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Elevated expression of glutaminase confers glucose utilization via glutaminolysis in prostate cancer

Tiejun Pan [a], Lei Gao [a,*], Guojun Wu [b], Guoqiu Shen [a], Sen Xie [a], Handong Wen [a], Jiarong Yang [a], Yu Zhou [a], Zhong Tu [a], Weihong Qian [a]

[a] Department of Urology, Wuhan General Hospital of Guangzhou Military Region, Wuhan, Hubei, China
[b] Department of Urology, Xijing Hospital, Fourth Military Medical University, Xi'an, China

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ABSTRACT

Cancer cells reprogram their metabolism towards aerobic glycolysis and elevated glutaminolysis, which contributes to the aggressive phenotype. Understanding how these metabolic pathways are regulated may provide critical targets for therapeutic intervention. Glutaminase (GLS1) is a key enzyme that converts glutamine to glutamate. In this study, we show the loss of GLS1 function by RNA interference or inhibitor diminished the rates of glucose utilization, growth and invasiveness of prostate cancer cells. We propose that GLS1 positively regulates glucose uptake in addition to glutaminolysis. Further, GLS1 involves the transcriptional repression of thioredoxin interacting protein (TXNIP), which is a potent negative regulator of glucose uptake and aerobic glycolysis. Most importantly, we provided direct evidence that elevated GLS1 expression was highly correlated with the tumor stage and progression in prostate cancer patients. Together, we defined a key role for GLS1 in coupling glutaminolysis of the TCA cycle with elevated glucose uptake and consequently the growth of prostate cancer cells. These data extends the role of GLS1 in regulating cell metabolism and the clinical utility of GLS1 inhibitors in the restriction of essential nutrients.

1. Introduction

Prostate cancer (PCA) is one of the most common malignant tumors in the world. Multiple biologic processes and genetic alterations are likely the underlying causes of PCA [1]. The aggressiveness of PCA and the lack of targeted therapies highlight the need to understand the pathways required for their development and progression.

Glucose and glutamine are abundant nutrients absolutely required for cell growth and survival [2]. The altered glucose metabolism in cancer cells is termed the Warburg effect, which describes the propensity for most cancer cells to take up glucose avidly and convert it primarily to lactate even with the availability of oxygen, hence termed "aerobic glycolysis" [3,4]. Glutamine is a major source for energy and nitrogen for biosynthesis, and a carbon substrate for anabolic processes in cancer [5]. Actively growing cells depend on glutaminolysis that catabolizes glutamine to generate ATP and maintain the mitochondrial function for metabolism, which is termed "glutamine addiction" [6]. Although the alterations in both nutrient sensing and growth factor signaling pathways in PCA have yet to be adequately investigated [7,8], less is known about how cells respond to nutrient changes and the outcome in coordinating cell growth.

The key enzyme in the conversion glutamine to glutamate is glutaminase (GLS), including two different subtypes: GLS1 (kidney-type) and GLS2 (liver-type) [9]. GLS1 is predominantly expressed in human PC3 prostate cancer cells [10]. Elevated expression of GLS1 has been observed in colorectal cancer [11] and breast cancer [12]. The intracellular glutamate is an important metabolic intermediate that connects with a wide variety of distinct biological processes, including the antioxidant glutathione synthesis, amino acid catabolism, and the conversion to α-ketoglutarate (α-KG) as a substrate for the TCA cycle. This process of glutaminolysis by GLS1 has been shown to mediate genetic events, and proposed as a critical step in targeting glutamine metabolism [13]. Further, multiple genetic factors have also been implicated in

Abbreviations: PCA, prostate cancer; BPH, benign prostatic hyperplasia; GLS, glutaminase; BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide; α-KG, α-ketoglutarate; TXNIP, thioredoxin interacting protein.

* Corresponding author at: Department of Urology, Wuhan General Hospital of Guangzhou Military Region, Wuhan, Hubei 430070, China.
E-mail address: biogaolei@126.com (L. Gao).

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the regulation of GLS1 expression and glutamine metabolism. For example, oncogenic transcriptional factor c-Myc positively regulates GLS1 expression and glutaminolysis through the repression of miR-23 [10]. Due to the critical function of GLS1 for cancer cell growth and survival, several small-molecule inhibitors of glutaminase have been developed, such as BPTES [14] and CB-839 [15], and their antitumor activities have been further evaluated. Especially, CB-839 is currently undergoing clinical investigation in certain types of cancers [15].

