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ORIGINAL ARTICLE – OPEN ACCESS

Downregulation of glutaminase 1 (GLS1) Inhibits Proliferation, Clonogenicity, and Migration of Aggressive MDA-MB-231 Breast Cancer Cells by Increasing p21 and Decreasing Integrin-β1 Expression

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ABSTRACT

Objective: Glutamine metabolism is an important pathway in cell proliferation and tumor progression. The first enzyme to be converted in the process of glutamine metabolism, glutaminase 1 (GLS1), exhibits increased expression in many types of cancer, including breast cancer. Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype with high glutamine metabolic activity. The aim of this research was to examine the effects on glutamine metabolism and carcinogenic properties following small interfering RNA (siRNA)-mediated inhibition of GLS1 in glutamine-dependent TNBC.

Materials and Methods: The effects on cell proliferation, migration, apoptosis, colony formation, and the cell cycle of MDA-MB-231 cells using different siRNAs targeting GLS1 were analyzed using an MTS assay, a wound-healing assay, clonogenic analysis, and annexin V and propidium iodide staining methods. The protein expression of GLS1, integrin beta 1 (β1), caspase-3, and p21 were examined using western blot analysis and flow cytometry.

Results: The findings revealed that cell viability, migration, and colony formation were significantly suppressed in MDA-MB-231 cells transfected with 2 different GLS1 siRNAs. Furthermore, the results of flow cytometry and western blot analysis demonstrated that knockdown of GLS1 induced arrest in the G0/G1 phase of the cell cycle through the p21 signaling pathway, but did not induce apoptosis.

Conclusion: GLS1 is needed for cell proliferation and promotes tumor progression and growth of MDA-MB 231 cells. siRNAs may provide a means to downregulate GLS1 and offer a promising target for breast cancer therapy.

Keywords: Apoptosis, cell cycle, GLS1, glutamine metabolism, triple negative breast cancer.

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INTRODUCTION

Triple-negative breast cancer (TNBC) is a hormone receptor-negative metastatic breast cancer that accounts for 15% to 20% of all breast cancer cases (1). TNBC is very aggressive in comparison with other breast cancer subtypes, given the tendency for distant tissue metastasis, development of metastases early in the disease, and the overall length of survival (1, 2). Since patients with TNBC are estrogen receptor-negative, progesterone receptor-negative, and human epidermal growth factor receptor 2 (HER2)-negative, HER2-targeted or endocrine therapy is not helpful. Chemotherapy is currently the only treatment approach for this patient group. New, specific treatment approaches are needed for the treatment of metastatic TNBC.

Cancer cells need biomolecules and energy to support unlimited growth capacity. Impaired energy metabolism is a primary feature of cancer (3). The cells must adjust their metabolism to secure nutrients. The reprogramming of glutamine metabolism has been referred to as glutamine addiction. Glutaminase is a phosphate-dependent mitochondrial enzyme involved in the first step of glutamine metabolism (4). This enzyme converts glutamine to glutamate and has 2 isoforms: glutaminase 1 (GLS1) and glutaminase 2 (GLS2) (5). GLS1 contributes to cancer progression via increased expression in various cancers, such as hepatocellular carcinoma, prostate, breast, and colorectal cancer (6–9). Inhibition of GLS1 or glutamine metabolism has been shown to reduce cell growth (proliferation) by inducing apoptosis in some cancer cells and animal models of cancer (10–12). In cancer cells exhibiting glutamine dependence, inhibition of the GLS1 enzyme may provide an effective mechanism for the treatment of these cancers.

Breast cancer is heterogeneous and the expression of proteins associated with glutamine metabolism can differ. Glutamine requirements vary across different breast cancer molecular subtypes. Some require only an exogenous source of glutamine and demonstrate glutamine addiction. TNBC and HER2+ breast cancer subtypes exhibit more glutamine addiction than other subtypes (13, 14). Glutamine metabolism appears to contribute to progression in breast cancer subtypes. Therefore, it may be that inhibition of glutamine metabolism via GLS1 could be a treatment target to stop tumor progression.

