

Enhanced Susceptibility to 5-Fluorouracil in Human Colon Cancer Cells by Silencing of GRP78

SEUNGPIL YUN¹, YONG-SEOK HAN^{2,3}, JUN HEE LEE⁴, SANGMIN KIM¹ and SANG HUN LEE^{2,3}

¹Neuroregeneration and Stem Cell Programs, Institute for Cell Engineering,
Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.;
²Medical Science Research Institute, Soonchunhyang University, Seoul Hospital, Seoul, Republic of Korea;
³Department of Medical Bioscience, Soonchunhyang University, Asan, Republic of Korea;
⁴Department of Pharmacology and Toxicology,
University of Alabama at Birmingham School of Medicine, Birmingham, AL, U.S.A.

Abstract. *Background:* Glucose-regulated protein (GRP78), also known as immunoglobulin heavy chain binding protein and as heat shock 70 kDa protein 5, is present in the endoplasmic reticulum (ER) membrane. GRP78 is generally expressed at low concentrations, but is increased by physiological stress. GRP78 is thought to protect against tissue or organ damage under pathological conditions, such as neurotoxic stress, myocardial infarction, or arteriosclerosis. In addition, in tumors, GRP78 expression is much higher than in normal tissues. Furthermore, high levels of GRP78 expression have been shown to increase the risk of malignancy and metastasis in prostate and colon cancer. Because both anticancer drugs and down-regulation of GRP78 expression inhibit cancer progression and growth, we hypothesized that down-regulation of GRP78 expression might lead to enhanced susceptibility of cancer cells to cytotoxic action of 5-fluorouracil (5-FU). *Materials and Methods:* GRP78 expression was suppressed in LoVo colon cancer cells by utilizing small-interfering RNA (si-GRP78), and the cells were subsequently used to study the antiproliferative and anticancer effects of 5-FU treatment. The signaling pathways responsible for the increase of LoVo cell susceptibility to 5-FU treatment after exposure to GRP78 siRNA were determined by western blot. *Results:* GRP78 silencing significantly inhibited cell viability and increased apoptosis of LoVo cells. Furthermore, combined treatment with 5-FU and GRP78 siRNA for 12 h reduced cell viability, and increased apoptosis and generation

reactive oxygen species more strongly than either of the two treatments applied separately. In order to examine the role of ER stress in increased susceptibility of LoVo cells to 5-FU after pretreatment with GRP78 siRNA, we analyzed expression levels of ER stress marker proteins, such as phosphorylated protein kinase-like endoplasmic reticulum kinase (PERK), phosphorylated eukaryotic initiation factor 2 alpha (eIF2 α), activating transcription factor 4 (ATF4), phosphorylated inositol-requiring enzyme 1 alpha (IRE1 α), phosphorylated p38, and C/EBP homologous protein (CHOP). Treatment with 5-FU alone increased the expression of ER stress marker proteins, whereas combined exposure to both 5-FU and GRP78 siRNA led to an even stronger effect on these markers. Similar to the pattern of modulation of ER stress protein expression, the levels of apoptosis-related proteins were also more strongly affected by combined exposure to 5-FU and GRP78 siRNA than by single treatments. In particular, expression of Bcl-2-associated X protein (BAX), cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase 1 (PARP1) were increased, whereas the expression of B-cell lymphoma 2 (BCL2) was reduced by these treatments. *Conclusion:* GRP78 silencing and incubation with 5-FU have synergistic effects on the inhibition of LoVo colon cancer cell growth via the induction of ER stress-dependent apoptosis.

The endoplasmic reticulum (ER) is a membranous organelle in eukaryotic cells involved in cellular homeostasis and apoptosis of cells in response to numerous physiological stresses (1). Consequently, dysregulation of ER functions has been correlated with various pathological states of cancer cells (1). Malignant tumors cause hypoxia, nutrient depletion, and generation of excess reactive oxygen species (ROS) of the surrounding microenvironment of the cell, perturbing the function of ER (2-4). Such cellular stress conditions activate the unfolded protein response (UPR) in ER, which is known to enhance cell survival by preventing

