▐▐▐ RESEARCH ARTICLE

Leukemia Cells Resistant to Glutamine Deprivation Express Glutamine Synthetase Protein

Glutamin Yoksunluğuna Dirençli Lösemi Hücreleri Glutamin Sentetaz Proteini Eksprese Eder

🅿 Burcu Yücel[1,2,3], 🅿 Saniye Ada[2,4]

[1]İstanbul Medeniyet University Faculty of Medicine, Department of Medical Biology, İstanbul, Turkey
[2]İstanbul Medeniyet University, Science and Advanced Technologies Research Center (BILTAM), İstanbul, Turkey
[3]Health Institutes of Turkey (TUSEB), Turkish Biotechnology Institute, İstanbul, Turkey
[4]İstanbul Medeniyet University Faculty of Medicine, Department of Medical Biochemistry, İstanbul, Turkey

▐▐▐ Abstract

Objective: Low glutamine levels have been shown in tumor environments for several cancer subtypes. Therefore, it has been suggested that cancer cells rewire their metabolism to adopt low nutrient levels for survival and proliferation. Although glutamine is a non-essential amino acid and can be synthesized de novo, many cancer cells including malignant hematopoietic cells have been indicated to be addicted to glutamine. This study aimed to investigate the proliferation of leukemia cell lines in glutamine-deprived conditions.

Materials and Methods: Cell proliferation of K562, NB-4, and HL-60 cells was determined by calculating cell numbers in normal vs. low glutamine media. Changes in mRNA expressions were investigated using qRT-PCR. The glutamine synthetase (GS)-encoding *GLUL* gene was knocked out (KO) in HL-60 cells using the CRISPR/Cas9 method and protein expression was evaluated with immunoblotting.

Results: The proliferation of all cell lines was decreased in glutamine-deprived medium. GS protein expression was increased in glutamine-limited medium although the mRNA level did not change. Increased protein expression was confirmed with inhibition of new protein synthesis by treating cells with cycloheximide. To further investigate the role of GS protein, the GS-encoding *GLUL* gene was KO in HL-60 cells using the CRISPR/Cas9 method. GS KO cells proliferated less compared to control cells in glutamine-limited medium.

Conclusion: Our results indicate that upregulated GS protein expression is responsible for glutamine addiction of leukemia cell lines. Exploiting the genetic and metabolic mechanisms responsible for GS protein expression could lead to the identification of new anti-cancer drug targets.

Keywords: Crispr/Cas9, Glutamine limitation, Glutamine synthetase, Leukemia

▐▐▐ Öz

Amaç: Farklı kanser alt tiplerinde tümör çevresinde düşük glutamin seviyeleri gösterilmiştir. Bu nedenle, kanser hücrelerinin hayatta kalmak ve çoğalabilmek için düşük besin seviyelerinde metabolizmalarını yeniden yapılandırdığı öne sürülmüştür. Glutamin esansiyel olmayan bir amino asit olmasına ve de novo sentezlenebilmesine rağmen, malign hematopoietik hücreler de dahil olmak üzere birçok kanser hücresinin glutamine bağımlı olduğu belirtilmiştir. Bu çalışmada lösemi hücre hatlarının glutaminden yoksun koşullarda çoğalmasının araştırılması amaçlandı.

Gereç ve Yöntemler: K562, NB-4 ve HL-60 hücrelerinin hücre proliferasyonu, normal ve düşük glutamin ortamında hücre sayıları hesaplanarak belirlendi. mRNA ifadelerindeki değişiklikler qRT-PCR kullanılarak araştırıldı. Glutamin sentetaz (GS) kodlayan *GLUL* geni, CRISPR/Cas9 yöntemi kullanılarak HL-60 hücrelerinde susturuldu ve protein ekspresyonu immünoblotlama ile değerlendirildi.

Bulgular: Tüm hücre hatlarının proliferasyonu, glutaminden yoksun ortamda azaldı. GS protein ekspresyonu, mRNA seviyesi değişmemesine rağmen, glutamin yoksun ortamda artmış olarak belirlendi. Artan protein ekspresyonu, hücrelere sikloheksimid ile muamele edilerek yeni protein sentezinin inhibisyonu immünoblotlama ile tespit edildi. GS proteininin rolünü daha fazla araştırmak için, GS kodlayan *GLUL* geni, CRISPR/Cas9 yöntemi kullanılarak HL-60 hücrelerde susturuldu. GS susturulmuş hücreler glutamin sınırlı ortamda kontrol hücrelerine kıyasla daha az çoğaldı.

