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Glutaminase 1 plays critical roles in myelodysplastic syndrome and acute myeloid leukemia cells

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Abstract.
BACKGROUND: Myelodysplastic syndrome (MDS) features bone marrow failure and a heightened risk of evolving into acute myeloid leukemia (AML), increasing with age and reducing overall survival. Given the unfavorable outcomes of MDS, alternative treatments are necessary. Glutamine, the most abundant amino acid in the blood, is metabolized first by the enzyme glutaminase (GLS).
OBJECTIVES: To investigate whether GLS is involved in the progression of MDS. The efficacy of GLS inhibitors (CB839 or IPN60090) and BCL2 inhibitor venetoclax was also examined.
METHODS: We employed GLS inhibitors (CB839, IPN60090) and the BCL2 inhibitor venetoclax, prepared as detailed. MDS and AML cell lines were cultured under standard and modified (hypoxic, glutamine-free) conditions. Viability, proliferation, and caspase activity were assessed with commercial kits. RT-PCR quantified gene expression post-shRNA transfection. Mitochondrial potential, ATP levels, proteasome activity, and metabolic functions were evaluated using specific assays. Statistical analyses (t-tests, ANOVA) validated the findings.
RESULTS: The glutamine-free medium inhibited the growth of MDS cells. *GLS1* expression was higher in AML cells than in normal control samples (GSE15061), whereas *GLS2* expression was not. Treatment of MDS and AML cells for 72 h was inhibited in a dose-dependent manner by GLS inhibitors. Co-treatment with the B-cell lymphoma 2 (BCL2) inhibitor venetoclax and GLS inhibitors increased potency. Cells transfected with GLS1 short hairpin RNA showed suppressed proliferation under hypoxic conditions and increased sensitivity to venetoclax.
CONCLUSIONS: Targeting glutaminolysis and BCL2 inhibition enhances the therapeutic efficacy and has been proposed as a novel strategy for treating high-risk MDS and AML.

Keywords: Glutaminolysis, GLS inhibitor, MDS, AML, BCL-2 inhibitor, hypoxia

1. Introduction

Myelodysplastic syndrome (MDS) is a clonal hematopoietic malignancy characterized by peripheral blood cytopenia and a high risk of transformation into acute myelogenous leukemia (AML) [1]. In MDS, dysplasia is observed in the bone marrow, along with anemia, neutropenia, and thrombocytopenia [2]. Clinically, patients with MDS who develop anemia often require regular blood transfusions to increase the number of healthy circulating red blood cells [3]. Several scoring systems can be used to predict the prognosis of patients with MDS. In general, these scoring systems include analyses of peripheral cytopenia, the percentage of blasts in the bone marrow, and cytogenetic features.

The treatment of MDS begins with risk stratification using validated tools such as the International Prognostic Scoring System (IPSS) or its revised version,

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the IPSS-R [4,5]. MDS can be classified into subtypes associated with low and high risks of conversion to acute myeloid leukemia, which can help in the selection of treatment [6]. Azacitidine is the current standard of care for high-risk MDS; however, the median overall survival has been shown to be 5.6 months, and the 2-year survival probability has been reported at 15% among patients with azacytidine failure [7]. Notably, allogeneic stem-cell transplantation and investigational agents have been associated with better outcomes compared with those of conventional clinical care [7]. Therefore, an alternative strategy is required to improve the prognosis of patients with MDS, especially older patients.

The Warburg effect occurs when proliferating cancer cells preferentially convert glucose to lactate instead of pyruvate in the tricarboxylic acid (TCA) cycle (also known as the Krebs cycle), even in the presence of oxygen [8]. Glutamine is the most abundant circulating amino acid in blood and muscle and is vital for many basic cellular functions in cancer cells, including the synthesis of metabolites that maintain mitochondrial metabolism [9]. Glutaminase is the initial enzyme in glutamine metabolism, catalyzing the conversion of glutamine to glutamic acid in cells. Glutaminases are crucial in the metabolism, growth, and proliferation of cancer cells. In mammalian cells, two paralogous genes exist: *GLS1* and *GLS2* [10]. Enhanced glutamine metabolism (glutaminolysis) is recognized as a hallmark of cancer and signifies an essential metabolic shift in cancer cells [9]. With an enriched understanding of tumor metabolism, the glutamine metabolic pathway has garnered significant interest in cancer research. This pathway produces adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), facilitating nucleotide and lipid synthesis in cancer [11]. Tumor cells depend on glutamine for growth, and GLS, with its GLS1 and GLS2 isoforms, is a mitochondrial enzyme necessary for glutamine catabolism [10]. GLS1 inhibitors have been explored in the treatment of various cancers [10].

In this study, we assessed glutaminolysis in MDS and AML cell lines. Older patients with AML generally exhibit a poor prognosis, even after treatment with a hypomethylating agent. Compared to those receiving azacitidine alone, overall survival is notably extended, and the incidence of remission is higher among patients treated with azacitidine plus the B-cell lymphoma 2 (BCL2) inhibitor venetoclax [12]. Given that BCL2 inhibitors are employed in AML management, we explored whether the combination of venetoclax and GLS inhibitors could enhance cytotoxicity against MDS and AML cell lines.

2. Materials and methods

2.1. Reagents

The GLS inhibitor, CB839 (telaglenastat), was obtained from Selleck Chemicals (Houston, TX, USA), whereas the other GLS1 inhibitor, IPN60090 dihydrochloride, was obtained from MedChemExpress LLC (Monmouth Junction, NJ, USA). The BCL-2 inhibitor, ABT-199 (Venetoclax), was purchased from Abcam (Cambridge, CB2 0AX, UK). The inhibitors were dissolved in dimethyl sulfoxide. All other reagents were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Cell lines and cell culture

The MDS cell line SKM-1 (monoblastic leukemia following MDS) and the AML cell lines MOLM-14 (acute monocytic leukemia: AML-M5a) and Kasumi-1 (8;21 chromosome translocation) were obtained from the Japan Research Bioresource Cell Bank (Ibaraki, Osaka, Japan). Additional AML cell lines, such as U937 (pro-monocytic model cell line), THP-1 (human monocytic leukemia cell line), and MV4-11 (biphenotypic B-myelomonocytic leukemia), were acquired from the American Type Culture Collection (Manassas, Virginia, USA). Another MDS cell line, MDS-L (with a deletion in the 5q chromosome), was graciously provided by Prof. Kaoru Toyama (Kawasaki Medical School, Kurashiki, Okayama, Japan). The cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. MDS-L cells were cultured in RPMI 1640 medium supplemented with 20% FBS. In some experiments, cell cultures were incubated under hypoxic conditions with 1% O_2 at 37°C in an atmosphere of 5% CO_2. Experiments were initiated 72 h after adaptation to hypoxic conditions.

