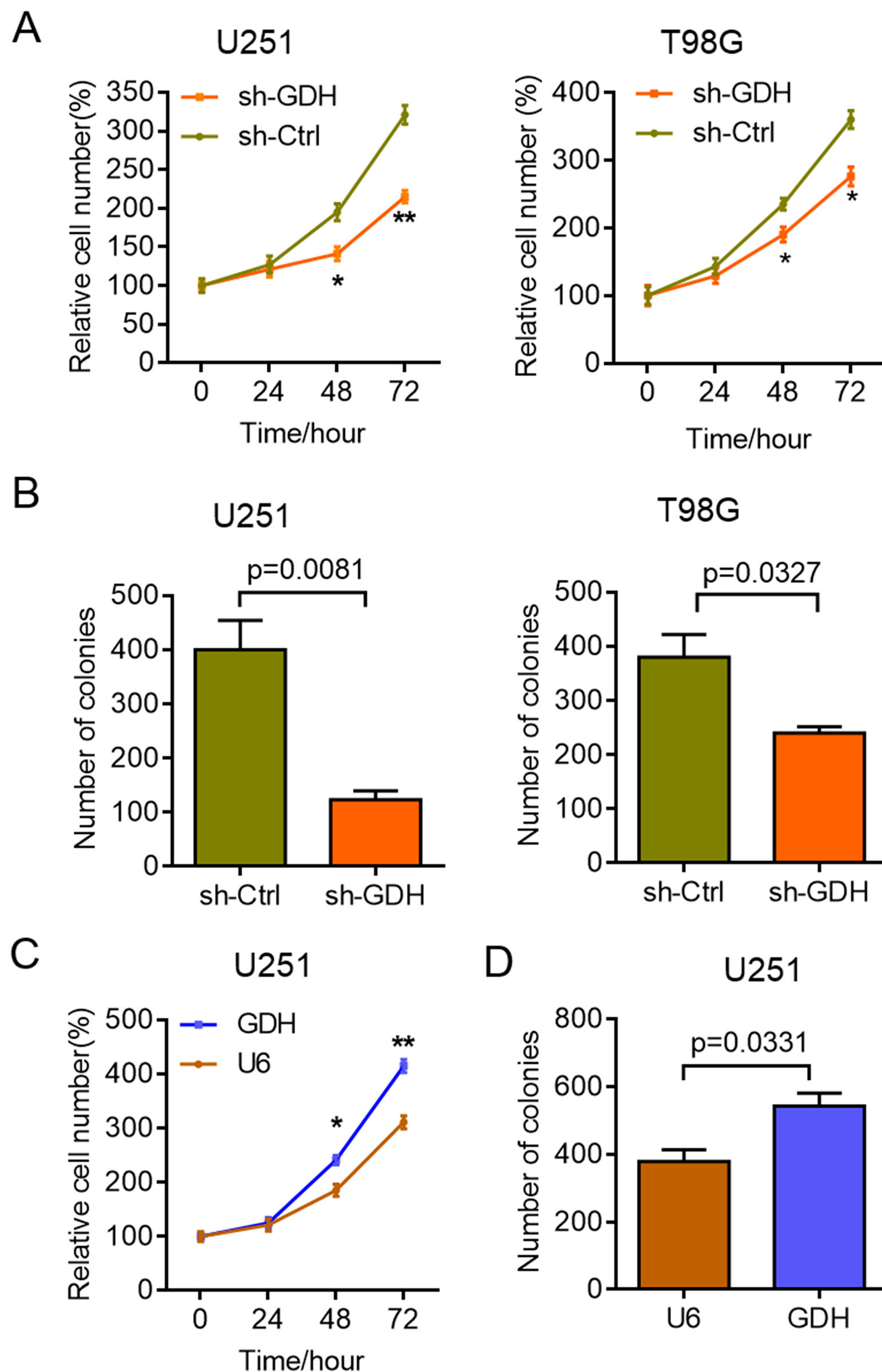

Figure 3: GDH promotes glioma cell proliferation and colony formation *in vitro.* A. GDH knockdown inhibits glioma cell growth. U251 and T98G cells were infected with lentivirus carrying sh-GDH or sh-Ctrl. **B.** GDH knockdown inhibits glioma cell colony formation. Quantitative analysis of colony numbers are shown. U251 and T98G cells with/without GDH stable knockdown were subjected to colony formation assay. **C.** GDH overexpression promotes glioma U251 cell proliferation. U251 cells were infected with lentivirus carrying human GDH or control vector. **D.** GDH overexpression promotes colony formation of glioma U251 cells. U251 cells with/without GDH stable overexpression were subjected to colony formation assay.