Sonuç: Sonuçlarımız, artmış GS protein ekspresyonunun lösemi hücrelerinde glutamin bağımlılığından sorumlu olduğunu göstermektedir. GS protein ekspresyonunun düzenlenmesinde görev alan genetik ve metabolik mekanizmaların aydınlatılması ile yeni kanser önleyici ilaç hedefleri tanımlanabilir.

Anahtar Sözcükler: Crispr/Cas9, Glutamin kısıtlaması, Glutamin sentetaz, Lösemi

Introduction

Leukemia is a malignant disease of bone marrow, characterized by increased proliferation and abnormal differentiation of blood progenitor cells [1]. Despite advances in therapy, the relapse rate is high, especially in elderly patients, and drug resistance is still a common problem [2]. Today, many genetic and epigenetic mechanisms that alter myeloid cell proliferation and differentiation have been defined [3]. However, deregulated metabolic pathways in abnormal myeloid cells are still under investigation as other cellular processes.

Tumor cells, including malignant hematopoietic cells, demand more nutrients as they rapidly proliferate. Glutamine is the second most consumed nutrient by cancer cells after glucose [4]. Scientists first discovered the importance of glutamine in cancer cell environments for its contribution to the synthesis of ATP and building blocks similar to glucose [5].

Glutamine is a non-essential amino acid and has diverse roles in cells including the biosynthesis of macromolecules, energy production, anti-oxidant defense, regulation of gene expression, and cell signaling pathways [6]. Glutamine is converted to glutamate via the glutaminase enzyme and glutamate produces α-ketoglutarate, an intermediate metabolite in the tricarboxylic acid (TCA) cycle. Recent studies both in vivo and in vitro have suggested that the amount of glutamine within the cancer cell environment is limited [7]. Therefore, it is speculated that cancer cells adopt their metabolism to the environment in order to survive and proliferate [8]. Indeed, various cancer cells have been found resistant to glutamine limitation or deprivation in numerous studies [9].

As most mammalian cells can synthesize glutamine de novo, it is surprising that many cancer cells cannot proliferate in glutamine-deprived conditions [10]. The glutamine synthetase (GS) enzyme catalyzes condensation of glutamate and ammonia in the presence of ATP. GS is encoded by the *GLUL* gene and its expression is shown to be regulated by several growth factors and oncogenic signals such as MYC and KRAS in a few cancer cell types. However, these regulations could be tissue-specific [11,12].

Recent studies indicate that exploitation of genetic resistance mechanisms of glutamine limitation and rewired glutamine metabolism could help identify new therapeutic targets for many cancer subtypes. In this study, we aimed to investigate whether leukemia cell lines K652, NB-4, and HL-60 are addicted to glutamine for proliferation. The reason to choose these cell lines was their different genetic compositions. The HL-60 cell line is null for *p53* tumor suppressor gene expression and positive for *cMYC* and *KRAS* oncogenes, while NB-4 cells have t(15,17) cytogenetic abnormality [13]. On the other hand, K562 cells express the brc/abl chimeric protein [13]. The role of GS

expression in glutamine addiction was also sought. For this purpose, the CRISPR/Cas9 genome editing method was applied to silence the *GLUL* gene and then cell proliferation was assessed in low glutamine medium.

Materials and Methods

Cell Culture

The K562, NB-4, and HL-60 leukemia cell lines were cultured in RPMI 1640 medium (Gibco, Thermo Fisher, USA) supplemented with 10% fetal bovine serum (FBS), 1% pen/strep, and 1% L-glutamine at 37 °C with 5% CO_2.

Glucose and Glutamine Limitation

For glutamine limitation experiments, RPMI 1640 medium without glucose and without glutamine (Cat. No. P04-17550, PAN-Biotech, Germany) was supplemented with 10 mM glucose, 10% FBS, and 1% pen/strep. Therefore, the amount of glutamine within the medium was approximately 50 µM, which is the amount in FBS (Gibco, Thermo Fisher). For the high glutamine counterpart, 2 mM glutamine was added to the same medium. To obtain a low glucose medium, RPMI 1640 medium without glucose and without glutamine (Cat. No. P04-17550, PAN Biotech) was supplemented with 1 mM glucose, 10% FBS, 1% pen/strep, and 2 mM glutamine. Cells were grown in the relevant medium for 3 days. At the end of the experiments, cells were counted on a hemocytometer. Cell proliferation in percentage was calculated in nutrient-limited media compared to normal media.