2.3. Data collection and processing

We retrieved microarray data from the GSE13159 and GSE19429 [13,14]. In the GSE13159 study, 2096 blood or bone marrow samples from patients with acute and chronic leukemia were hybridized to Affymetrix HG-U133 Plus 2.0 GeneChips. The GSE19429 dataset included 183 patients with MDS and 17 healthy controls. Bone marrow samples were obtained, and CD34+ cells were isolated from patients with MDS and

healthy controls. Data analysis was performed using the GEO2R website, an interactive tool for comparing various sample groups in a GEO series. The data were downloaded in the SOFT format, converted to XLS files, and analyzed using Microsoft Office Excel (Microsoft Corporation, Redmond, WA, USA). Differentially expressed genes were identified based on an adjusted P value (< 0.05) and a log2 fold change ($\geqslant 1.0$ or $\leqslant -1.0$).

2.4. Cell proliferation assay

Cells (2×10^5/ml) were treated with the indicated concentrations of Venetoclax, CB839, or IPN60090 for 72 h. A glutamine-free RPMI 1640 medium was used for the glutamine-deprived experiments. Cell viability was measured using the Cell Counting Kit-8 (Dojindo Laboratories, Mashiki, Kumamoto, Japan) or CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. The absorbance at 450 nm or luminescence was analyzed using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA).

2.5. Caspase 3/7 activity

The Caspase Glo 3/7 assay kit was obtained from Promega (Madison, WI, USA) and used to measure caspase activity according to the manufacturer's instructions. After 48 h of incubation with the indicated concentrations of venetoclax, CB839, or IPN60090, the luminescence of each sample was analyzed using an EnSpire Multimode Plate Reader.

2.6. Cytotoxicity assay

Cells were incubated for 72 h with the indicated concentrations of venetoclax, CB839, or IPN60090. The cytotoxicity was determined based on the release of lactate dehydrogenase (LDH) release. A cytotoxicity LDH assay kit with a water-soluble tetrazolium salt was obtained from Dojindo Laboratories. An EnSpire Multimode Plate Reader was used to measure the amount of LDH released from the dead cells.

2.7. Quantitative real-time reverse transcription polymerase chain reaction analysis (RT-PCR)

Total RNA was extracted from myeloma samples using an RNAqueous-4PCR Kit (Life Technologies Japan KK, Minato-ku, Tokyo, Japan) and reverse transcribed using a First-Strand cDNA Synthesis Kit (OriGene Technologies, Rockville, MD, USA). RT-PCR was performed using a Roche Light Cyber 2.0 detection system (Roche Diagnostic Gmbh, Minato-ku, Tokyo, Japan). Specific GLS1, GLS2, and β-actin primers were purchased from Takara Bio, Inc. (Otsu, Shiga, Japan). Specific gene expression was quantified using a SYBR Green PCR Kit (Roche) according to the manufacturer's protocol.

2.8. Short-hairpin RNA (shRNA) transfection

Transfection with short hairpin RNA (shRNA) was performed as described previously [15]. Briefly, the mammalian GLS1 lentiviral vector and control shRNA vector were obtained from VectorBuilder Japan Inc. (Yokohama, Kagawa, Japan). SKM-1 cells were cultured in a six-well culture dish for 24 h in RPMI 1640 medium with 8 g/mL polybrene (hexadimethrine bromide) (Merck KgaA) and infected with lentiviral vectors. The medium was replaced with a fresh, complete medium the following day. RT-PCR was performed to determine GLS1 expression levels.

2.9. Mitochondrial membrane potential

A mitochondrial Staining Kit (Merck KGaA) was used to analyze the mitochondrial membrane potential (MMP) according to the manufacturer's protocol [16]. After 72 h of incubation with venetoclax, CB839, or IPN60090, JC-1 monomers and aggregates were analyzed using an EnSpire Multimode Plate Reader.

2.10. ATP assays

The indicated concentrations of CB839 and IPN60090 were applied to the MDS and AML cells for 24 h. In some experiments, cells were cultured in glutamine-free RPMI 1640 medium for 72 h. Intracellular ATP concentrations were assessed using the Cell ATP Test Reagent Kit Ver. 2 from TOYO B-Net (Tokyo, Japan), following the manufacturer's instructions. Luciferase activity was subsequently measured with an EnSpire Multimode Plate Reader (PerkinElmer).

2.11. Enzyme-linked immunosorbent assays

SKM-1 cells were grown in RPMI medium under or without hypoxic conditions. After 24 h, the cells were collected and stored at $-80°$C. A 20S Proteasome Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to measure proteasome activity. The lu-

minescence was measured using an EnSpire Multimode Plate Reader (Perkin Elmer). Nicotinamide adenine dinucleotide phosphate (NADP) was analyzed using the NADP/NADPH Assay Kit-WST (Dojindo Laboratories) in accordance with the manufacturer's instructions. After 24 h of incubation with the indicated concentrations of CB839 or IPN60090, absorbance was measured at 450 nm using an EnSpire Multimode Plate Reader. The extracellular rate of oxygen consumption (OCR) plate assay kit and glycolytic assay (extracellular acidification) kit were obtained from Dojindo Laboratory and Abcam (Cambridge, UK) and analyzed according to the manufacturer's instructions.

2.12. Colony assay

The colony assay was performed as described previously [17]. Briefly, 1×10^2 shRNA transfectant SKM-1 cells were plated in triplicate in six-well plates containing a methylcellulose medium (MethoCultTM Express # 04437; StemCell Technologies, Vancouver, Canada) under hypoxic conditions at 37°C in an atmosphere of 5% CO_2 and 1% O_2 at 37°C. Colony counts were determined using an EVOSTM FL Digital Inverted Fluorescence Microscope 7 days after plating (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Results are presented as mean and standard error.

2.13. Statistical analyses

All data are expressed as the mean ± SD from three independent experiments and were analyzed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). The student's two-sided t-test was employed to assess the significance of differences between two independent groups. For comparisons among multiple independent groups and a single control group, a one-way analysis of variance (ANOVA) followed by either Bonferroni or Dunnett's post hoc test was utilized. Additionally, two-way ANOVA with Dunnett's or Šídák's multiple comparisons test was applied for statistical analyses where indicated. Statistical significance was established at $p < 0.05$. Notably, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ were considered significant.