GDH inhibited the activity of GPx1 (Figure 5E and 5F). We next knocked down GPx1 in U251 cells (Figure 5G). GPx knockdown increased mitochondrial ROS level and blocked the effect of GDH knockdown on mitochondria ROS level (Figure 5H), indicating that GPx1 contributes to the function of GDH in regulating redox homeostasis. We finally tested whether α-KG supplement could rescue the effect of GDH knockdown on GPx1 activity and mitochondrial ROS. The results demonstrate that methyl-α-KG treatment increased GPx1 activity and reduced mitochondrial ROS level in U251 cells (Supplementary Figure S4), indicating thatα-KG contributes to the role of GDH in mitochondrial oxidative stress.

Inhibition of GDH inhibits glioma growth

The above findings demonstrated that GDH regulates bioenergetics and redox homeostasis, and acts as an oncogenic factor for glioma. These evidence indicate that GDH may serve as a potential candidate target for glioma therapy. We therefore designed genetic and pharmacological strategies to inhibit the function of GDH for glioma treatment.

Sirt4, a mitochondrial member of the Sirtuin family and an ADP-rybosylase, was reported to inhibit the activity of GDH [12]. Therefore, we first genetically overexpressed Sirt4 in glioma U251 cells stalely (Figure 6A). We found that Sirt4 expression was downregulated in human glioma tissues (Supplementary Figure S5). Sirt4 overexpression significantly inhibited GDH activity and reduced intracellular α-KG level (Figure 6B and 6C). Next, U251 cells with Sirt4 stable overexpression were subjected to cell proliferation and colony formation assay, the results showed that Sirt4 overexpression repressed U251 proliferation and colony formation (Figure 6D). We also examined the effects of Sirt4 on tumor growth using subcutaneous xenograft model of U251 cells in mice. We found that U251 cell growth was markedly reduced by Sirt4 overexpression (Figure 6F).

We further determined whether pharmacologic inhibition of GDH activity could also repress glioma development. We inhibited GDH activity with a reported inhibitor, EGCG, in U251 cells (Figure 7A and 7B). EGCG treatment reduced intracellular α-KG level (Figure 7C), and repressed U251 proliferation and colony formation (Figure 7D and 7E). Strictly, EGCG treatment could inhibit the growth of U251 cells *in vivo* (Figure 7F). These findings indicated that inhibition of GDH with EGCG inhibited the development of glioma. However, EGCG targets a group of enzymes that use NADPH as a cofactor