In this study, we focused on the role of GLS1 in coordinating the utilization of glucose and glutamine, and its potential association with PCA clinical features. We demonstrated for the first time that elevated GLS1 expression was highly correlated with the tumor stage and progression in PCA patients. GLS1 up-regulates glucose uptake through glutaminolysis in glutamine addicted PC3 cells. Further, GLS1 inhibits transcriptional activation of thioredoxin interacting protein (TXNIP), which is a potent negative regulator of glucose uptake and aerobic glycolysis [16]. Together, these observations highlight the critical associations of GLS1, glucose uptake and tumor progression, and suggest that glutaminase inhibitors may provide therapeutic benefit in PCA treatment via blocking both glucose and glutamine utilization.

2. Materials and methods

2.1. Cell culture and conditions

PC3 cells were maintained at 37 °C under 5% CO_2 in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum. Nutrient depletion studies were performed using glucose and glutamine-free medium without pyruvate. Reconstituted media for all experiments was supplemented with 10% FBS, 2 mM sodium pyruvate, and when needed, glucose or glutamine was added into the media to the final concentrations, 25 and 2 mM, respectively.

2.2. Reagents

2-NBDG (N13195) was from Invitrogen. BPTES (SML0601) was from Sigma–Aldrich. ONTARGETplus SMARTpool GLS1 siRNA (L-004548-01-0005) and ONTARGETplus Non-targeting pool (D-001810-10-5) were from Dharmacon (Subsidiary of GE Healthcare). TXNIP siRNA (sc-44943) was from Santa Cruz Biotechnology (Santa Cruz, California).

2.3. Clinical collection of prostatic samples

A total of 144 PCA and BPH samples were collected, comprised of 37 BPH and 107 PCA samples. The collection and use of human prostatic tissues were approved by the Fourth Military Medical University medical ethics committee. All patients provided written informed consent. Each tumor was scored for pathological stage based on the Gleason system.

2.4. IHC analysis

IHC was performed using the avidin-biotin-peroxidase method. All sections were deparaffinized in xylene and dehydrated using a concentration gradient of alcohol. After non-specific binding was blocked, the slides were incubated with anti-GLS1 antibody in phosphate-buffered saline at 4 °C overnight. Biotinylated goat IgG (1:400, Sigma) was incubated with the sections for 1 h at room temperature and detected with a streptavidinperoxidase complex. The brown color indicative of peroxidase activity was developed by incubating with 0.1% 3,3-diaminobenzidine and 0.05% H_2O_2 for 5 min at room temperature.

2.5. Evaluation of IHC staining

Both the intensity and the extent of immunological staining were analyzed semi-quantitatively. Sections with no labeling or fewer than 5% labeled cells were scored as 0. 15–25%, 25–50%, 50–75% or $\geqslant$75% of labeled cells were scored as 1, 2, 3, 4 respectively. The staining intensity was scored similarly, with 0 used for negative staining, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive. The scores for the percentage of positive cells and for the staining intensity were multiplied to generate an immunoreactive score for each specimen. The product of the quantity and intensity scores were calculated such that a final score of 0 indicated no expression, 1–4 indicated weak expression, 5–8 indicated moderate expression and 9–12 indicated strong expression. Each sample was examined separately and scored by two pathologists. The photos were collected through light microscopy and SPOT Advanced Software (Olympus, Nagano, Japan).

2.6. Cell growth assays

Cells were seeded into each well of a 96-well plate with five replicates for each group at each time point. For MTT assay, 20 ml of MTT solution (5 mg/ml) was added to each well, and after 4 h of incubation, the medium was aspirated and DMSO was added to each well. The OD values were determined at 570 nm using a Sunrise microplate reader (Tecan, Groedig, Austria). To measure viability by direct counting, cells were collected and stained with 0.4% Trypan Blue. Cells excluding and taking up dye were counted on a hemocytometer under phase contrast microscopy.

2.7. In vitro invasion assays

Cell invasions were examined with polycarbonate transwell filters containing 8 mm pores (Becton Dickinson Labware, USA). Cells were seeded in serum-free media on the upper side of a transwell chamber that was coated with Matrigel (BD Biosciences, USA). The cells were allowed to migrate toward media containing 10% fetal bovine serum for 24 h. After the incubation period, the cells on the lower side of the membrane were fixed, stained with crystal violet, and counted. The invasion indices were calculated as the mean number of cells in 10 random fields at $\times$20 magnification.