3. Results

3.1. Analysis of glutamine in MDS cell line and gene expression in MDS or AML samples

Glutamine is typically considered a nonessential amino acid under normal physiological conditions, yet it is classified as conditionally essential in certain contexts [18]. In cancer, glutamine plays a critical role in bioenergetics, biosynthesis, tumor growth, and antioxidant production through glutaminolysis [19]. Consequently, we initially investigated the function of glutamine in the MDS cell line SKM-1. The omission of glutamine from the RPMI 1640 medium and subsequent incubation for 48 h or 72 h led to reduced cell proliferation and enhanced cytotoxicity compared to conditions with glutamine (Fig. 1A and 1B). Additionally, Caspase 3/7 activity was elevated (Fig. 1C). ATP is recognized as the molecular unit of intracellular energy currency [20]. Glutamine-driven oxidative phosphorylation is a major source of ATP. Therefore, we evaluated intracellular ATP levels and found that the amount of ATP decreased (Fig. 1D). The cells were cultured in a glutamine-depleted medium, resulting in the observation of inhibited cell proliferation after 24 h (Fig. 1E). Many malignant tumor cells exhibit glutamine addiction [10]. Consequently, we examined the expression of *GLS* genes, namely *GLS1* (or *GLS*) and *GLS2*, utilizing a public functional genomics database (GSE13159) [13]. From the Gene Expression Omnibus (National Center for Biotechnology Information, Bethesda, MD, USA) database (GSE13159), it was noted that *GLS1* gene expression in AML cells was elevated compared to that of normal control samples (Fig. 1F). However, *GLS2* expression did not show an increase in either MDS or AML cells (Fig. 1G). Subsequently, we assessed *GLS1* and *GLS2* expression in MDS subtypes using data from GSE19429 [14]. The analysis from GSE19429 revealed that the expression levels of *GLS1* and *GLS2* remained unchanged across the MDS staging conducted using the French American British classification of refractory anemia (RA) and RA with excess blasts (RAEB) (Fig. 1H).

3.2. GLS1 gene expression and activity of venetoclax under hypoxic condition

O_2 is a crucial determinant of cell metabolism and gene expression [21]. The local oxygen tension in the bone marrow is notably low [22]. Consequently, we investigated the gene expression of *GLS1* and *GLS2* in SKM-1 cells under hypoxic conditions. The expression of *GLS1* increased compared to that of normoxic conditions, whereas *GLS2* expression remained stable (Fig. 2A). We observed that cell proliferation was reduced in glutamine-free medium under hypoxic conditions relative to normoxic conditions (Fig. 2B). Additionally, under hypoxic conditions, 20S proteasome

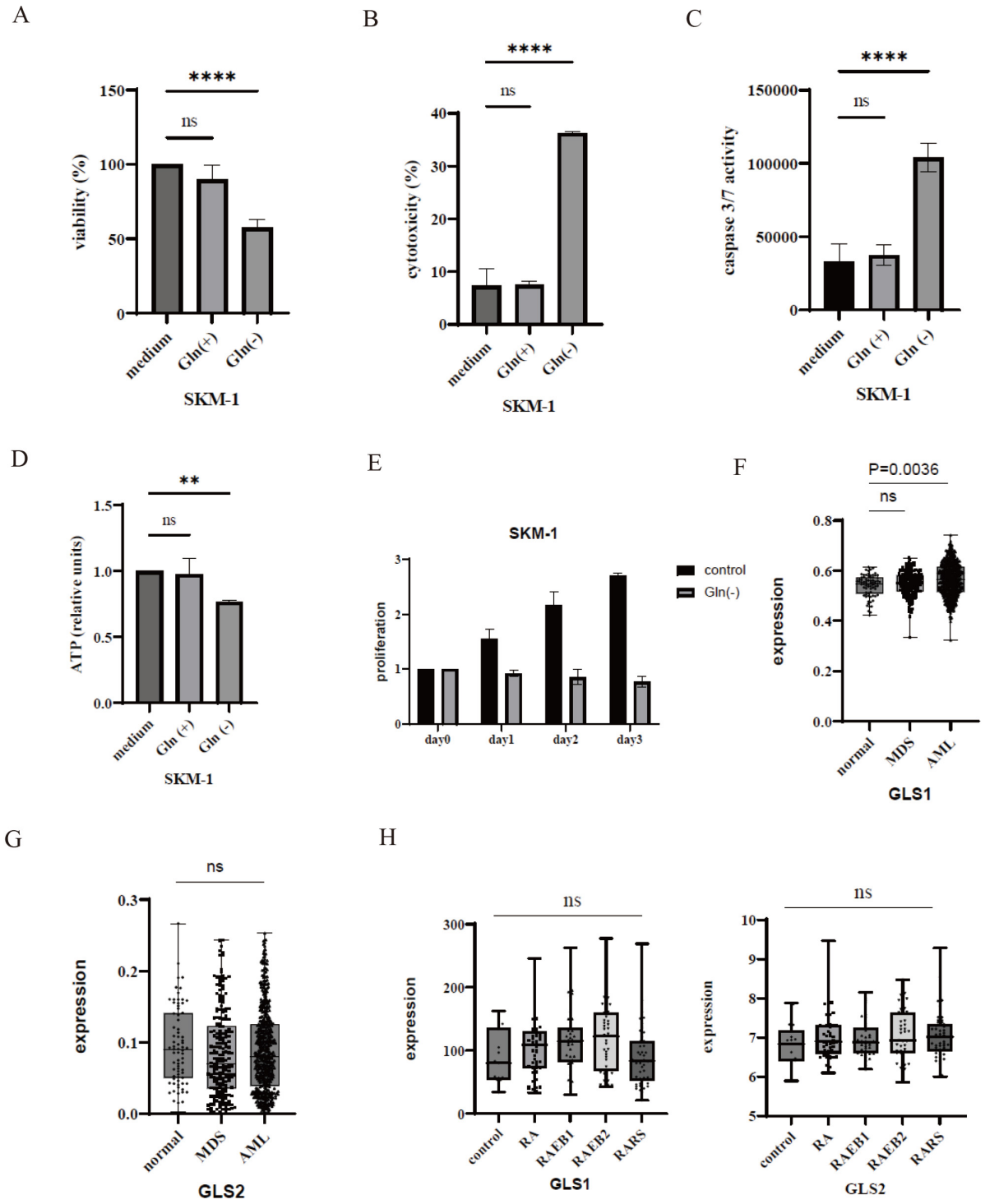

Fig. 1. Glutaminolysis of SKM-1 and gene expression of GLS1 and GLS2 in MDS and AML cells. SKM-1 cells were incubated in RPMI 1640 medium with or without glutamine for 48 h or 72 h. Cell viability (A), cytotoxicity (B), caspase 3/7 activity (C), and intracellular ATP levels (D) were evaluated. $^{**}p < 0.01$, $^{****}p < 0.0001$ were compared to control. ns, not significant. (E) The cells were cultured in a glutamine-depleted RPMI1640 medium. The cell viability was evaluated. (F, G, H) Expression of *GLS1* and *GLS2*. Validation of *GLS1* and *GLS2* was performed using GEO data (GSE13159 and GSE19429); ns, not significant.