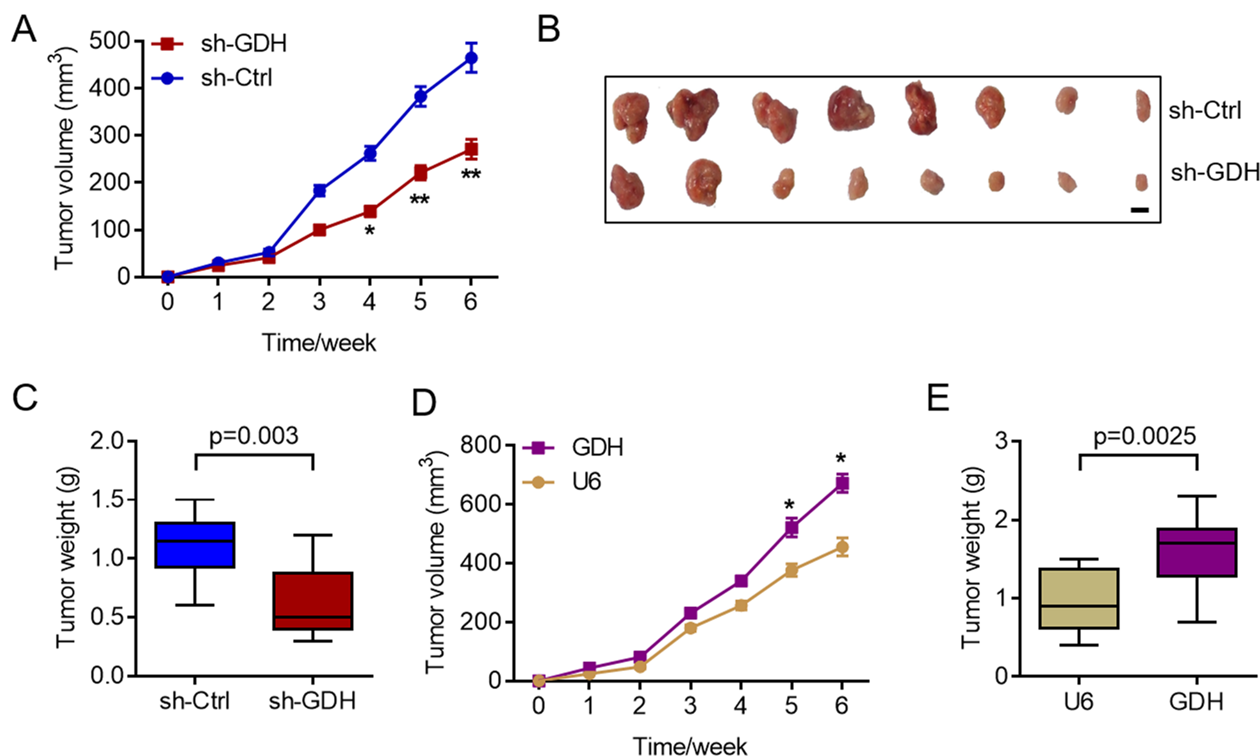

Figure 4: GDH facilitates glioma growth *in vivo*. A. Tumor growth curve *in vivo*. U251 cells with/without GDH stable knockdown were subjected to xenograft study. N=8 in each group. *p<0.05,**p<0.01 *vs.* sh-Ctrl. **B.** Representative picture showing glioma size *in vivo* at six weeks. Bar= 2 cm. **C.** GDH knockdown inhibits glioma U251 cell growth *in vivo*. Tumor weight was evaluated at the end of the experiment. N=8 in each group. **D.** Tumor growth curve *in vivo*. U251 cells with/without GDH stable overexpression were subjected to xenograft study. N=8 in each group. *p<0.05 *vs.* U6. **E.** GDH overexpression promotes glioma U251 cell growth *in vivo*. Tumor weight was evaluated at the end of the experiment. N=8 in each group.

[13-15]. Jin *et al*. [6] currently identified R162 (Figure 8A), a purpurin analog, as a potential GDH inhibitor. R162, which is more cell-permeable than purpurin due to its allyl group, demonstrated inhibitory effects on mitochondrial GDH activity and decreased intracellular α-KG level in U251cells (Figure 8B and 8C). Finally, we found that R162 treatment repressed U251 cell growth *in vitro* and *in vivo* (Figure 8D-8F).

Taken together, either genetic or pharmacological targeting GDH attenuated glioma development.

DISCUSSION

Three enzymes (GDH, GOT2 and GPT2) are reported to convert glutamate to α-KG [5]. Interestingly, our evidence showed that GDH maintains the physiological levels of α-KG in glioma cells (Figure 1), which is in consistent with the condition in breast cancer and leukemia [6], indicating that this mechanism may present a common phenomenon in cancer cells. We found that the expression level and activity of GDH were much higher in glioma cells compared with normal astrocyte cells, which may be accounted for higher intracellular α-KG in glioma cells (Figure 1).