Correspondence to: Sang Hun Lee, Ph.D., Soonchunhyang Medical Science Research Institute, Soonchunhyang University, Seoul Hospital, 59, Daesagwan-ro (657 Hannam-dong), Yongsan-gu, Seoul 140-887, Republic of Korea. Tel: +82 027099029, Fax: +82 027925812, e-mail: ykckss1114@nate.com, jhlee0407@sch.ac.kr

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cells from entering apoptotic or senescence programs induced by cancer-related dysfunction (5). Likewise, there are various proteins in the ER membrane that act to prevent cell death caused by cancer. Specifically, previous studies suggested a potential role of glucose-regulated protein (*GRP*) 78 gene in UPR-related anti-apoptotic processes: i) *GRP*78 was found to be consistently overexpressed in human colorectal cancer (CRC); ii) human CRC cells with overexpression of *GRP*78 exhibited increased survival, proliferation, and metastasis (6). In addition, it has been demonstrated that *GRP*78 promotes resistance to chemotherapeutic agents (7, 8). In light of these findings, further studies addressing the role of *GRP*78 in cancer progression are necessary. In particular, increasing attention has been paid to the precise relationship between *GRP*78 and CRC, which is one of the most commonly diagnosed cancers in the world, with 1.3 million new cases diagnosed annually (9). Colon cancer and its prognosis is generally unfavorable because CRC is particularly malignant and resistant to many therapeutic drugs, mostly due to the loss of apoptosis signaling (9). However, despite its malignancy, CRC and its mechanisms have not been sufficiently elucidated. Previous studies have documented a potential role of the pro-survival property of *GRP*78 in inducing tumor growth and, possibly, in conferring drug resistance in colon cancer cells (10). In some tumors, overexpression of the *GRP*78 gene accompanied an increase in tumorigenicity and drug resistance (11-13). As a result, it has been hypothesized that overexpression of *GRP*78 might be involved in the pathogenesis of colorectal carcinomas (14). Association of *GRP*78 protein functions with colon cancer was specifically studied in cell lines (10). However, the role of *GRP*78 in the regulation of apoptosis has never been studied in LoVo colon cancer cells as far as we are aware. In the present study, we therefore sought to fill this gap in the literature and elucidated the relationship between down-regulation of *GRP*78 and cell death in human LoVo colon cancer cells.

Chemotherapy continues to play a vital role as an adjuvant therapy in CRC, and 5-fluorouracil (5-FU) remains a drug of choice for patients with CRC (15). Specifically, 5-FU kills cancer cells by increasing ROS generation and inducing ER stress, promoting, in turn, cancer cell apoptosis (16-19). However, there have been many cases in which drug-resistant tumor cells emerged after 5-FU treatment (17-19). Because resistance to the induction of apoptosis is a prominent cause of chemotherapy failure, strengthening pro-apoptotic action by using combination therapy is currently the goal of many novel treatments. Recently, a potential correlation between 5-FU effects and *GRP*78 activity has been of interest in the literature. Because *GRP*78 is involved in the cell survival pathway, and *GRP*78 overexpression is implicated in drug resistance, a potentially useful treatment would enhance the 5-FU apoptotic pathway activity by

concomitantly down-regulating *GRP*78. Such a combination of genetic regulation of a pro-survival protein with a drug treatment that induces cellular apoptosis may be a more effective mode of CRC therapy, as CRC tumors exhibit particularly high levels of drug resistance and poor prognosis. Thus, in response to increased attention on combination anticancer therapies, our study aimed to affect cancer cell viability at both the genetic and pharmacological levels. Furthermore, we hypothesized that we would see a synergistic impact on the induction of apoptosis in LoVo CRC cell line.

Materials and Methods

Cell culture. LoVo human colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l glucose) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C and in humidified atmosphere of 95% O₂ and 5% CO₂ in an incubator.

Transfection with siRNA. LoVo cells were grown to 70% confluence in 60-mm culture plates and washed twice with phosphate-buffered saline (PBS). The cells were transfected for 48 h with 100 nM SMART pool siRNAs specific to *GRP*78 mRNA or non-targeting siRNA (scramble siRNA) using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) in serum-free DMEM, according to the manufacturer's protocols.