This study examined the role of GLS1, an important enzyme in glutamine metabolism, and its potential utility as a target to reduce MDA-MB-231 cell proliferation.

MATERIALS and METHODS

Study Design

The MDA-MB-231 cells used in this research were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C in 5% CO_2. The cells were passaged after achieving 80% proliferation in a medium of Dulbecco's Modified Eagle's Medium (DMEM) F12 and 1% penicillin/streptomycin.

Transfection with siRNA

Two forms of small interfering RNA (siRNA) targeting the GLS1 gene and a control siRNA that does not suppress any gene (GLS1#1: SASI-HS01-71581, GLS1#2: SASI-HS01-71573, Control: SIC001; MilliporeSigma, Burlington, MA) were purchased. The cells were transfected with the siRNAs with the HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The siRNA concentration to be transfected was based on previous studies (15, 16).

Western Blot Analysis

The cells were seeded at a density of $3.5x10^5$ in 25 cm^2 flasks in 4 mL of medium. After 24 hours of incubation,they were transfected with 50 nM of the siRNAs. At the conclusion of a 72-hour transfection period, the cells were harvested and protein isolation was performed using the ReadyPrep protein extraction kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. A DC Protein Assay kit (BioRad, Hercules, CA) was used to determine the protein concentration and was measured using an enzyme linked immunosorbent assay reader at 750 nm. Western blot analysis was performed as described in previous studies (15, 16). Protein expression was examined using GLS1 (Proteintech Group, Rosemont, IL, USA), integrin β1 (Proteintech Group, Rosemont, IL, USA), caspase-3 (Proteintech Group, Rosemont, IL, USA), and p21 (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. Anti-mouse β-actin primary and secondary antibodies (Proteintech Group, Rosemont, IL, USA) were used as a loading control.

Cell Viability

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] analysis was conducted to define the viability and proliferation of cells after transfection according to a previously described method (16).

Colony Formation Assay

Colony formation analysis of the transfected cells was performed as previously described (16).

Wound-Healing Assay

A wound-healing (scratch) assay was used as described in previous studies to determine whether the migration of cells was affected after transfection with siRNAs (15, 16).

Analysis of Apoptosis

Once 300,000 cells had been seeded into 25 cm^2 flasks, a Muse Annexin V kit (MilliporeSigma, Burlington, MA) was used according to the manufacturer's instructions to detect apoptosis in the cells after transfection with siRNAs.