Isocitrate dehydrogenase 1 (IDH1) belongs to the family of isocitrate dehydrogenase enzymes which is comprised of three members: IDH1, IDH2 and IDH3. All IDH enzymes catalyze the oxidative decarboxylation of isocitrate to α-KG with the reduction of either $NADP^+$ or NAD^+ to generate NADPH or NADH respectively [16]. Mutations in the IDH1 gene in a large subset of human low-grade gliomas [17, 18]. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype and contributes to glioma development [19]. Here we identify the role of the mitochondrial α-KG regulator, GDH, in glioma. GDH expression and activity were up-regulated in human glioma tissues. Strictly, high GDH expression predicted poor overall survival in patients with glioma (Figure 2). These findings indicate that both cytoplasmic and mitochondrial α-KG levels are critical for maintaining cell fate in human glioma. However, it remains to determine whether some GDH mutations exist in patients with glioma and how GDH expression is regulated remains unknown.

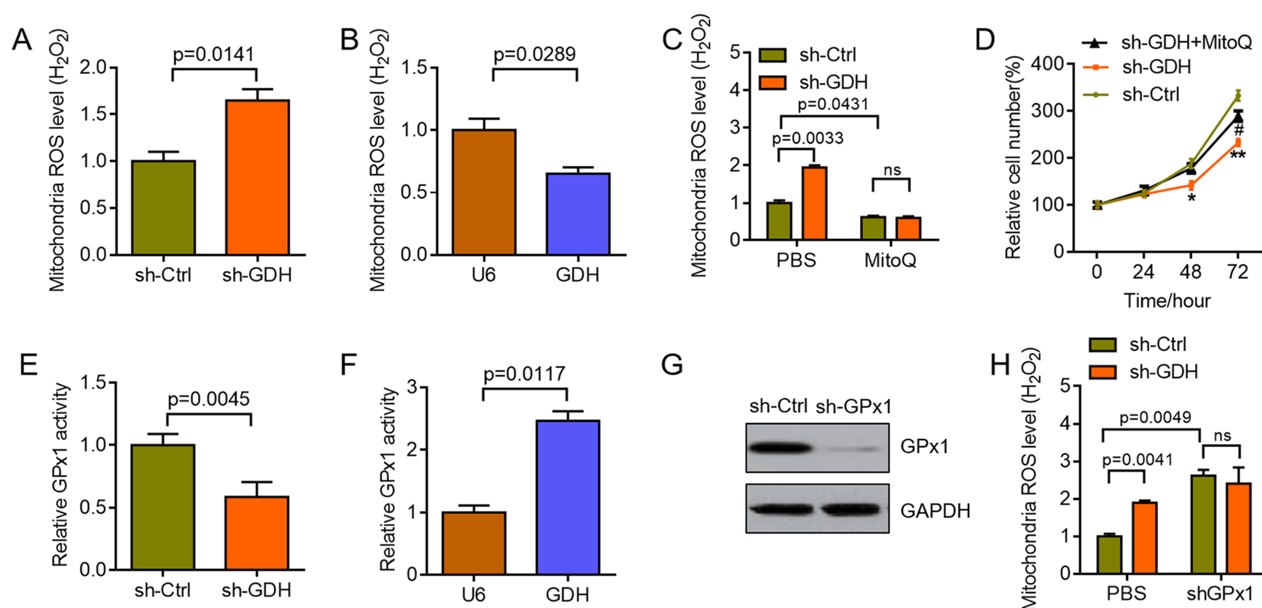

Figure 5: GDH contributes to redox homeostasis in cancer cells. A. GDH knockdown up-regulates mitochondrial ROS level. U251 and T98G cells were infected with lentivirus carrying sh-GDH or sh-Ctrl for 24 hours and then mitochondrial ROS were determined. **B.** GDH overexpression down-regulates mitochondria ROS level. U251 and T98G cells were infected with lentivirus carrying human GDH or empty control for 24 hours and then mitochondrial ROS were determined. **C.** MitoQ blocks the effects of GDH knockdown on mitochondrial ROS. U251 and T98G cells were pretreated with 100nM mitoQ for 24 hours and then infected with lentivirus carrying sh-GDH or sh-Ctrl for 24 hours and then mitochondrial ROS were determined. **D.** MitoQ blocks the effects of GDH knockdown on cell proliferation. U251 and T98G cells were pretreated with 100nM mitoQ for 24 hours and then infected with lentivirus carrying sh-GDH or sh-Ctrl. *p<0.05,**p<0.01 *vs.* sh-Ctrl. # indicates p<0.05 of sh-GDH *vs.* sh-GDH+mitoQ. **E.** GDH knockdown promotes GPx activity. U251 and T98G cells were infected with lentivirus carrying sh-GDH or sh-Ctrl for 24 hours and then GPx activity was determined. **F.** GDH overexpression inhibits GPx activity. U251 and T98G cells were infected with lentivirus carrying human GDH or empty control for 24 hours and then GPx activity was determined. **G.** Western blot showing GPx knockdown in U251 cells. U251 cells were infected with lentivirus carrying sh-GPx or sh-Ctrl for 24 hours and then GPx protein level was determined. **H.** GPx knockdown blocks the effects of GDH knockdown on mitochondrial ROS. U251 cells were infected with lentivirus carrying sh-GDH, sh-GPx or sh-Ctrl for 24 hours and then mitochondrial ROS were determined.