Cell viability assay. LoVo cells were cultured in a 96-well culture plate with 5,000 cells/well. LoVo cells were then exposed to si-*GRP*78 or scrambled si-RNA. 5-FU (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide, filter-sterilized using a 0.45-µm pore filter (Sartorius Biotech GmbH, Göttingen, Germany), and stored as 500 mg/ml stock at 4°C until use. The cells were treated with 25 µM 5-FU at 37°C for 48 h, and then cell survival assay was performed. The assay used was a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the conversion of the tetrazolium salt to formazan by mitochondrial NAD(P)H-dependent oxidoreductases. Formazan levels were quantified by measuring the absorbance at 575 nm by using a microplate reader (BMG labtech, Allmendgrün, Ortenberg Germany).

Propidium iodide/annexin V flow cytometric analysis. In order to examine the level of apoptosis of LoVo cells transfected with scrambled si-RNA or si-*GRP*78 in the presence or absence of 5-FU, cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (De Novo Software, Los Angeles, CA, USA) and evaluated using a Cyflow Cube 8 FACS (Sysmex Partec, Görlitz, Germany). Data were analyzed using standard FSC Express software (De Novo Software).

Dihydroethidium staining. Dihydroethidium (DHE; Sigma-Aldrich) was used to measure superoxide anion levels in LoVo cells transfected with scrambled si-RNA or si-*GRP*78 in the presence or absence of 5-FU. The cells were incubated with 10 µM DHE for 30 min at 37°C. After washing with PBS three times, samples were

analyzed by flow cytometry (Cyflow Cube 8 FACS; Sysmex Partec). Data were analyzed using standard FSC Express software (De Novo Software).

Western blot analysis. Total cellular protein of LoVo cells was extracted using RIPA lysis buffer (Thermo Fisher Scientific). Cell lysate containing 20 µg of protein was separated *via* 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for antibody probing. After washing with TBST (10 Mm Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6), membranes were blocked with 5% skimmed milk for 2 h and then incubated with primary antibodies against *GRP78*, phosphorylated-protein kinase-like endoplasmic reticulum kinase (PERK), total PERK, phosphorylated-eukaryotic initiation factor 2 alpha (eIF2α), total eIF2α, activating transcription factor 4 (ATF4), phosphorylated-inositol-requiring enzyme 1 alpha (IRE1α), total IRE1α, phosphorylated-c-Jun N-terminal kinases (JNK), total JNK, phosphorylated-p38, total p38, C/EBP homologous protein (CHOP), B-cell lymphoma 2 (BCL2), BCL2-associated X protein (BAX), cleaved caspase-3, cleaved poly (ADP-ribose) polymerase 1 (PARP1), and β-actin (all from Santa Cruz Biotechnology, Dallas, TX, USA). After the incubation of the membranes with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), bands were detected using enhanced chemiluminescence reagents (Amersham Biosciences, Little Chalfont, UK) in a dark room.

Statistical analysis. Results are expressed as the mean±standard error of the mean (SEM). All experiments were analyzed by one-way analysis of variance. Comparisons of results in three or more groups were made using the Bonferroni-Dunn test. Differences were considered statistically significant when the *p*-value was less than 0.05.

Results

GRP78 silencing enhanced 5-FU-induced cell death of LoVo colon cancer cells. Before evaluating potential synergistic effects of 5-FU and *GPR78* down-regulation on cancer cell viability, the efficiency of *GRP78* silencing was verified by immunoblot analyses, which demonstrated a significant reduction of *GRP78* expression in cells transfected with si-*GRP78* (Figure 1A). To assess the effects of 5-FU and *GRP78* silencing applied individually and in combination on LoVo cell viability and apoptosis, MTT and annexin V/PI apoptosis assays using flow cytometry were performed. As shown in Figure 1B, treatment with 5-FU reduced the viability of LoVo cells. Combined action of 5-FU and *GPR78* siRNA reduced cell viability even further (Figure 1B). Indeed, treatment with 5-FU significantly increased the percentage of early and late apoptotic cells to 23.83% compared to that in control cells (3.11%). In addition, in cells transfected with si-*GRP78*, early and late apoptotic cells comprised only 5.37%. The percentage of apoptotic cells was much higher in cell cultures transfected with si-*GRP78* and treated with 5-FU (42.72%) (Figure 1C and D). These results suggest that *GPR78* down-regulation in combination with the treatment with 5-FU has a strong anticancer effect in a colon cancer model.