WST-1 Cell Viability Assay

Cells were plated in a 96-well plate at a concentration of 20,000 cells/mL in the relevant medium.

Plates were incubated at 37 °C with 5% CO_2. On day 3, 10 µL of WST-1 solution (Cayman Chemical, USA) was added into wells and cells were further incubated for 4 h. Absorbance was measured at 452 nm on a plate reader (MultiScan GO, Thermo Fisher). Relative fold change of cell viability was presented as percentage comparing absorbances to normal medium.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RNA was isolated using the NucleoSpin RNA Isolation Kit (Macherey-Nagel, Germany) and concentrations were measured. One microgram of RNA was reverse-transcribed using the High Capacity RNA-to-cDNA Synthesis Kit (Applied Biosystems, USA) according to the manufacturer's protocol. qPCR was performed on the QIAGEN RotorGene Q system using SYBR Green (Applied Biosystems) with the following primers: GLUL-Forward: TGGGAACTGGAATGGTGC, Reverse: CGTTGATGTTGGAGGTTTCATG; GLUD1-Forward: CTGTTGGTGAGTCTGATGGG, Reverse: TCTGGGTGCGTTGGATTTG; GLS-

Forward: GAAAGAGTACTGAGCCCTGAAG, Reverse: GTTGCCCATCTTATCCAGAGG; RPLP0-Forward: AGCATCTACAACCCTGAAGTG, Reverse: AGCAAGTGGGAAGGTGTAATC. The primers used in this study were newly designed with the Genome Browser and Primer 3 website [14].

Immunoblotting

Cell pellets were obtained by centrifugation and rinsed with ice-cold PBS. Pellets were lysed with RIPA buffer supplemented with protease (Complete, Roche, Germany) and phosphatase inhibitors (PhosStop, Roche). After centrifugation at 4 °C and 12,000 rpm for 5 min, the supernatants were collected and protein concentrations were measured with the BCA Protein Assay Kit (Pierce, Thermo Fisher). Bovine serum albumin was used as the protein standard and protein concentrations were adjusted to 1 mg/mL. Samples were run on 12% SDS gel and transferred to a PDVF membrane (Millipore, USA). Immunoblotting was done as previously described and images were captured on the Azure C300 gel imaging system.

Generation of GS Knockout Cell Line

To generate lentiviral knockout (KO) constructs, the annealed oligonucleotides given in Table 1 were cloned into the plentiCRISPR-Cas9 v2 vector using the T4 ligase (NEB, USA). Transfection-grade plasmids were used for viral packaging with Hek293T cells. Viral supernatant at a multiplicity of infection of 1 was used to transfect HL-60 cells with the spinfection method in the presence of 8 μg/mL polybrene. Transfected cells were selected with puromycin for 3 days. After selection, cells were cultured to obtain enough cells to plate in a 96-well plate for single cell cloning. Each well containing a single cell was marked and followed to obtain colonies. Each colony then grew further and GS protein expression was controlled with immunoblotting.

Inhibition of Protein Synthesis

New protein expression of GS under glutamine-limited conditions was evaluated using cycloheximide. Cells were treated with 0, 10, and 20 μM cycloheximide both in normal and low glutamine media for 48 h and then cell pellets were collected for immunoblotting.

Table 1. sgRNA list.	
GLUL gRNA1F	caccGATTGCGGGGACTAATGCCG
GLUL gRNA1R	aaacCGGCATTAGTCCCCGCAATC
GLUL gRNA2F	caccGAGCCATCGAAATTCCACTC
GLUL gRNA2R	aaacGAGTGGAATTTCGATGGCTC
Control sgRNA_2F	caccgATTATACCTCTTCACAGCTG
Control sgRNA_2R	aaacCAGCTGTGAAGAGGTATAATc

Statistical Analysis

Graphs were drawn and statistical analysis was performed using GraphPad V7. Student's t-test with Welch's correction was applied to compare two groups. All experiments were performed at least two independent times with similar results. A viability assay was performed with quintuple technical replicates.

Results

Cell Proliferation Decreased in Low Glutamine Medium

K562, NB-4, and HL-60 leukemia cell lines were cultured in 50 μM glutamine for 3 days. Compared to the control group, the cell number was decreased in 50 μM glutamine for all cell lines (Figures 1A and 1B), and these changes were statistically significant for all cell lines. Although NB-4 and HL-60 cells were found to be less proliferated in low glutamine media, a total decrease was observed in all cell lines with different genetic backgrounds.