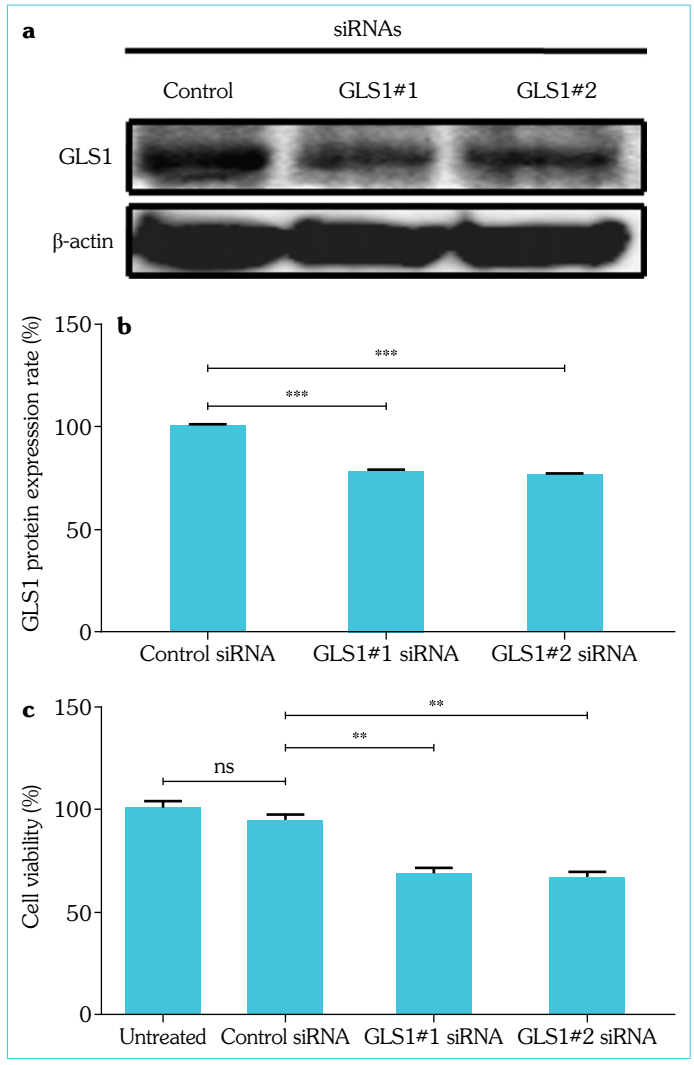

Figure 1. Glutaminase (GLS1) knockdown with targeted small interfering RNAs (siRNAs) inhibited cell viability and colony formation in MDA-MB 231 cells. (a, b) Western blot analysis results demonstrate significant reduction in GLS1 protein expression level with GLS1#1 and GLS1#2 siRNAs; (c) MTS assay results show evaluation of proliferation/cell viability. Data were presented as mean±SD. **p<0.01; ***p<0.001

Analysis of Cell Cycle

The cell cycle was assessed using a Muse Cell Cycle Analysis kit (MilliporeSigma, Burlington, MA). After seeding $3.5x10^5$ cells in culture flasks, they were transfected with 50 nM of the siRNAs for 72 hours and the cells were analyzed using a Muse Cell Analyzer (MilliporeSigma, Burlington, MA) in accordance with the manufacturer's protocol.

Statistical Analysis

Each laboratory analysis was repeated at least 3 times to ensure statistical validity. Prism 6 software (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analyses. The results were shown as mean±SD and a 2-sample unpaired t-test was used to compare the mean of independent groups. A p value of <0.05 was considered statistically significant.

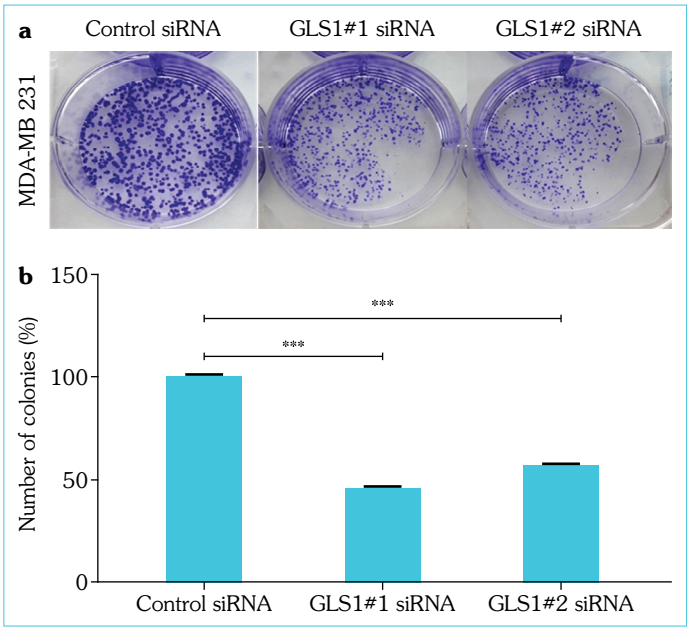

Figure 2. Effects of glutaminase (GLS1) knockdown with targeted small interfering RNAs (siRNAs) on colony formation. (a) Crystal violet staining evaluation of colony formation; (b). Colony density measurement. The data were presented as mean±SD, ***p<0.001

RESULTS

Inhibition of GLS1 Protein Decreased Proliferation and Colony Formation in MDA-MB 231 Cells

MDA-MB-231 cells were transfected with 2 different GLS1 siRNAs and a control siRNA, and 72 hours later, the isolated total protein was evaluated using western blot analysis. The expression of GLS1 protein in cells transfected with GLS1 siRNAs was suppressed compared with that of cells transfected with the control siRNA ($p<0.001$; Fig. 1a, b). The western blot results indicated that the GLS1 siRNAs provided an effective knockdown of GLS1 expression at the protein level and could be used to assess GLS1-mediated effects.