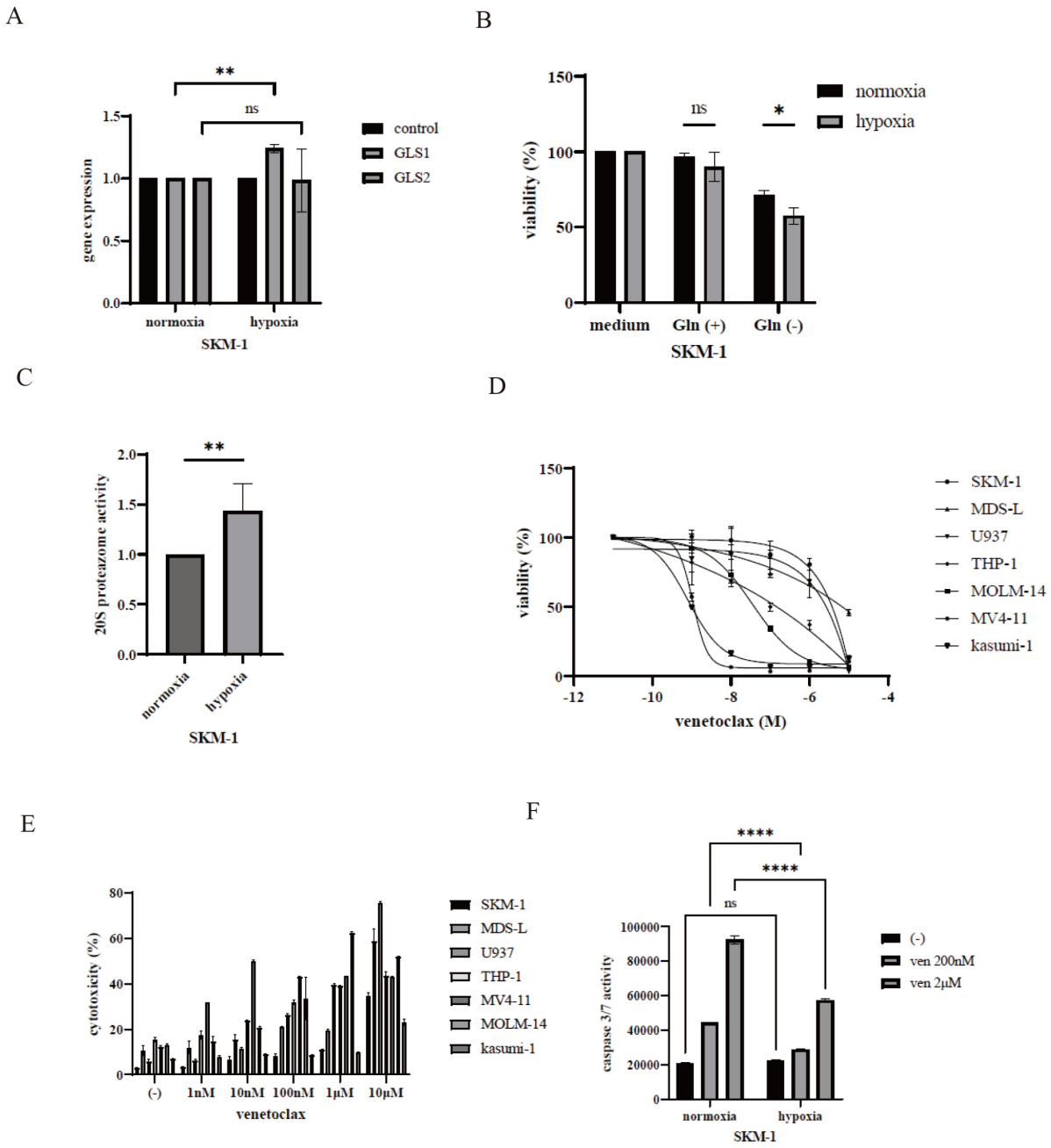

Fig. 2. GLS1 gene expression and activity of venetoclax under hypoxic conditions. (A) SKM-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS with or without hypoxia for 24 h. Gene expressions of *GLS1* and *GLS2* were evaluated by RT-PCR. $^{**}p < 0.01$ was compared to control. ns, not significant. (B) SKM-1 cells were incubated with RPMI 1640 medium with or without glutamine and/or hypoxia condition for 72 h. Cell viability was analyzed by cell counting kit-8. $^{*}p < 0.05$ was compared to normoxia. ns, not significant. (C) SKM-1 cells were cultured with or without hypoxia for 24 h. 20S proteasome activity was analyzed. $^{**}p < 0.01$ was compared to normoxia. (D) MDS and AML cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS with the indicated concentrations of venetoclax for 72 h in normoxia. Cell growth was evaluated using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay Kit or Cell Counting Kit-8. (E) MDS and AML cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS with the indicated concentrations of venetoclax for 72 h in normoxia. Cell growth was evaluated using the Cytotoxicity LDH Assay Kit. (F) SKM-1 cells were treated with the indicated concentrations of venetoclax for 48 h. Caspase 3/7 activity was analyzed using the Caspase Glo 3/7 Assay Kit. $^{****}p < 0.0001$ were compared to normoxia condition. ns, not significant.

activity was enhanced (Fig. 2C). We then assessed the efficacy of various concentrations of the BCL-2 inhibitor venetoclax in MDS and AML cell lines under normoxic conditions. Venetoclax inhibited the proliferation of MDS and AML cells in a dose-dependent manner (Fig. 2D). Subsequently, we conducted a cytotoxicity analysis using LDH-based assays to determine the percentage of dead cells. MDS and AML cell lines were exposed to specified concentrations of venetoclax for 72 h under normoxic conditions. Moreover, venetoclax dose-dependently increased the percentage of cytotoxic cells (Fig. 2E). However, caspase 3/7 activity decreased in response to venetoclax under hypoxic conditions (Fig. 2F).