2.8. Western blotting

Cells were collected and lysed. Protein concentrations were measured using the BCA protein assay kit from Thermo Scientific (Waltham, MA, USA). Forty micrograms of protein were separated by SDS-PAGE and transferred onto Hybond ECL nitrocellulose membranes. Western blotting analyses were performed using the following primary antibodies at a dilution of: 1:1000 for anti-GLS1 (Abcam, Cambridge, England); 1:1000 for anti-TXNIP (Medical and Biological Laboratories, Worburn, Massachusetts), 1:5000 for anti-β-actin (Sigma, St. Louis, MO). Secondary antibodies were chosen according to the species of origin of the primary antibodies and detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA) or the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

2.9. Quantitative real-time PCR (qRT-PCR)

cDNA was synthesized from RNA using the PrimeScript RT-PCR Kit (TAKARA, Dalian, China). PCRs were performed using specific primers. The mean Ct values for the target genes were normalized to the mean Ct value for the endogenous control RPL19. The ratio of mRNA expression of target gene versus RPL19 was defined as 2 (−DCt). Primer sequences of genes for PCR are the following: TXNIP

(forward 5-TGGTGGATGATGTCAATACCCCT-3, reverse 5-ATTGGCA AGGTAAGTGTGGC-3), GLUT1 (forward 5-AACTCTTCAGCCAGGGTC-CAC-3, reverse 5-CACAGTGAAGATGATGAAGAC-3 or RPL19 (forward 5-ATGTATCACAGCCTGTACCTG-3, reverse 5-TTCTTGGTCTCTTCCTC CTTG-3).

2.10. ATP analysis assay

ATP Bioluminescence Assay Kit CLS II (Roche Applied Science, Madison, Wisconsin) was used to determine the intracellular ATP levels. After treatment, cells were lysed and ATP levels were measured according to the manufacturer's protocol.

2.11. Glucose uptake assay

Cells were cultured in indicated conditions in the figures. For glucose uptake assays, 100 μM fluorescent glucose analog 2-NBDG (Invitrogen) was added to the media for 1.5 h. Fluorescence was measured using a BD FACScan.

2.12. Statistical analyses

The data are expressed as the means ± SD Statistical analyses using Student's *t*-test for independent groups was performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA) for Windows. Associations between GLS1 expression and categorical variables were analyzed by the Kruskal–Wallis test. $P < 0.01$ was considered as statistically significant.

3. Result

3.1. GLS1 is highly expressed in prostate cancers and correlates with clinicopathological factors

GLS1 catalyzes glutaminolysis through the conversion of glutamine to glutamate. To evaluate the clinical significance of GLS1 in prostate cancer, we examined GLS1 expression in benign prostatic hyperplasia (BPH) tissues from 37 patients and PCA from 107 patients. Immunohistochemical (IHC) analysis showed that GLS1was highly expressed in PCA tissues compared with BPH tissues (Fig. 1A). Statistical analysis showed that the rate of GLS1 positive expression in PCA specimens was 63.55% (68/107), which was significantly higher than that in BPH tissues 24.32% (9/37) (Fig. 1B). To determine the clinical significance of elevated GLS1 expression in PCA patients, we further examined the correlation between GLS1 protein expression and clinicopathological factors, including age, Gleason scores and TNM stage in PCA patients. The results demonstrated that the expression of GLS1 was positively correlated with Gleason scores and TNM stage (Supplementary Table 1). That is to say, an increase in Gleason score or TNM stage generally meant an increase in the percentages and levels of GLS1 positive expression.

3.2. Glutamine is a more important energy source in PC3 cells

Malignant cancer cells always require glutamine for survival, and exhibit a phenotype of "glutamine addiction" [6]. To better understand how glucose and glutamine coordinate cell growth, we determined the biological changes of human PC3 prostate cancer cell line under different nutrient conditions. The growth and viability of PC3 cells was diminished significantly by glutamine withdrawal and moderately with glucose withdrawal (Fig. 2A and B). The dose dependency of cell growth on glutamine was also observed in the presence or absence of glucose (Fig. 2C). Glucose and glutamine starved cells didn't grow any more, indicating both nutrients are necessary for cell survival (Fig. 2A–C). Accordingly, glutamine withdrawal also resulted in a more significant decrease in ATP levels than glucose withdrawal (Fig. 2D). These results suggested that glutamine as an energy source is more important than glucose in PC3 cells.