It was also observed that the viability of the MDA-MB 231 cells transfected with GLS1#1 and GLS1#2 siRNA was significantly reduced in comparison with cells transfected with the control siRNA ($p<0.01$; Fig. 1c). In addition, colony formation decreased with the application of both of the siRNAs in comparison with the control ($p<0.001$; Fig. 2a, b). These results suggest that GLS1 plays a significant role in the growth of MDA-MB 231 cells.

Knockdown of GLS1 Reduced Migration in MDA-MB 231 Cells

A wound-healing test was performed to assess whether siRNA-mediated inhibition of GLS1 affected cell migration of the MDA-MB 231 cells. The scratch wound area in the cells transfected with the control siRNA was completely closed at 48 hours, while the migration in cells transfected with the GLS1 siRNAs was limited, indicating that inhibition of GLS1 expression reduced migration in these cells (Fig. 3a, b). Integrin β1 expression was also assessed using western blot analysis. Integrin β1 is a transmembrane glycoprotein that mediates cell-extracellular matrix interaction and has

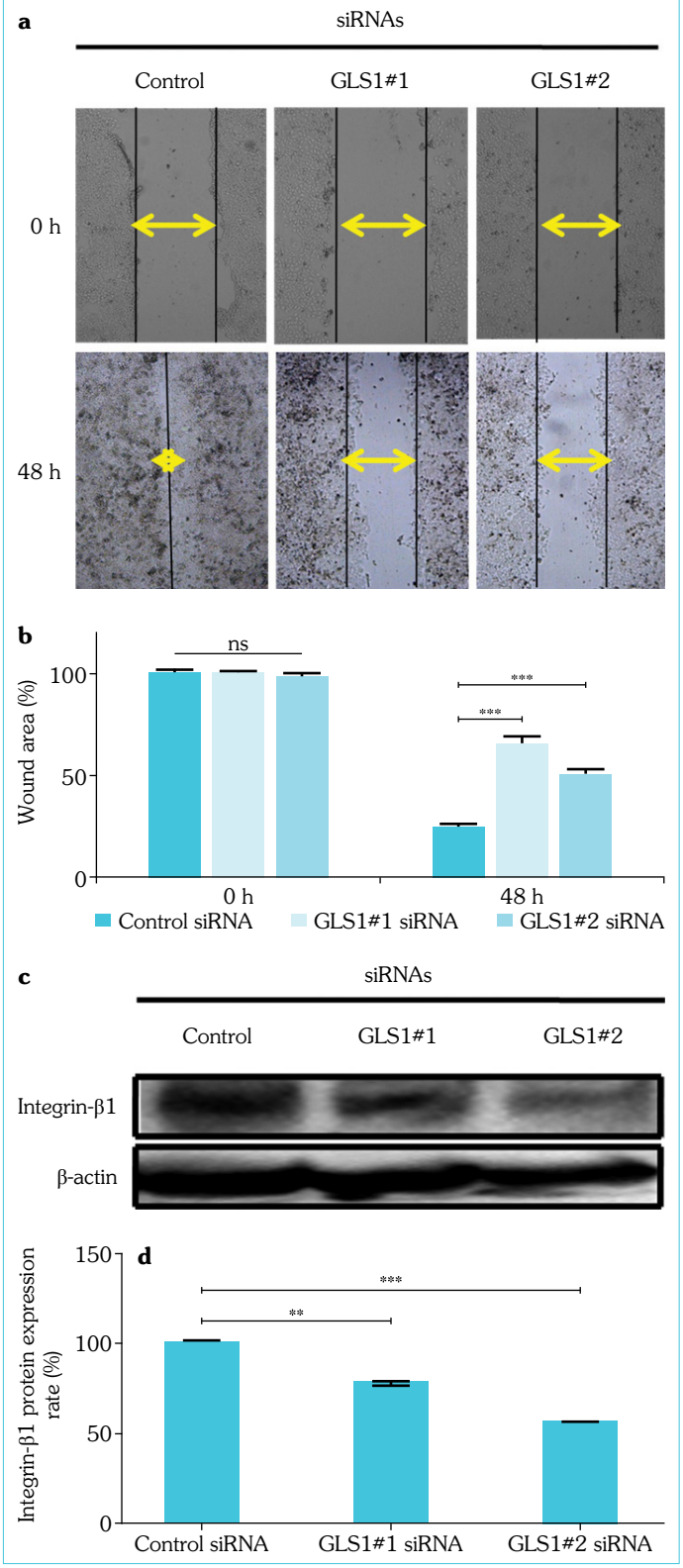

Figure 3. Effects of glutaminase (GLS1) knockdown with targeted small interfering RNAs (siRNAs) on cell migration. (a). Cells that migrated to the scratch test wound area; (b) bar graph of migration percentages from 3 independent experiments. Data are expressed as mean±SD; (c,d). Integrin β1 levels after inhibition of GLS1 with siRNAs. Data were presented as mean±SD. **p<0.01; ***p<0.001