3.3. Activity of GLS inhibitors in MDS and AML cell lines

From the GSE15061 dataset, *GLS1* expression increased in MDS and AML samples. CB839 and IPN60090 are GLS1 inhibitors. Thus, we investigated the efficacy of these inhibitors in MDS and AML cell lines. Our results demonstrated that both CB839 and IPN60090 significantly inhibited the proliferation of all MDS and AML cell lines in a dose-dependent manner (Fig. 3A, 3B). As glutamate is crucial for the biosynthesis of amino acids, nucleotides, lipids, and the reducing equivalents of NADPH [11], we further assessed the impact of GLS1 inhibitors on NADPH production. Our findings indicated that the levels of both NADPH and NADP+ were notably reduced by the treatments with GLS1 inhibitors, CB839 and IPN60090, in a dose-dependent fashion (Fig. 3C, 3D, 3E, 3F). Additionally, the total amount of ATP was decreased following the treatments with CB839 and IPN60090 (Fig. 3G). Oxygen is primarily consumed during ATP production via mitochondrial oxidative phosphorylation. Consequently, the OCR in the cells serves as an indicator of mitochondrial function. The extracellular acidification rate (ECAR) refers to the release of acidic substances by cells into the extracellular environment because of metabolic processes. Therefore, we focused on OCR and ECAR in this study: the former decreased, while the latter did not change after CB839 treatment (Fig. 3H).

3.4. Venetoclax and CB839 or IPN60090 inhibited the growth of the MDS and AML cell lines

Next, we performed a cell proliferation assay to assess the effectiveness of Venetoclax, CB839, and IPN60090 in MDS and AML cells. In a phase 1 study, the blood concentration of CB-839 reached 3.1 μM [23]. Moreover, in prior animal experiments, blood concentrations of IPN60090 exceeded 100 μM [24]. The median time for venetoclax to reach peak concentration occurs 6 h post-administration, with blood levels reaching 2.39 μM at the 400 mg dosage [25]. Consequently, 200 nM venetoclax, 200 nM CB-839, and 1 μM IPN60090 were selected for the combination dosing experiment. Compared with each drug alone, co-treatment with venetoclax and CB839 or IPN60090 inhibited cell proliferation in the MDS and AML cell lines SKM-1, MDS-L, U937, and THP-1 (Fig. 4A). The Chou-Talalay method is widely utilized to assess synergistic effects of drug combinations [26]. To measure the extent of interaction, a Combination Index (CI) was employed. Our findings indicated that the CI was less than 1, suggesting that the drug combination exhibited synergistic effects. Additionally, Caspase 3/7 activity increased (Fig. 4B). In the mitochondria, GLS1 and GLS2 hydrolyze glutamine to glutamate, which serves as a precursor of multiple metabolites. Mitochondrial function is a critical indicator of overall cell health, as highlighted by the association between mitochondrial dysfunction and various diseases, including cancer [27]. Therefore, we examined the MMP using a mitochondrial staining kit. We found that the MMP levels decreased after co-treatment with venetoclax, CB839, and IPN60090 (Fig. 4C).

3.5. Knockdown of GLS1 increased venetoclax sensitivity under hypoxia

Given the crucial role of *GLS1* in converting glutamine to glutamate, *GLS1* may play a significant role in the survival of MDS and AML cells. SKM-1 cells were stably transfected with expression vectors carrying shRNAs targeting *GLS1* (shGLS1) or non-targeting shRNAs, utilizing a standard lentiviral construct. Gene expression efficiency was assessed by RT-PCR (Fig. 5A). Cells were incubated at a final concentration of 1×10^5 cells/ml, and cell proliferation was subsequently evaluated. We observed that cell proliferation was lower in shRNA-transfected SKM-1 cells than in cells transfected with control shRNA under hypoxic conditions (Fig. 5B). The colony formation assay, an in vitro quantitative method for evaluating cell survival, measures the ability of a single cell to grow into a colony [28]. We then conducted colony formation assays to investigate the impact of *GLS1* inhibition. The number of colonies was lower in GLS1 shRNA-transfected SKM-1 cells under hypoxic con-