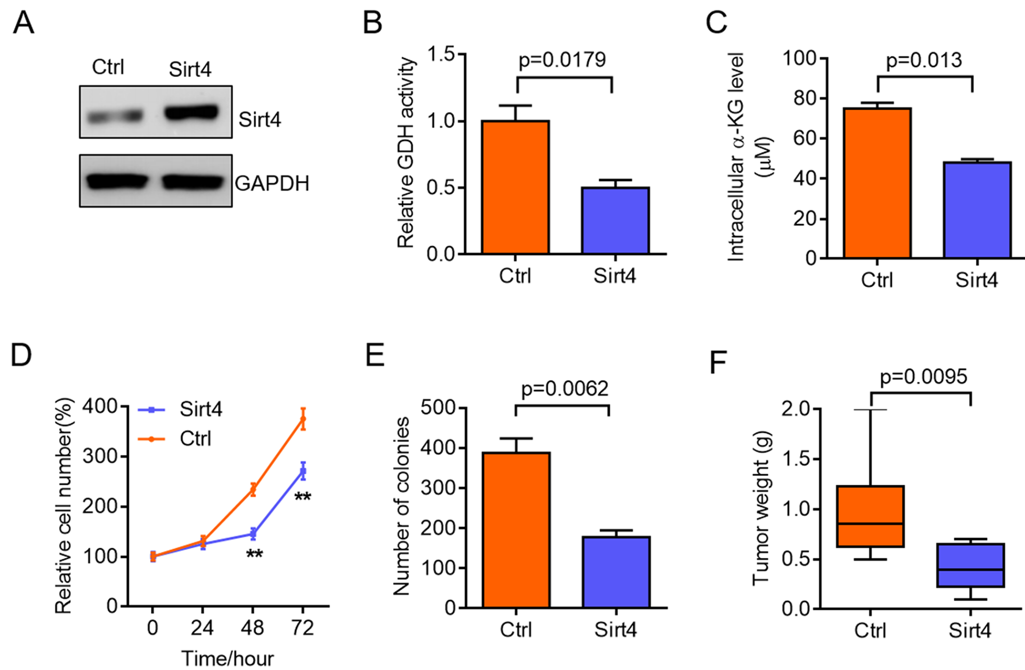

Figure 6: Sirt4 inhibits GDH activity and represses glioma cell growth. A. Western blot showing Sirt4 overexpression in U251 cells. U251 cells were infected with lentivirus carrying human Sirt4 for 24 hours. **B.** Sirt4 overexpression inhibits GDH activity in U251 cells. U251 cells were infected with lentivirus carrying human Sirt4 for 24 hours. **C.** Sirt4 overexpression downregulates cellular α-KG level in U251 cells. U251 cells were infected with lentivirus carrying human Sirt4 for 24 hours. **D.** Sirt4 overexpression inhibits cell proliferation. U251 cells were infected with lentivirus carrying human Sirt4 or empty control and subjected to cellular proliferation assay. **E.** Sirt4 overexpression inhibits colony formation. U251 cells stably carrying human Sirt4 or empty control were subjected to colony formation assay. **F.** Sirt4 overexpression inhibits U251 cell growth *in vivo*. Xenograft study was performed with U251 stably expression human Sirt4 or empty control. Tumor weight was examined at the experimental endpoint. N=8 in each group.