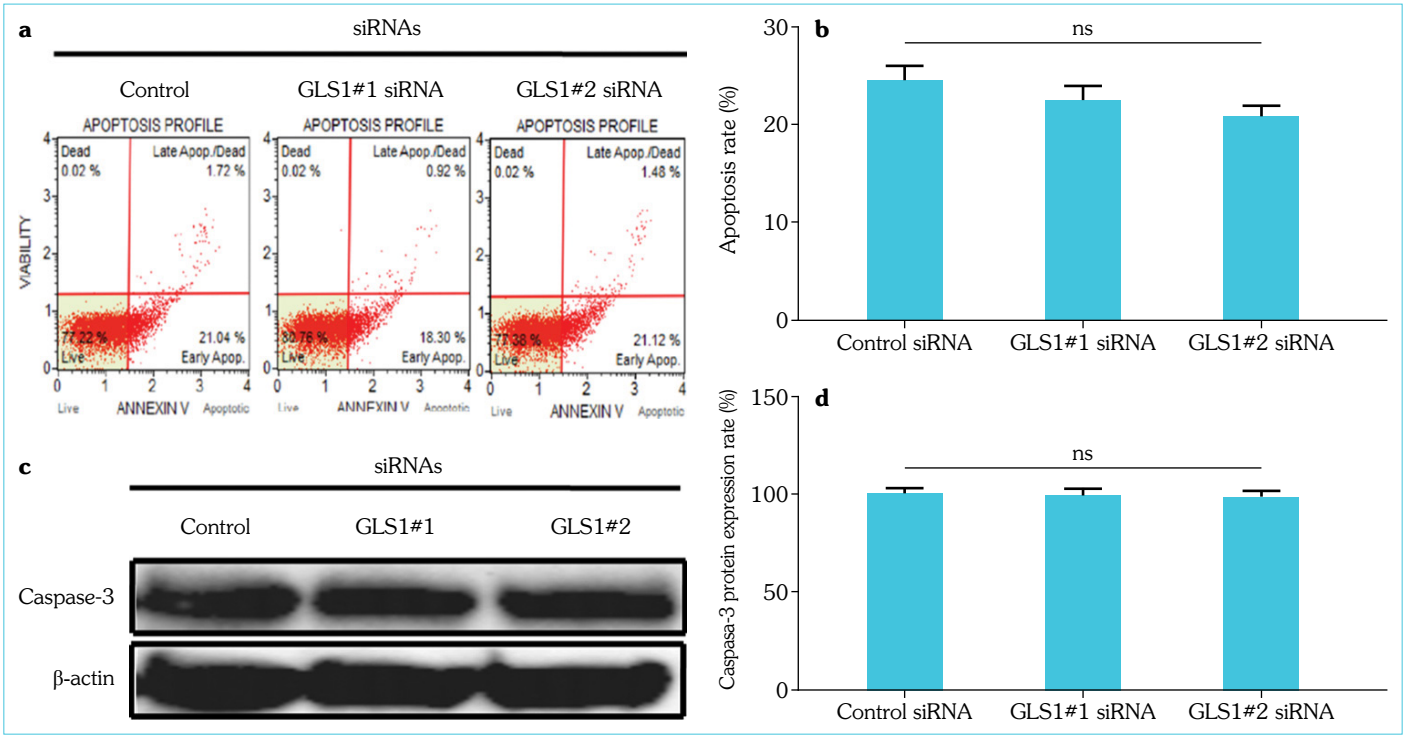

Figure 4. Effects of Glutaminase (GLS1) knockdown with targeted small interfering RNAs (siRNAs) on apoptosis. (a) Annexin V/PI staining assay results; (b) Percentage of apoptotic cells. Data were presented as the mean±SD; (c,d) Inhibition of GLS1 with 2 different siRNAs did not change the total caspase-3 protein expression. Data were presented as mean±SD. NS: Not significant

a role in cell proliferation, apoptosis, invasion, and migration in cancer (17). The findings of this study indicated that the inhibition of GLS1 with siRNAs greatly decreased the expression of integrin β1 (GLS1#1: $p<0.01$, GLS1#2: $p<0.001$; Fig. 3c, d).

Inhibition of GLS1 Did Not Induce Apoptotic Death in MDA-MB 231 Cells

An annexin V/PI staining assay used to examine whether GLS1 had any effect on the survival of MDA-MB231 cells transfected with GLS1-specific siRNAs indicated that downregulation of GLS1 did not induce apoptosis in the MDA-MB-231 cells ($p>0.05$, Fig. 4a, b). A western blot analysis revealing protein expression of caspase-3 confirmed the results. In the process of apoptosis, initiator caspases (e.g., caspase-8, -9) are activated and stimulate proteolysis of effector caspases (e.g., caspase-3), which cause apoptosis by cleaving to multiple cellular substrates (18). Knockdown of GLS1 by GLS1 siRNAs did not appear to alter the total caspase-3 protein level in the cells ($p>0.05$; Fig. 4c, d). The results of the apoptosis assay showed that suppression of GLS1 did not trigger apoptotic cell death in MDA-MB-231 cells.