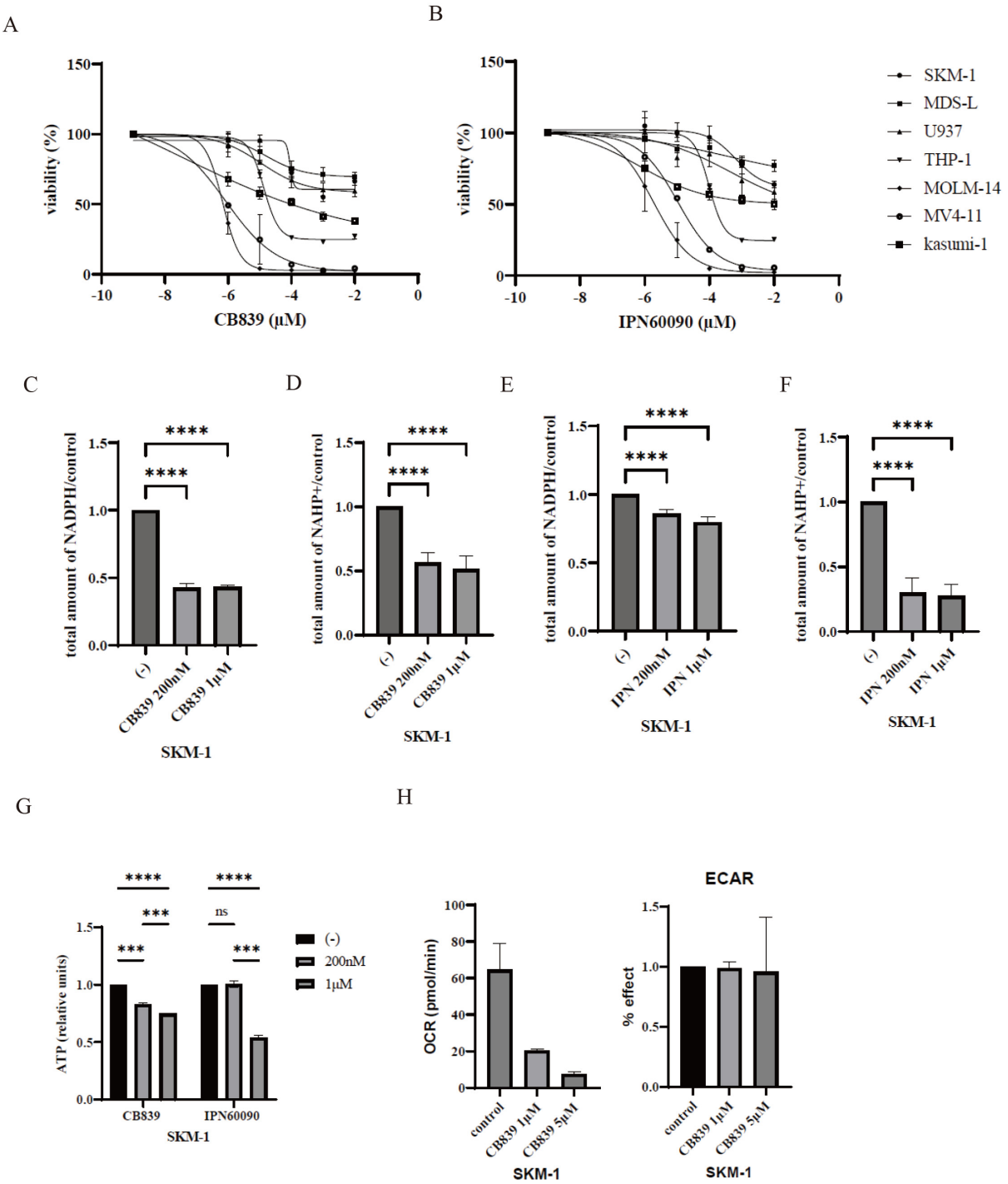

Fig. 3. Activity of GLS1 inhibitors in MDS and AML cells. (A, B) MDS and AML cell lines were cultured with the indicated concentrations of CB839 or IPN60090 for 72 h under hypoxia. Cell growth was evaluated using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay Kit or Cell Counting Kit-8. (C, D, E, F) MDS and AML cell lines were cultured with the indicated concentrations of CB838 or IPN60090 for 24 h. The total amount of NADPH or NADP+ was analyzed by using NADP/NADPH Assay Kit-WST. (G) SKM-1 cells were incubated with the indicated concentration of CB839 or IPN60090 for 72 h. Intracellular ATP levels were determined using the "Cell" ATP Assay Reagent Ver. 2 Kit. $^{***}p < 0.001$, $^{****}p < 0.0001$ were compared to control. ns, not significant. (H) SKM-1 cells were incubated with the indicated concentration of CB839 for 24 h. The extracellular OCR and the extracellular acidification were analyzed.

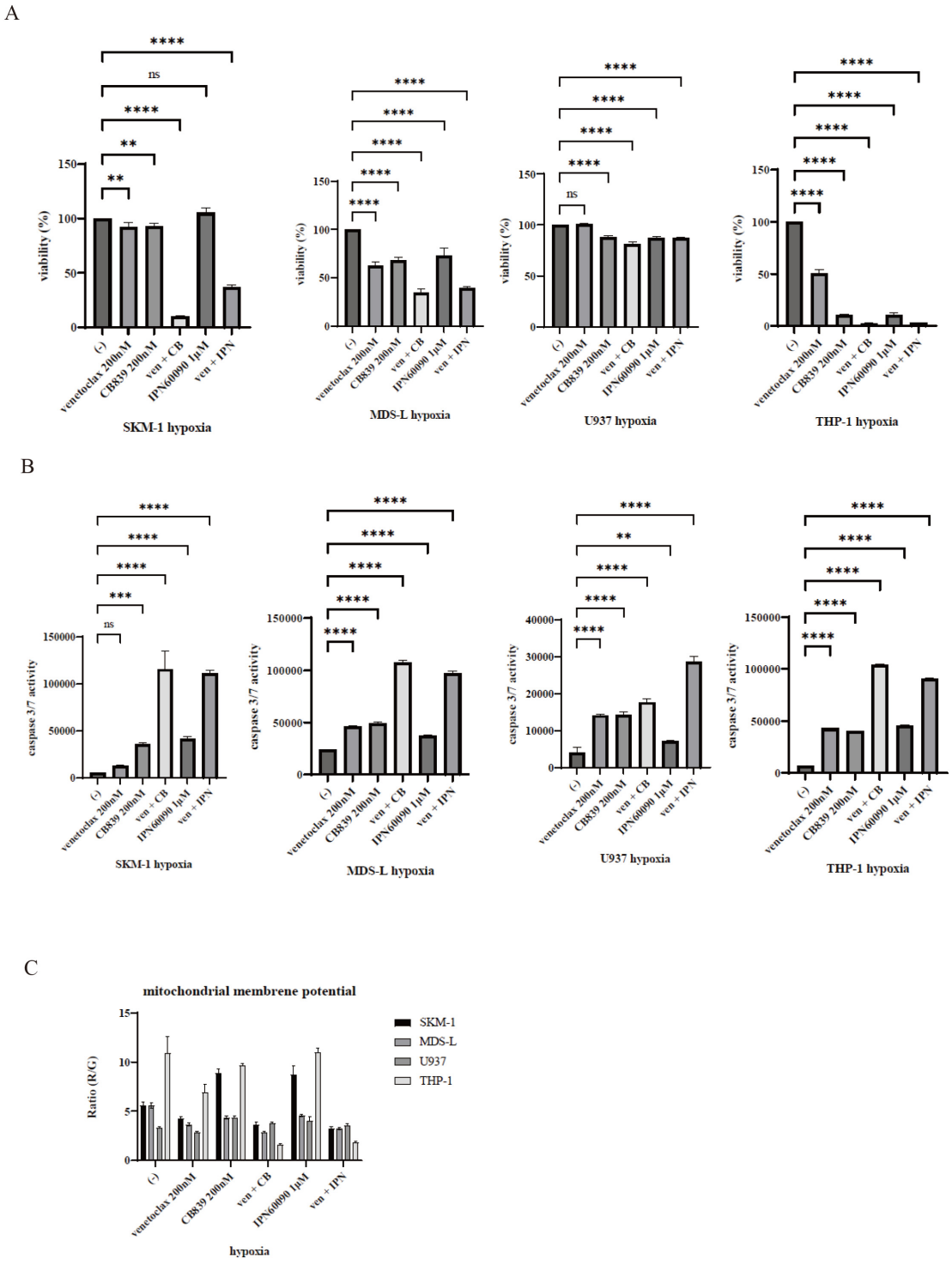

Fig. 4. Co-treatment with venetoclax and CB839 or IPN60090 induced cytotoxicity in the MDS and AML cells. SKM-1, MDS-L, U937, and THP-1 cell lines were cultured with the indicated concentrations of venetoclax, CB839, or IPN60090 under hypoxic conditions for 48 h or 72 h. Cell proliferation (A) and caspase 3/7 activity (B) were analyzed. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ were compared to control. ns, not significant. (C) SKM-1, MDS-L, U937, and THP-1 cells were treated with the indicated concentrations of venetoclax and/or CB839 or IPN60090 under hypoxia for 48 or 72 h. MMP was analyzed using a mitochondrial staining kit.