3.3. GLS1 dictates glucose uptake and cell survival via glutamine

Since the inhibition of glucose uptake by glutamine was observed in pancreatic cancer cells in the previous report [13], we wonder whether that regulatory mechanism is widespread including prostate cancer. PC3 cells in the absence of glucose and glutamine had elevated glucose uptake (Fig. 3A). By contrast, cells grown in medium containing glucose but lacking glutamine showed low glucose uptake. Addition of increasing concentrations of glutamine to the growth medium resulted in a dose-dependent increase in glucose uptake. Due to the association of GLS1 expression with PCA malignancies, we determined the metabolic responses of PC3 cells to GLS1 expression. Expectedly, the reduction of GLS1 by RNA interference (siGLS1) markedly diminished the cell growth and internal ATP levels (Fig. 3B and C). The glucose uptake were also dramatically decreased by siGLS1, suggesting that GLS1 regulates glucose uptake through glutamine (Fig. 3D). We next used a glutaminase inhibitor BPTES as tool to complement GLS1 knockdown approaches. Consistent with the above results, BPTES blocked cell growth, glucose uptake and internal ATP levels dose-dependently (Fig. 3E–G). Moreover, reduction of GLS1 by

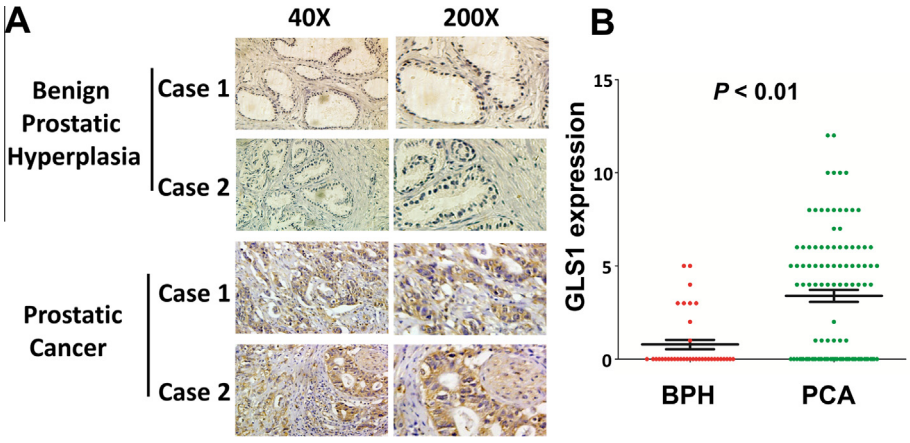

Fig. 1. GLS1 is highly expressed in human prostate cancer tissues. (A) Immunohistochemistry staining of GLS1 in human BPH or PCA tissues from different patients (Case 1 or Case 2). Original magnification: 40× or 100×. (B) Relative expression level of GLS1 in human tissue samples. Student's *t*-test was applied for statistical analyses.