Knockdown of GLS1 Resulted in Arrest of MDA-MB231 Cell Cycle in the G0/G1 Phases

A significant proportion of MDA-MB 231 cells transfected with GLS1#1 and GLS1#2 siRNAs demonstrated arrested development in the G0/G1 phase of cell cycle (GLS1#1: $p<0.05$, GLS1#2: $p<0.01$; Fig. 5a, b). More cells transfected with the control siRNA reached the S phase in comparison with those transfected with GLS1-targeted siRNAs ($p<0.01$; Fig. 5a, b). There was no statistically significant difference in the number that achieved the G2/M phase ($p>0.05$; Fig. 5a, b). The results suggest that the decrease in the proliferation of MDA-MB 231 cells transfected with GLS1#1 and GLS1#2 siRNAs was related to a halt of cell division at the G0/G1 phase due to a reduction in energy resulting from GLS1 inhibition. p21 is a cyclin-dependent kinase (CDK) inhibitor protein that mediates cell cycle progression through cyclin D-CDK4/6 and cyclin E-CDK2 complexes, which may arrest cells at the G1/S and G2/M transitions of the cell cycle (19). Western blot analysis used to assess the p21 protein expression indicated that inhibition of GLS1 with the siRNAs increased the expression of p21 protein (Fig. 5c, d; GLS1#1: $p<0.01$, GLS1#2: $p<0.001$).