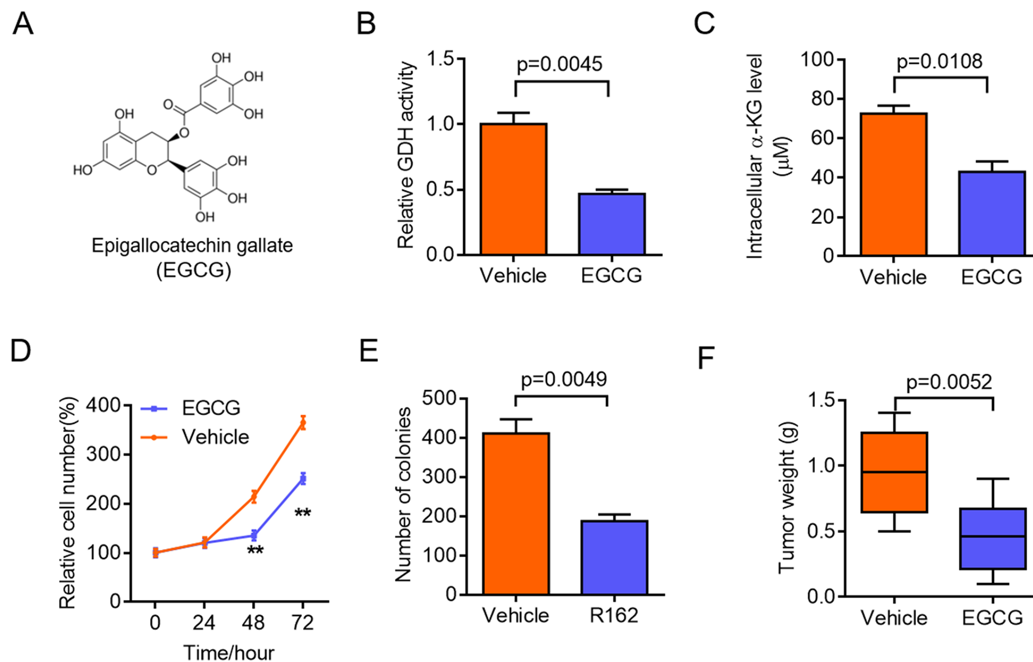

Figure 7: EGCG inhibits GDH activity and represses glioma cell growth. A. Chemical structure of EGCG. **B.** EGCG inhibits GDH activity in U251 cells. U251 cells were treated with EGCG (50 μM) for 24 hours and then GDH activity was determined. **C.** EGCG downregulates cellular α-KG level in U251 cells. U251 cells were treated with EGCG (50 μM) for 24 hours and then intracellular α-KG level was determined. **D.** EGCG inhibits cell proliferation of U251 cells. U251 cells were treated with EGCG (50 μM) for indicated 0, 24, 48 and 72 hours. **E.** EGCG inhibits colony formation of U251 cells. U251 cells were treated with EGCG (50 μM). **F.** EGCG inhibits U251 cell growth *in vivo*. Mice were treated with EGCG (50 mg/kg/day). Tumor weight was examined at the experimental endpoint. N=8 in each group.