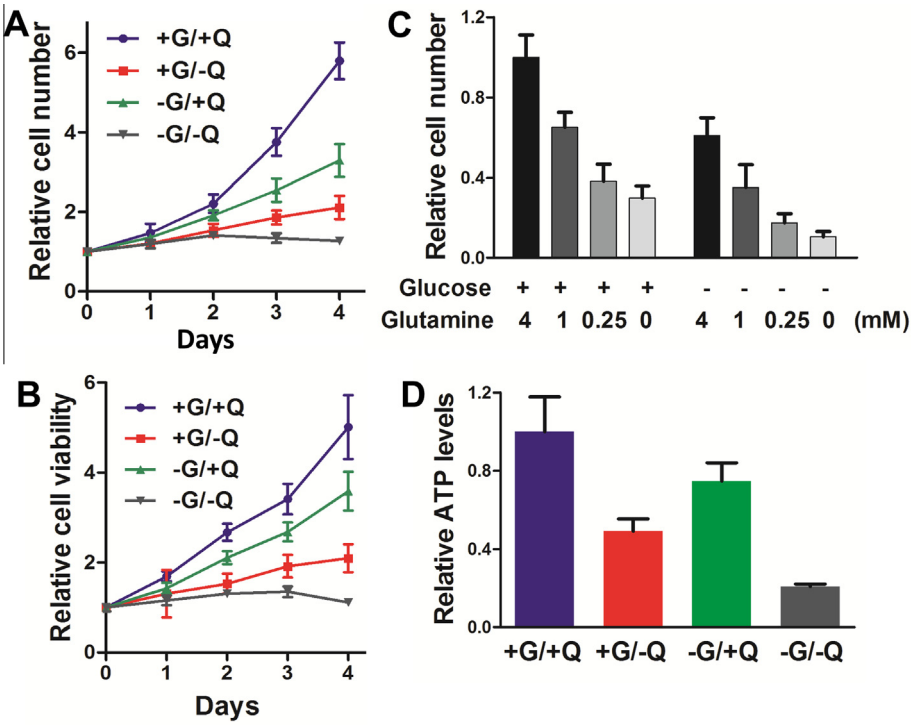

Fig. 2. Glutamine is a more important energy source in PC3 cells. (A and B) PC3 cells were then grown in medium lacking glucose "G" and/or glutamine "Q" as indicated over a 4-day time period. Relative cell numbers (A) or cell viabilities (MTT assay) (B) were determined. (C) Cells were cultured in medium with or without glucose and indicated dose of glutamine. Cell numbers were determined after 4 days. (D) Cells were cultured in normal medium or medium without glucose or glutamine for 48 h and harvested for ATP assay. The results shown were relative ATP levels per microgram total protein normalized to the control normal medium group. (A–D) The data are presented as the means ± SD from triplicate analyses.

either siRNA or BPTES significantly reduced the invasive capacity of PC3 cells (Fig. 3H and I). Collectively, these results demonstrate the pleiotropic role of glutamine in tumor growth, invasion and glucose uptake is dependent on GLS1 activity and glutaminolysis.

Glutamine enters the TCA cycle by being converted to glutamate and subsequently to α-ketoglutarate (α-KG). We then sought to restore α-KG levels in BPTES treated cells to determine whether this would ameloriate growth inhibition. Exposing cells to the cell permeable 3-trifluoromethylbenzyl (TFMB)-α-KG, significantly reduced growth inhibition of PC3 cells by BPTES (Fig. 3J). TFMB-α-KG also stimulated glucose uptake that was repressed by BPTES (Fig. 3K). Therefore, glutaminase inhibition decreased α-KG levels, potentially altering intermediary metabolism and consequently inhibiting the cell glucose uptake and cell survival in advance.

3.4. GLS1 represses TXNIP expression

Reduction of GLS1 decreased cell glucose uptake, raising the possibility GLS1 can contribute elevated glycolysis by a non-canonical mechanism in prostate cancer cells. Since TXNIP is a potent negative regulator of glucose uptake, we therefore determined whether GLS1 stimulates glucose uptake through the repression of TXNIP. TXNIP protein was dramatically induced by glucose, and this induction was repressed by glutamine (Fig. 4A). qRT-PCR analysis also revealed that TXNIP was up-regulated by 10.1-fold in +G/-Q growth medium. By contrast, it was only induced 3.4-fold in +G/+Q growth medium, indicating that glutamine suppressed glucose induced TXNIP expression in transcriptional levels (Fig. 4B). Furthermore, the reduction of GLS1 by siRNA or BPTES remarkably increased TXNIP mRNA and protein expressions, suggesting glutamine catabolism through GLS1 is critical for the induction of TXNIP and the repression of glucose uptake in advance (Fig. 4C–F). It has been reported that TXNIP restricts glucose uptake via the reduction of GLUT1 [17]. Here we observed that GLS1 siRNA or BPTES decreased GLUT1 expression, whereas TXNIP knockdown significantly reversed the reduction of GLUT1 (Fig. 4G and H). These observations document that GLS1 represses TXNIP expression and further induces glucose uptake via GLUT1 in PC3 cells. TXNIP repression by GLS1 or glutamine suggests a mechanism that coordinates the response to glucose and glutamine.

4. Discussion

Cancer cell metabolism is altered compared with normal tissue, which contributes to the initiation and progression of tumors. Therapies that target various aspects of cell metabolism are currently being developed and primarily focused on glucose metabolism [18]. In spite of that, cancer cells with elevated aerobic glycolysis always exhibit increased dependence on glutamine for growth and survival [18]. GLS1 plays a critical role in catalyzing glutaminolysis, and connects with a wide variety of distinct biological processes through the conversion of glutamine to glutamate [9]. Here, we defined a key role for GLS1 in coupling glutaminolysis of the TCA cycle with elevated glucose uptake and consequently the growth of prostate cancer cells.