DISCUSSION

Cancer cells disrupt mechanisms controlling cell proliferation and adjust energy metabolism in order to promote their growth and survival (3). One of the changes to the central metabolism of most cancer cells is the disruption of glutamine metabolism. Some cancer cells, such as TNBC cells, which lack progesterone and estrogen receptors and have a low/no HER2 expression, need glutamine to proliferate (13). GLS1 is expressed in all mammalian tissues except liver tissue and is associated with invasive activities and tumor growth in many types of cancer (20).

Inhibition of glutaminolysis, or GLS1 activity, has been shown to reduce tumor growth in cancer cells and in animal models of cancer, as well as induce apoptosis in some studies (7, 11, 12, 21). Glutamine restriction suppresses cellular growth, stops the

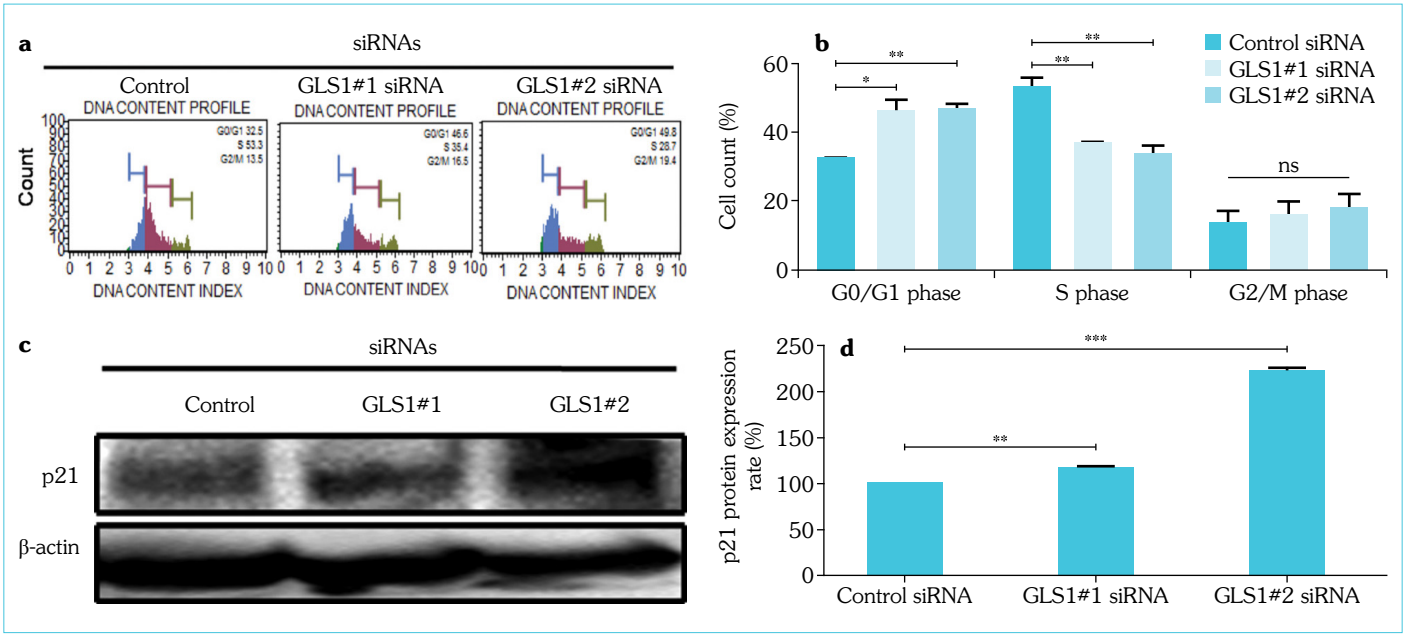

Figure 5. Effects of glutaminase (GLS1) knockdown with targeted small interfering RNAs (siRNAs) on cell cycle stage. (a,b) The percentage of cells in cell cycle phases. The data were presented as the mean±SEM from 3 experiments; (c,d) Increased p21 protein expression. Data were presented as mean±SD. *p<0.05; **p<0.01; ***p<0.001. NS: Non-significant

cell cycle at the G1 phase, and causes disruptions in important pathways, such as the mammalian target of rapamycin pathway, leading to an increase in the production of reactive oxygen species (22, 23). However, it is not yet precisely known how GLS1 inhibition affects the carcinogenic properties of cancer cells. Additional studies are needed to discover the full potential therapeutic role of GLS1 in cancer treatment.