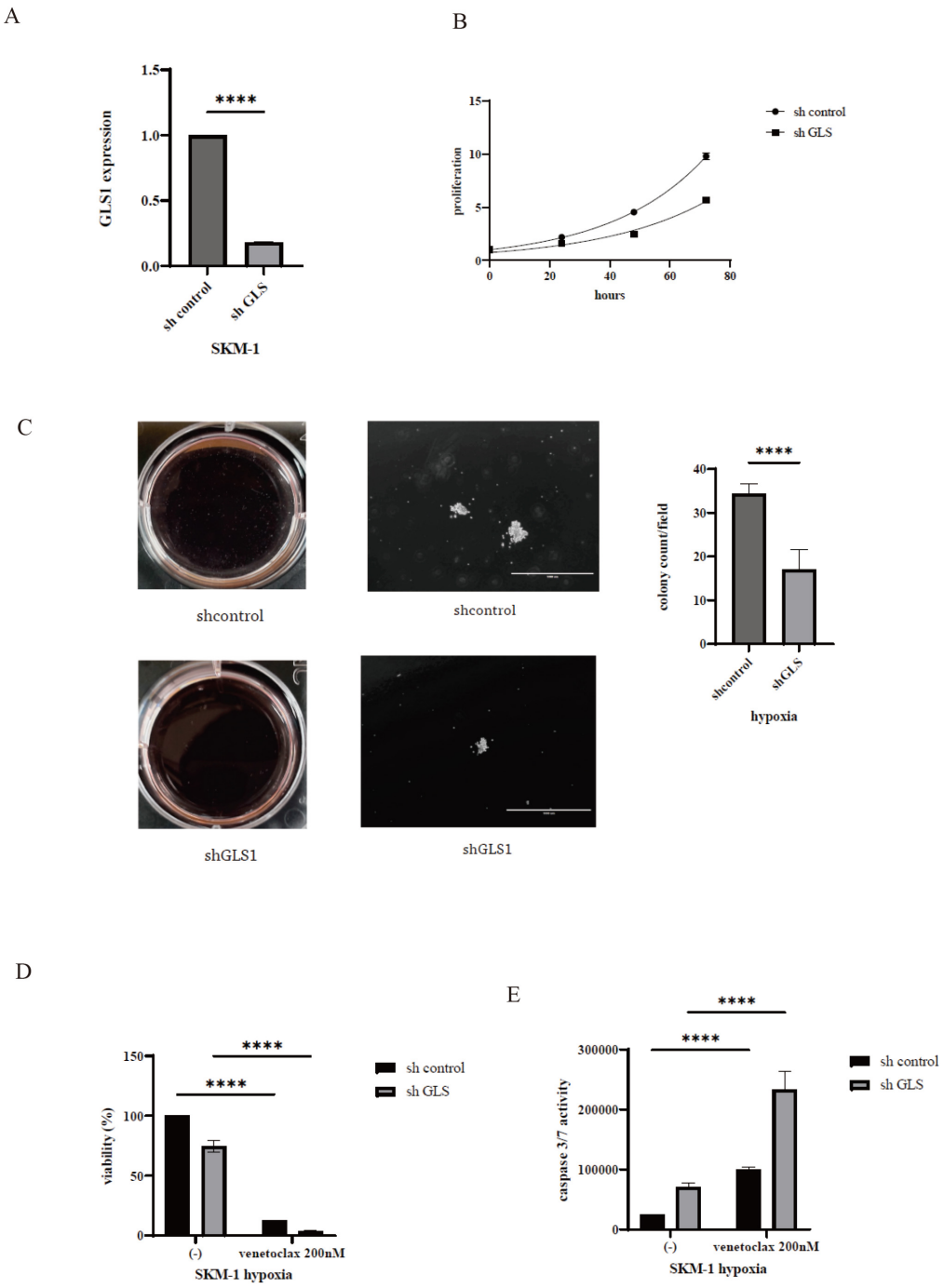

Fig. 5. Knockdown of *GLS1*-induced venetoclax activity under hypoxia. (A) Gene expression of *GLS1* was analyzed by RT-PCR assay. $^{****}p <$ 0.0001 were compared to control shRNA transfectant cells. (B) Cellular proliferation of shRNA-transfected SKM-1 cells was evaluated using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay Kit or Cell Counting Kit-8. (C) 1×10^2 shRNA transfectant SKM-1 cells were plated under hypoxia for 7 days. The colonies on each dish were photographed using a digital camera and counted using an EVOS^{TM} FL Digital Inverted Fluorescence Microscope. The quantitative graph displays the colonies formed, and representative images are shown. Scale bar: 1,000 μm. $^{****}p < 0.0001$ were compared to control shRNA transfectant cells. The results represent three independent experiments. (D, E) ShRNA transfectant SKM-1 cells were incubated with the indicated concentration of venetoclax with or without hypoxia for 48 or 72 h. Cell viability (D) and caspase 3/7 activity (E) were evaluated. $^{****}p < 0.0001$ were compared to control.

ditions (Fig. 5C). Further, we assessed the efficacy of venetoclax under these conditions. In the GLS1 shRNA-transfected SKM-1 cells, cell viability was notably decreased by venetoclax compared to that in control shRNA-transfected cells (Fig. 5D), and Caspase 3/7 activity was elevated (Fig. 5E). These findings suggest that GLS1 expression influences both cell proliferation and venetoclax sensitivity.

4. Discussion

We investigated *GLS1* activity in MDS and AML cell lines. Our study demonstrated an increase in *GLS1* expression in AML samples, as evidenced by GEO data. Furthermore, our findings reveal that *GLS1* expression is augmented under hypoxic conditions. Glutaminolysis, the process through which cells convert glutamine into TCA cycle metabolites via multiple enzymes, is central to this observation [9].