In order to fully assess GLS1 clinical significance, we firstly evaluated GLS1 expression in the tissues from BPH and PCA patients. Our study demonstrated for the first time elevated GLS1 expression in PCA tissues, which suggested GLS1 might associate with the initiation and progression of prostate cancer. GLS1 expression was higher in T3/T4 tumors, and tumors with higher Gleason scores. Thus, the highly expression of GLS1 always indicates the malignant PCA phenotype in patients, and GLS1 could be considered as an early diagnostic and potential target in cancer therapy.

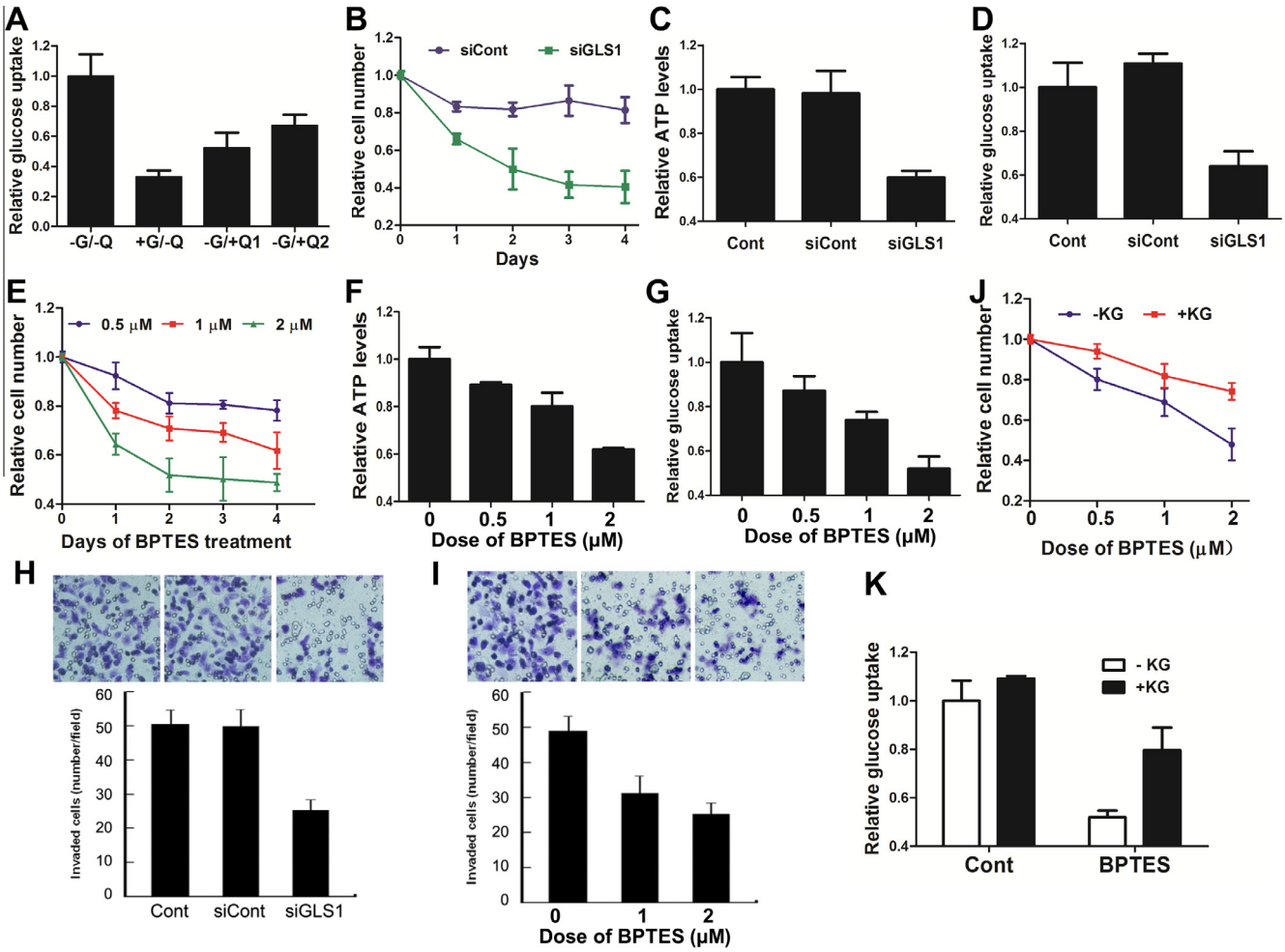

Fig. 3. GLS1 dictates glucose uptake and cell survival via glutamine. (A) PC3 cells were cultured under the indicated growth conditions for 24 h and glucose uptake was determined. (B) Cell growth in control cells (Cont) or cells transfected with siRNA for GLS1 (siGLS1) or control siRNA (siCont). The results shown were growth inhibition normalized to the control group. (C and D) Cells were transfected with or without GLS1 siRNA. 48 h after transfection, cells were harvested for ATP assay (C) or glucose uptake assay (D). (E) PC3 cells were grown in the presence of BPTES for the indicated number of days. The cell growth was normalized to its growth in medium without BPTES treatment. (F and G) The internal ATP levels (F) or glucose uptake (G) were determined in PC3 cells following a 24 h treatment with the indicated dose of BPTES. (H and I) Invasive behavior of untreated cells and cells treated with GLS1 siRNA or BPTES. The invasion indices were further calculated. (J) Cells were cultured with medium with or without 2 mM α-KG and indicated dose of BPTES. Cell numbers were determined after 2 days. (K) The glucose uptake was determined in PC3 cells following a 24 h treatment with or without 2 mM α-KG and 2 μM BPTES. (A–K) The data are presented as the means ± SD from triplicate analyses.

Especially, GLS1 inhibitor CB-839 is undergoing clinical investigation in certain types of cancers [15]. Our study extends the application of drugs targeting GLS1 in cancer therapy, suggesting the promising role of GLS1 in PCA treatment.