GRP78 silencing enhanced 5-FU-induced accumulation of ROS. In order to determine the relationship between *GRP78* expression and 5-FU-induced generation of ROS, we examined the latter using DHE, a small-molecule fluorescent ROS probe specific for O₂⁻ (20), under silencing of *GRP78* expression in colon cancer cells. The reaction between O₂⁻ and DHE generates a highly specific red fluorescent product, 2-hydroxyethidium [2-OH-E(+)], shifting its excitation and emission peak wavelengths from 350 and 400 nm to 518 and 605 nm, respectively (21, 22). LoVo cells were pre-transfected with *GRP78* siRNA and then exposed to 5-FU for 12 h. DHE fluorescence intensity at 518 and 605 nm was then quantified using flow cytometry. Treatment with either 5-FU or si-*GRP78* alone increased DHE-positive cell numbers (Figure 2A and B). In addition, combined treatment with 5-FU and si-*GRP78* led to a stronger increase in the number of DHE-positive cells than in the case of both individual treatments (Figure 2A and B). These results suggest that down-regulation of *GRP78* expression has a synergistic effect on 5-FU-induced ROS accumulation.

GRP78 silencing enhanced 5-FU-induced ER stress. It is known that during ER stress, the expression of ER stress-related proteins changes in ER membrane due to the activity of PERK, IRE1α, and ATF (23). Thus, we assumed that combined exposure to 5-FU treatment and *GRP78* silencing would strongly increase expression of ER stress marker proteins in colon cancer cells. As shown in Figure 3A and B, our assumption was correct, and the exposure to the combination of 5-FU and si-*GRP78* increased PERK phosphorylation, eIF2α phosphorylation, and ATF4 expression stronger than either of the two treatments applied individually. Furthermore, as shown in Figure 4A and B, combined treatment with 5-FU and si-*GRP78* also increased IRE1α phosphorylation, p38 phosphorylation, and CHOP expression more potently than did 5-FU or si-*GRP78* alone. These results suggest that *GRP78* silencing further enhances the consequences of ER stress caused by 5-FU.

GRP78 silencing enhanced 5-FU-induced apoptosis of human colon cancer cells. As demonstrated above, *GRP78* silencing increased the number of apoptotic colon cancer cells after treatment with 5-FU. However, because cell viability and apoptosis assay data cannot provide clues to the mechanistic explanations for the sensitization to 5-FU action in the background of *GRP78* silencing, we sought to examine changes in the expression of several apoptosis-related proteins in colon cancer cells in greater detail. We reveal that incubation with 5-FU led to a decrease in the expression of anti-apoptotic protein BCL2, whereas expression levels of the pro-apoptotic proteins BAX, cleaved caspase-3, and cleaved PARP1 were increased by that treatment (Figure 5A and B). In addition, we found that