GLS, GLUL, and GLUD1 mRNA Expressions in Low Glutamine Media

Glutaminase is encoded by the GLS gene and the cDNA has three isoforms. The used primer pair was designed to target common sequences of these three isoforms. Glutamate dehydrogenase catalyzes the reaction of glutamate conversion to α-ketoglutarate and has two isoforms: mitochondrial and cytoplasmic. The mitochondrial enzyme is encoded by the GLUD1 gene (Figure 2A). mRNA expressions of GLS and GLUD1 for glutamine catabolism and GLUL for de novo glutamine synthesis were analyzed using qRT-PCR (Figures 2B, 2C, and
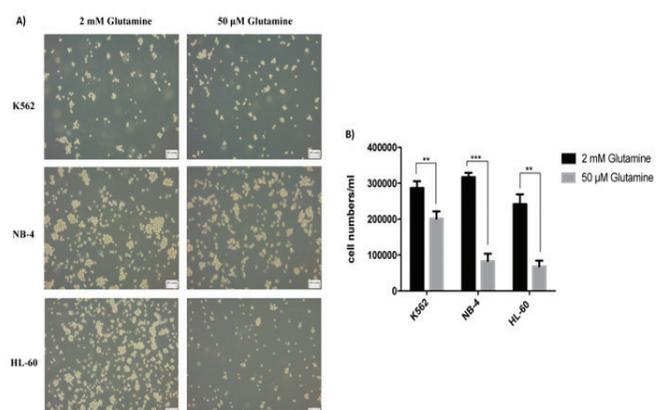

Figure 1. Cell proliferation of leukemia cells in normal (2 mM) and low glutamine (50 μM) media. **A)** Investigation of cell confluence under light microscopy at 10^x magnification. **B)** Cell proliferation calculated in cell numbers. Cells were plated at a concentration of 20,000 cells/mL and cultured for 72 h. Afterward, cells were washed and counted on a hemacytometer. NB-4 and HL-60 cell numbers were dramatically decreased in low glutamine medium. Data are presented as mean ± SD. **p<0.01, ***p<0.001.

2D). No statistically significant changes in mRNA expressions were found in low glutamine media for the three cell lines. *GLUL* mRNA expression was slightly decreased in low glutamine compared to normal media for K562 and HL-60 cells; however, these changes were not statistically significant.
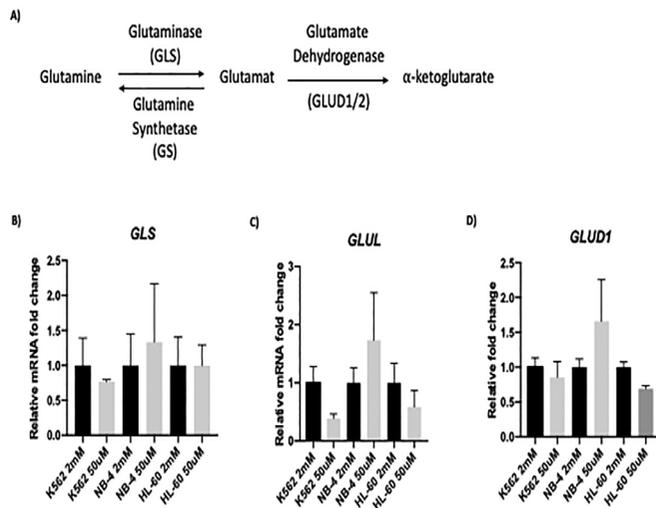

Figure 2. Glutaminase (*GLS*), glutamine synthetase (*GLUL*), and glutamine dehydrogenase 1 (*GLUD1*) mRNA expressions. **A)** Glutamine catabolizes to α-ketoglutarate via GLS and GLUD1/2 enzymes. **B)** *GLS* relative mRNA expression, **C)** GS-encoding relative *GLUL* mRNA expression, and **D)** *GLUD1* relative mRNA expression levels in 2 mM and 50 μM glutamine-containing medium. Data are presented as mean ± SD. No significant change was found in mRNA expression for the three genes.
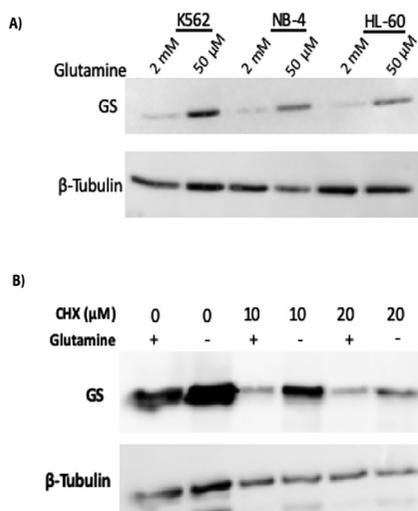

Figure 3. Immunoblotting of GS protein in leukemia cells. **A)** GS protein expression was elevated for all three cell lines in low glutamine medium. **B)** The HL-60 cell line was treated with 10 and 20 μM cycloheximide (CHX) in low glutamine medium to examine upregulated GS protein synthesis. GS protein synthesis was gradually decreased at increased CHX doses in low glutamine.