Since a variety of nutrients enter the TCA cycle in a variety of manners, the restriction of each nutrient may have different effects on cell growth. In rapidly growing cells, glucose-derived citrate preferentially participates in the synthesis of fatty acids required for cell growth. Thus the glutaminolysis is critical to compensate for the reduced TCA intermediates [2]. Recent study has also stressed the importance of glutaminolysis in maintaining the malignant phenotype [10]. Our experiments show that PC3 cells are more sensitive to the deprivation of glutamine than that of glucose, indicating the phenotype of glutamine addiction, which was well documented and has been a target of interest for therapy [18,19]. Furthermore, our study also demonstrated a significant coordination between glucose and glutamine, and the utilization of glutamine functions upstream of glucose uptake. Therefore, in low glutamine condition cell growth is held in check by limiting the glucose availability.

Due to the association of GLS1 expression with PCA malignancies, we determined the role of GLS1 in cell survival. Interestingly, GLS1 reduction with siRNA or BPTES led to a significant reduction in glucose uptake as well as a decrease of ATP levels, thus inhibiting cell growth and progression. We suggest that GLS1 controls the coordination between glutamine and glucose, and restricts cell survival as a metabolic checkpoint. Glutamine enters the TCA cycle by being converted to glutamate and subsequently to α-KG, indicating BPTES can inhibit glutaminase activity and further lower glutamate and α-KG levels. Expectedly, the restoration of α-KG rescued the rate of glucose uptake and cell growth. This result raises the possibility that glutamine-dependent intermediates in TCA cycle may contribute to the regulation of glucose utilization as well.

To further investigate the underlying mechanism of GLS1 conferring glucose utilization, we analyzed the genes downstream of glutamine-dependent intermediates. Since there is evidence that glutamine dictates glucose uptake by regulating transcriptional activity of MondoA and the expression of TXNIP [13], we therefore determined whether manipulating GLS1 can influence TXNIP expression. TXNIP has pleiotropic roles as a tumor suppressor, such as controlling redox status via binding and inhibition of thioredoxin, enhancing the stability of p27Kip1 and destabilizing hypoxia inducible factor 1α (HIF1α) [20,21]. Especially, TXNIP can negatively regulate glucose uptake and aerobic glycolysis