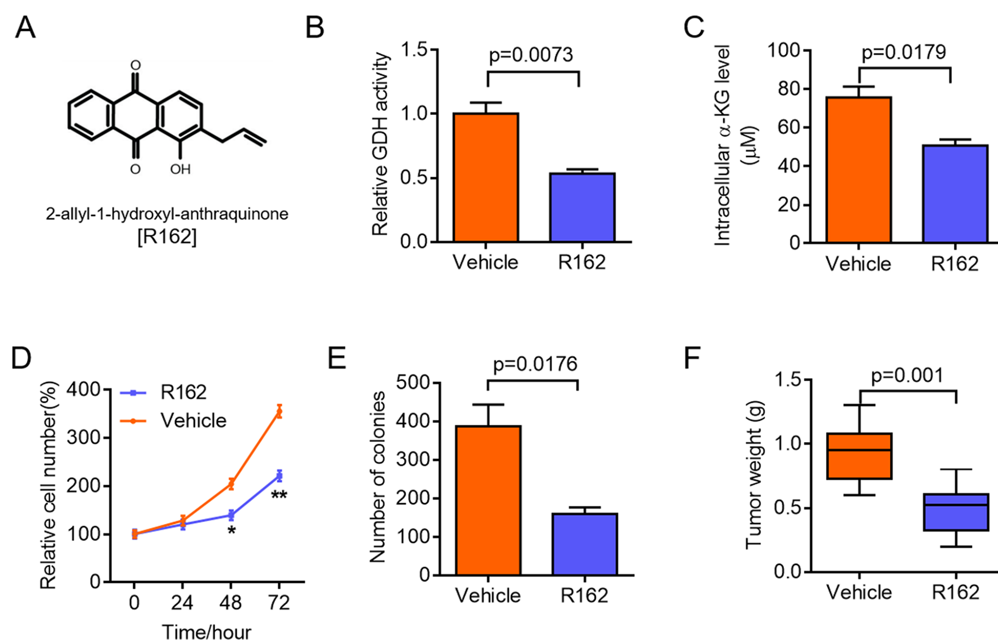

Figure 8: R162 inhibits GDH activity and represses glioma cell growth. **A.** Chemical structure of R162. **B.** R162 inhibits GDH activity in U251 cells. U251 cells were treated with R162 (20 μM) for 24 hours and then GDH activity was determined. **C.** R162 downregulates cellular α-KG level in U251 cells. U251 cells were treated with R162 (20 μM) for 24 hours and then intracellular α-KG level was determined. **D.** R162 inhibits cell proliferation of U251 cells. U251 cells were treated with R162 (20 μM) for indicated 0, 24, 48 and 72 hours. **E.** R162 inhibits colony formation of U251 cells. U251 cells were treated with R162 (20 μM). **F.** R162 inhibits U251 cell growth *in vivo*. Mice were treated with R162 (30 mg/kg/day). Tumor weight was examined at the experimental endpoint. N=8 in each group.

Glutamine serves as an alternative carbon source for both bioenergetics and anabolic biosynthesis in addition to glucose. Consistently, our results showed that suppression of GDH resulted in decreased glutamine uptake, which may render cancer cells more dependent on glycolysis and more sensitive to stress conditions such as glucose deprivation. Moreover, GDH deficiency resulted in decreased biosynthesis of ATP. The GDH knockdown cells showed decreased cell proliferation and tumor growth (Figure 3 and 4), suggesting that metabolic defects due to GDH deficiency may eventually contribute to these changes. Interestingly, a previous report showed that glioblastoma cells require GDH to survive impairments of glucose metabolism or Akt signaling [20]. Our findings showing that GDH regulates redox homeostasis through GPx1 shed insights into the current understanding of the biological functions of GDH and reveal a distinct crosstalk between glutaminolysis and redox maintenance (Figure 5). Our findings, along with others [3, 6, 21, 22], showcase the complicated signaling properties in cancer cells that coordinate metabolic and cell signaling networks to provide ultimately optimized proliferative advantages to cancer cells. Taken together, these findings implicate that GDH plays a critical role in α-KG-mediated biological function, including not only bioenergetics but redox homeostasis in glioma cells.

Anyhow, GDH expression and activation contribute to glioma development, indicating that GDH may be a potential target for glioma therapy. Targeting GDH by shRNA, Sirt4 overexpression or small molecule inhibitors EGCG and R162 led to reduced intracellular α-KG level in glioma cells. GDH inhibition also repressed glioma cancer cell proliferation and tumor growth *in vitro* and *in vivo* (Figures 6-8). In addition, a current work reported that inhibitor (AGI-5198) of mutant IDH1 also delays growth and promotes differentiation of glioma cells [23]. These findings implicate that maintaining α-KG to a relative lower level by inhibition its regulators may serve as a promising therapeutic strategy for glioma treatment.

In conclusion, both GDH expression and activity are up-regulated in glioma, which leads to poor survival. GDH regulates bioenergetics and redox homeostasis in glioma cells and serves as an oncogenic factor for human glioma. Genetic or pharmacological inhibition of GDH activity may serve as a promising strategy for treatment of glioma.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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