Malignant cells reprogram their metabolic machinery to meet the demands of malignant transformation and progression [29]. GLS1, a mitochondrial enzyme, catalyzes the hydrolysis of glutamine to glutamate, supporting the rapid proliferation of tumor cells. In this study, we observed that the proliferation of MDS cells was glutamine-dependent. The agents CB839 and IPN60090 diminished the NADPH pool, thereby inhibiting glutamine metabolism and the proliferation of both MDS and AML cells. Consequently, MDS and AML cells exhibit a significant reliance on glutamine for ATP production. Beyond ATP synthesis, mitochondria perform a variety of critical functions dependent on metabolism, including maintaining cellular redox balance, calcium homeostasis, inflammatory signaling, and apoptosis [27]. Thus, GLS1 significantly contributes to ATP production by supplying glutamate to MDS and AML cells. Importantly, hypoxia is a critical component of the bone marrow and hematopoietic stem cell niche [22]. Under hypoxic conditions, the bone marrow microenvironment may support the maintenance of MDS and AML cells through enhanced glutamine metabolism. BCL2 family proteins are crucial in the intrinsic mitochondrial apoptotic pathway [30]. Preclinical studies have demonstrated that venetoclax induces apoptosis in malignant cells reliant on BCL2 for survival. Moreover, venetoclax monotherapy has demonstrated modest efficacy in AML [12]. One study revealed that the remission rate was higher in previously untreated AML patients receiving azacitidine plus venetoclax than in those receiving only azacitidine. Notably, venetoclax combined with azacitidine is the standard of care for newly diagnosed AML patients who are ineligible for intensive chemotherapy [12]. Furthermore, we demonstrated that co-treatment with venetoclax and CB839 or IPN60090 inhibited the proliferation of MDS and AML cells under hypoxic conditions.

Previous studies have shown that *GLS1* expression is significantly higher in hepatocellular carcinomas [31] and is correlated with lymph node metastasis and advanced clinical stages of colorectal cancer [32]. Moreover, GLS1 levels are positively associated with the stage, metastasis, and disease severity of various cancers. *GLS1* is often overexpressed in highly proliferative cancer cells to meet the increasing demand [11]. Thus far, glutaminolysis and GLS1 have been identified as significant targets in cancer patients, including those with MDS and AML. Emerging evidence has revealed that inhibitors of these pathways could provide a beneficial strategy for leukemia therapy. Novel inhibitors of glutaminases, crucial enzymes in glutamine metabolism, are targeting glutamine addiction as a viable strategy for the treatment of MDS and AML. Given its well-documented effects on cellular energy, tumor progression, and redox homeostasis over recent decades, GLS1 has emerged as a potential therapeutic target in certain cancers. A series of inhibitors has been developed to treat patients with glutamine-dependent cancers.

CB839, a small-molecule GLS1 inhibitor used in clinical trials, has demonstrated safety, favorable PK/PD, and antitumor activity [33]. It exhibits promising results in metastatic renal cell carcinoma when combined with cabozantinib or everolimus [23,34] and inhibits the growth of *PIK3CA*-mutant colorectal cancer in combination with 5-FU in xenograft models [35]. Moreover, CB839 selectively targets JAK2-V617F mutant hematopoietic stem cells [36]. The discovery of the GLS1 inhibitor led to the development of IPN60090, a novel selective inhibitor currently undergoing phase 1 trials [24]. Clinical trials continue to explore the potential of glutaminase inhibition as a therapeutic strategy for malignancies such as MDS and AML. The analysis of MDS samples was not possible in this study due to the limited availability of primary samples. Significant discrepancies were noted in the glutaminase gene expression data between MDS cell lines and clinical samples versus normal samples from published datasets. Future studies should address this issue using clinical samples.

Combining CB839 with venetoclax has been shown to enhance cell death in MDS and AML. Both CB839 and IPN60090, when paired with venetoclax, exhibit synergistic antiproliferative effects. GLS1 knockdown

cells demonstrated increased sensitivity to venetoclax, and the combination of CB839 and venetoclax was particularly effective under hypoxic conditions. These results suggest that targeting both GLS1 and BCL2 may offer a promising therapeutic strategy for patients with MDS and AML. Additionally, this study revealed that venetoclax sensitivity decreased under hypoxic conditions; however, co-treatment with GLS inhibitors and venetoclax inhibited cell proliferation in these conditions. Further, GLS1 shRNA reduced cell growth and enhanced venetoclax sensitivity under hypoxic conditions, indicating that combination therapy could potentially overcome venetoclax resistance in the bone marrow. These findings underscore the potential clinical benefits of combining these treatments to improve outcomes for patients with MDS and AML in hypoxic tumor environments.

5. Conclusions

Our findings indicate that GLS1 inhibitors possess significant antileukemic effects in MDS and AML cell lines. Concurrent targeting of BCL2 and GLS1 enhances therapeutic efficacy and may represent a novel approach for patients with high-risk MDS and AML. Our study offers promising clinical evidence supporting the development of candidate drugs for treating MDS and AML. Further efforts in drug development are warranted to capitalize on these therapeutic benefits.

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Data availability statement

All datasets and materials generated for this study are included in the manuscript.

Conflict of interest

A.G. received research funding from Eisai Co., Ltd.; Ono Pharmaceutical Co., Ltd.; Taiho Pharmaceutical Co., Ltd.; Takeda Pharmaceutical Co., Ltd.; Nippon Shinyaku Co., Ltd.; Chugai Pharmaceutical Co., Ltd.; MSD K.K.; Otsuka Pharmaceutical Co., Ltd.; Sumitomo Pharma Co., Ltd.; Nippon Shinyaku Co., Ltd.; Bayer Yakuhin, Ltd.; Daiichi Sankyo Co., Ltd.; and Nihon Pharmaceutical Co., Ltd. A.G. reports honoraria from Novartis Pharma K.K., Alexion Pharmaceuticals, Inc., Eisai Co., Ltd., Ono Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Nippon Shinyaku Co., Ltd., Chugai Pharmaceutical Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Sumitomo Pharma Co., Ltd., Daiichi Sankyo Co., Ltd., Nihon Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., Janssen Pharmaceutical K.K., Pfizer Japan Inc., and Sanofi K.K.. A.G. reports consulting fees from PharmaEssentia Japan K.K.; Chugai Pharmaceutical Co., Ltd.; and Alexion Pharmaceuticals, Inc.. In addition, A.G. reported participation in the data safety monitoring board or advisory board of PharmaEssentia Japan K.K., Chugai Pharmaceutical Co., Ltd., and Alexion Pharmaceuticals Inc. S.O. reported no conflicts of interest.

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