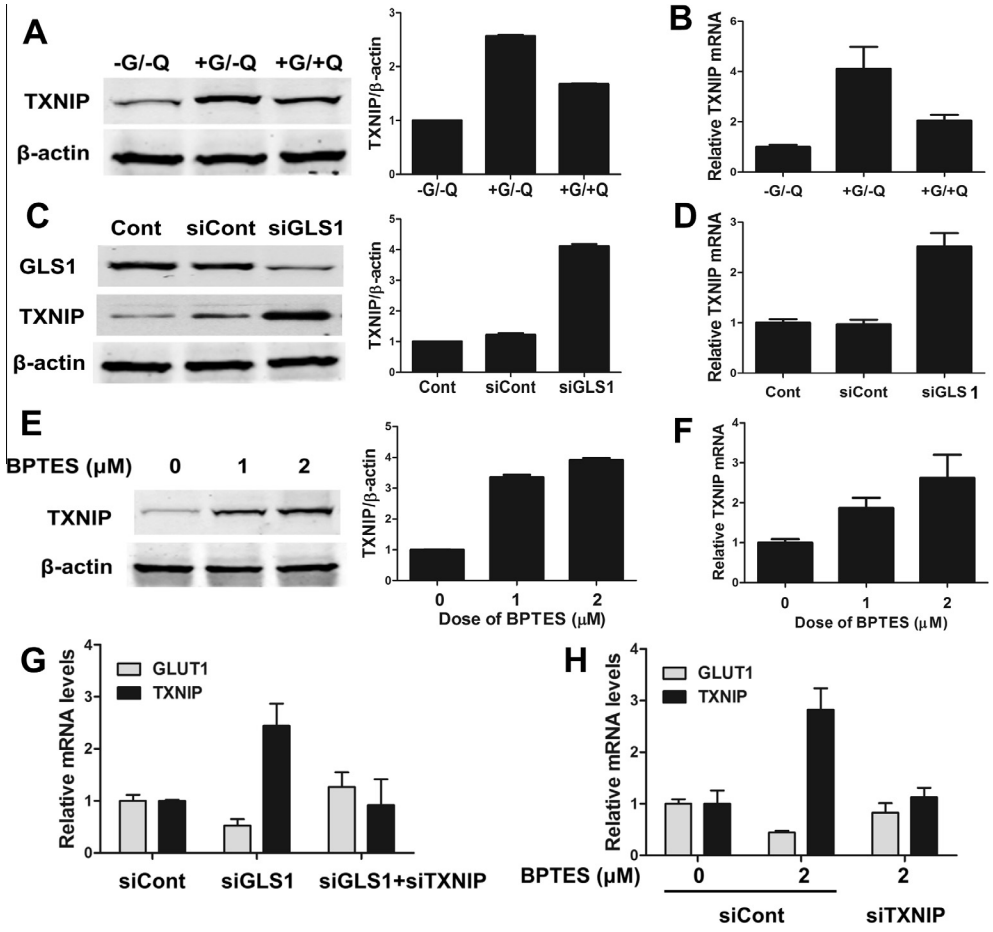

Fig. 4. GLS1 represses TXNIP expression. Western blotting and qRT-PCR were used to determine the relative expression of TXNIP in PC3 cells under the indicated growth conditions for 24 h (A and B), or following a 48 h treatment with transfected GLS1 siRNA (C and D), or following a 24 h treatment of indicated dose of BPTES (E and F). (G and H) qRT-PCR was performed for GLUT1 or TXNIP expressions in cells transfected with indicated siRNA for 48 h or treated with BPTES for additional 24 h. In A, C and E, the blots of TXNIP were quantified normalized to β-actin. In B, D, F, G and H, the data are presented as the means ± SD from triplicate analyses.

[16]. Our experiments showed that TXNIP was up-regulated by glucose and repressed by glutamine, and GLS1 inhibition remarkably induced TXNIP transcriptional expression. Further, there is evidence that energy stress like ATP/ADP ratios results in TXNIP phosphorylation and degradation by AMP-dependent protein kinase (AMPK), which results in an increase in GLUT1 levels [17]. We propose that the drop of ATP content by GLS1 reduction will in turn activates AMPK which reduces TXNIP, and therefore stimulates glucose transport. Expectedly, GLUT1 expression was repressed by GLS1 reduction, and reversed by TXNIP knockdown. It is thus indicating the elevated GLS1 levels support cell growth by stimulating glucose transport and additional glucose uptake by repressing the glucose-induced activation of TXNIP.

Cellular metabolism is incredibly dynamic and appears to compensate for changes in intermediary metabolism. Thus there is a debate that whether glutaminolysis inhibition will be effective as a single arm therapy [22]. Our work provides the evidence that the elevated expression of GLS1 in prostate cancer plays key roles in controlling the coordination between glutamine and glucose. The blockade of glutamine metabolism also restricts glucose uptake. As such, Targeting GLS1 may lead to the inhibition of both glycolysis and glutaminolysis, and that will be especially effective at restricting highly glycolytic cancer cells. These data will extend the role of GLS1 in regulating cell metabolism and the clinical utility of GLS1 inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.105.

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