In this study, we determined that the inhibition of GLS1 with GLS1 targeted siRNAs reduced the proliferation, clonogenicity, and migration in triple-negative MDA-MB 231 cells. The results appear to confirm that GLS1 contributes to proliferation, migration, and colony formation in MDA-MB 231 cells.

It has been reported that glutamine metabolism, or GLS1 activity, reduced cancer cell proliferation by inducing an apoptosis mechanism in some cancers, such as head and neck cancer (12), colorectal cancer (7), ovarian cancer (24), acute myeloid leukemia (AML) (10), hepatocellular carcinoma (11), and xenograft tumor models generated by injecting AML cells (21). Reports have also demonstrated suppression using small molecule inhibitors or RNA interference (20, 22). Gross et al. (6) examined the glutaminase inhibitor CB-839 in TNBC cell lines and found that this inhibitor, which has multiple cytotoxic effects on enzymes and transporters using glutamine, induced apoptosis of MDA-MB-231 cells (6). In contrast, Lampa et al. (25) demonstrated that inhibition of GLS1 with short hairpin RNA did not induce apoptosis in breast cancer MDA-MB-231 cells (25). Similarly, our study indicated that downregulation of GLS1 via GLS1-siRNAs inhibited the proliferation of MDA-MB 231 cells, but did not induce apoptosis, according to annexin V apoptosis analysis and apoptotic protein caspase-3 expression findings.

Glutamine limitation has been reported to suppress cellular growth and stop cell growth in the G0/G1 phase of the cell cycle in studies of non-small-cell lung cancer and ovarian cancer (22, 26). Our findings that the cell cycle of MDA-MB 231 cells was arrested in the G0/G1 phase with inhibition of GLS1 was consistent with this earlier research. In addition, we observed that the expression level of p21 protein, an important modulator of the cell cycle, increased in MDA-MB 231 cells with GLS1 inhibition. The results of this study indicated that the suppression of glutamine metabolism blocked the energy source of the cell and led to a halt at the G0/G1 phase.

In studies of lung, pancreatic, ovarian, and colorectal cancers, GLS1 has been shown to contribute to colony formation (26–29), migration, and invasion (24, 27–30). Our findings also showed that GLS1 was involved in proliferation, colony formation, and migration of MDA-MB 231 cells. Furthermore, we demonstrated that downregulation of GLS1 decreased integrin-β1 expression in MDA-MB-231 cells. Integrin-β1 appears to be an important mediator in breast cancer progression and initiation (17). Our findings suggest that GLS1 contributes to the expression of mediators promoting cell proliferation and migration in MDA-MB-231 cells.

CONCLUSION

Evaluation of other studies of GLS1 along with the results of our study indicate that GLS1 appears to have a critical function in cancer cell proliferation, cellular survival, migration, and tumorigenesis. GLS1 plays an important part in glutamine-addicted cancers due to its role in cellular bioenergetic pathways and the first step of glutamine energy metabolism, which plays a role in biomolecule synthesis. Downregulation of GLS1 to inhibit aggressive progression may be a molecular target for therapy of breast cancer cells such as MDA-MB 231 cells with high glutamine metabolism.

Ethics Committee Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – SA, HDA, ZH; Design – SA, HDA, ZH; Supervision – SA, HDA, ZH; Resource – SA, HDA, ZH; Materials – SA, HDA, ZH; Data Collection and/or Processing – SA, HDA, ZH; Analysis and/or Interpretation – SA, HDA, ZH; Literature Search – SA, HDA, ZH; Writing – SA, HDA, ZH; Critical Reviews – SA, HDA, ZH.

Conflict of Interest: The authors have no conflict of interest to declare.

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