Increased GS Protein Expression in Low Glutamine Condition

Regulation of GS protein expression in the low glutamine condition was assessed by immunoblotting. It was found that GS expression was upregulated in all cell lines in low glutamine medium. To further evaluate increased protein synthesis, HL-60 cells were treated with 10 and 20 μM protein synthesis inhibitor CHX for 48 h in low glutamine media. We found that new GS protein synthesis was decreased upon increased CHX concentration. The GS protein level was almost the same at a concentration of 20 μM CHX. This indicates that the leukemia cell lines used in this study upregulate GS protein expression under low glutamine conditions.

GS Expression Is Important for Response to Low Glutamine

To further investigate whether upregulation of GS expression is important for cell proliferation, we knocked out the *GLUL* gene in HL-60 cells using the CRISPR/Cas9 genome editing method. Two GS KO isogenic HL-60 cell lines were obtained with the use of two different sgRNAs (Figure 4A). Using these cells, cell proliferation was assessed in low glutamine media compared to normal media. We detected that GS KO cells proliferated significantly less in low glutamine conditions in comparison to control cells (Figure 4B). Cell proliferation of KO cells was below 25%, whereas it was almost 30% for control cells. We did not observe any change in cell proliferation of GS KO cells in normal medium (data not shown). Whether GS expression also impacts cell proliferation in conditions of low glucose and low glucose with low glutamine was also determined. GS KO and GS-expressing cells proliferated at similar rates in low glucose medium (Figure 4C). However, in low glucose/low glutamine medium, the GS KO cell proliferation rate was lower compared to control cells.

Discussion

In addition to ATP synthesis, glutamine plays important roles in many pathways for proliferation of cancer cells [15]. Decreased glutamine levels in tumor environments have been shown for different tumor types [16]. Therefore, cancer cells rewire their metabolisms to adapt to low glutamine levels for survival and proliferation [17]. Many tumor cells including hematopoietic cancer cells have been identified as addicted to glutamine for proliferation and survival [18,19,20]. In this study, we found that K562, HL-60, and NB-4 leukemia cell lines are dependent on glutamine for proliferation. Cell counts were found to be lower in low glutamine media for all three cell lines (Figure 1). Glutamine addiction has been shown to be independent of the amount of glucose in the tumor microenvironment [11]. It was shown that the amount of intermediate TCA cycle metabolites altered in the P483B Burkitt lymphoma cell line in glutamine-deprived conditions was not connected to the amount of glucose

[11]. Similar results were obtained with the HL-60 AML cell line and cell proliferation was restored when oxaloacetic acid was added to glutamine-deprived medium [21]. Furthermore, glutathione levels were found to decrease while reactive oxygen species levels increased in glutamine-deprived conditions [21], suggesting a possible suppression of oxidative phosphorylation. In this study, we also showed that proliferation of leukemia cells in glutamine-deprived conditions is independent of glucose level (Figure 4C). In addition to glutamine limitation, the use of glutaminase inhibitors to inhibit cellular use of glutamine (glutaminolysis) has demonstrated similar results and increased apoptosis in AML and ALL cell lines [22]. Targeting glutaminolysis has also been indicated to impart anti-leukemic properties by altering proliferation and differentiation of blast cells in different AML cell lines [23]. These results suggest that glutamine is important not only for ATP synthesis but also for intermediate metabolites as building blocks in cells. In this study, proliferation of K562, NB-4, and HL-60 leukemia cell lines was decreased in low glutamine medium. However, the proliferation rate of the K562 cell line was higher in comparison to NB-4 and HL-60 cells (almost 50%).

The GS enzyme is suggested to play a role in glutamine addiction [24]. GS, encoded by the *GLUL* gene, and increased *GLUL* expression have been associated with poor prognosis in glioblastoma [25] and hepatocellular carcinoma [26]. It has been reported that basal-type breast cancer cells have low levels of *GLUL* expression and they are more addicted to glutamine as compared to luminal-type breast cancer cells [27]. Moreover, *GLUL* expression has been shown to be regulated by cMYC in basal-type cells, suggesting that glutamine addiction could be tissue-specific [28].

In addition to cMYC, different oncogenic signals have been associated with the glutamine addiction of many cancer cells [29]. Post-translational modification of GS is done by mTORC1 in pancreatic cancer cells. It has been determined that mTORC1 stabilizes and inhibits proteasomal degradation of the GS protein [30]. On the contrary, in liver cells, *GLUL* inhibition by methionine sulfoximine did not alter the mTOR signaling pathway [31]. In another study, L-asparaginase was found to increase GS expression by inhibiting mTORC1 in some AML cell lines and GS knockdown enhanced drug-induced apoptosis in these cells, suggesting that GS expression could also be important for drug response in AML [32]. In this study, we found that GS protein expression is increased in the K562, HL-60, and NB-4 leukemia cell lines in low glutamine media (Figure 3A), although mRNA levels were not changed significantly (Figure 2). These data suggest that GS protein synthesis could be upregulated in low glutamine conditions. To test this hypothesis, we treated cells with CHX, a protein synthesis inhibitor drug, and observed that the GS protein level was not elevated in the low glutamine medium upon treatment (Figure 3B). Furthermore, the GS-encoding *GLUL* gene was knocked out using the CRISPR/Cas9 genome editing method in this study. Two KO lines and one control GS isogenic line were cultured in low glutamine medium, and we found that GS KO cells proliferated less compared to control cells. Similarly, control cells proliferated more in low glucose/low glutamine medium compared to GS KO cells. On the other hand, knocking out GS did not affect cell proliferation in low glucose/normal glutamine medium (Figure 4C).
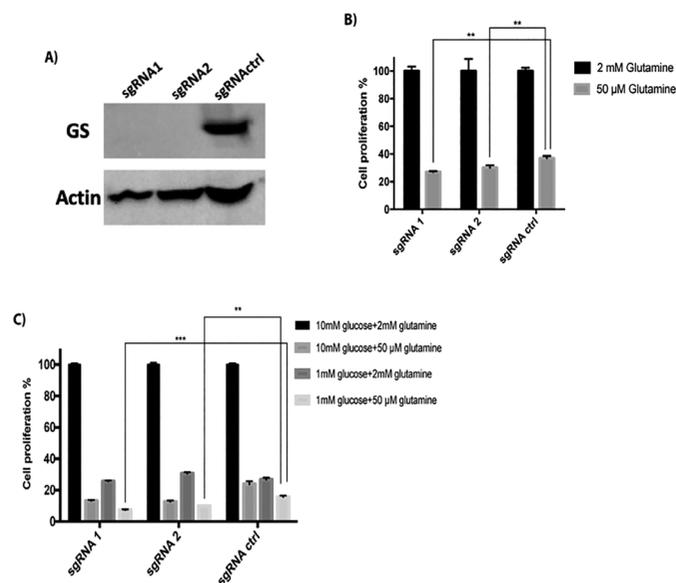
Conclusion

Our data suggest that increased GS protein expression is important for glutamine addiction in leukemia cells. To our knowledge, this is the first study showing elevated GS levels in leukemia cells in glutamine-deprived conditions. Although K562, NB-4, and HL-60 cells have different genetic abnormalities as mentioned in the introduction, GS expression was elevated in all cell lines in low glutamine conditions. This indicates that other genetic mechanisms might regulate GS protein expression rather than known genetic abnormalities such as cMYC and KRAS [33]. Further studies utilizing mechanisms involved in the regulation of GS protein expression in AML cells will contribute to the development of new therapeutics.

Figure 4. Proliferation of *GLUL* knockout (KO) cell lines in low glutamine and low glucose medium. **A)** Generating *GLUL* KO cell lines. Immunoblotting of GS shows two KO cell lines generated with two different sgRNAs. **B)** GS KO cells proliferated less in low glutamine medium compared to control cells. **C)** Downregulated GS protein expression did not change cell proliferation in low glucose/normal glutamine medium. However, in low glucose/low glutamine medium, cell proliferation was lower compared to control cells, suggesting that GS downregulation sensitizes leukemia cells only to glutamine restriction. Data are presented as mean ± SD. **p<0.01, ***p<0.001.

Authorship Contributions

Concept: B.Y.; Design: B.Y.; Data Collection or Processing: S.A.; Analysis or Interpretation: B.Y., S.A.; Literature Search: B.Y.; Writing: B.Y., S.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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