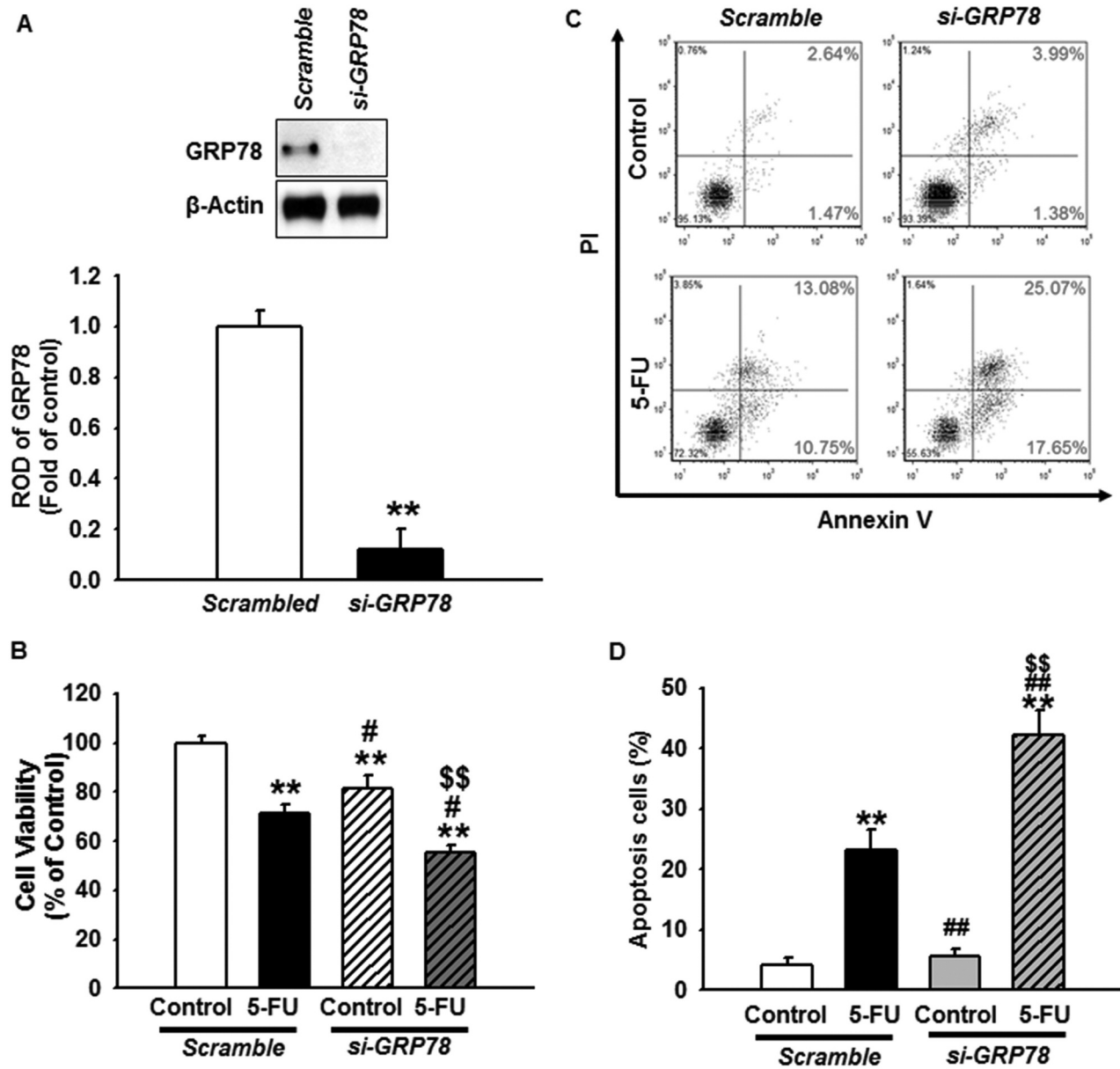


Figure 1. Effect of glucose-regulated protein (GRP78) silencing on 5-fluorouracil-induced LoVo colon cancer cell death. A: Efficacy of reduction of basal GRP78 level by GRP78 siRNA (si-GRP78) was determined by western blot. Cells were pretreated with si-GRP78 or non-targeting scrambled siRNA for 48 h. Total protein was extracted and immunoblotted with an antibody to GRP78. The amount of β -actin was used as an internal loading control. Bar graph illustrates mean (\pm SEM) normalized GRP78 levels in analyzed samples. B: LoVo cells were pre-transfected with si-GRP78 for 48 h and then treated with 25 μ M 5-fluorouracil (5-FU) for 48 h. Cell viability was measured using a modified MTT assay. Data are expressed as the mean \pm SEM. C: Apoptosis of LoVo cells was measured using propidium iodide (PI)/annexin V staining and flow cytometry. PI/annexin V double-negative cells were considered live cells, PI-negative/annexin V-positive cells were considered early apoptotic cells, and PI/annexin V double-positive cells were considered late apoptotic cells. D: Standard quantification of PI/annexin V-positive apoptotic cells. Data are expressed as the mean \pm SEM. Statistical significance is indicated as follows: ** p <0.01 vs. control, # p <0.05 and ## p <0.01 vs. scrambled siRNA+5-FU, \$\$ p <0.01 vs. si-GRP78+5-FU.

combined treatment with si-GRP78 and 5-FU caused more pronounced changes in the above-mentioned parameters than those observed in single treatment groups (Figure 5A and B).

Discussion

Anticancer drugs and GRP78 are known to modulate cancer progression and growth. In the present study, we showed that

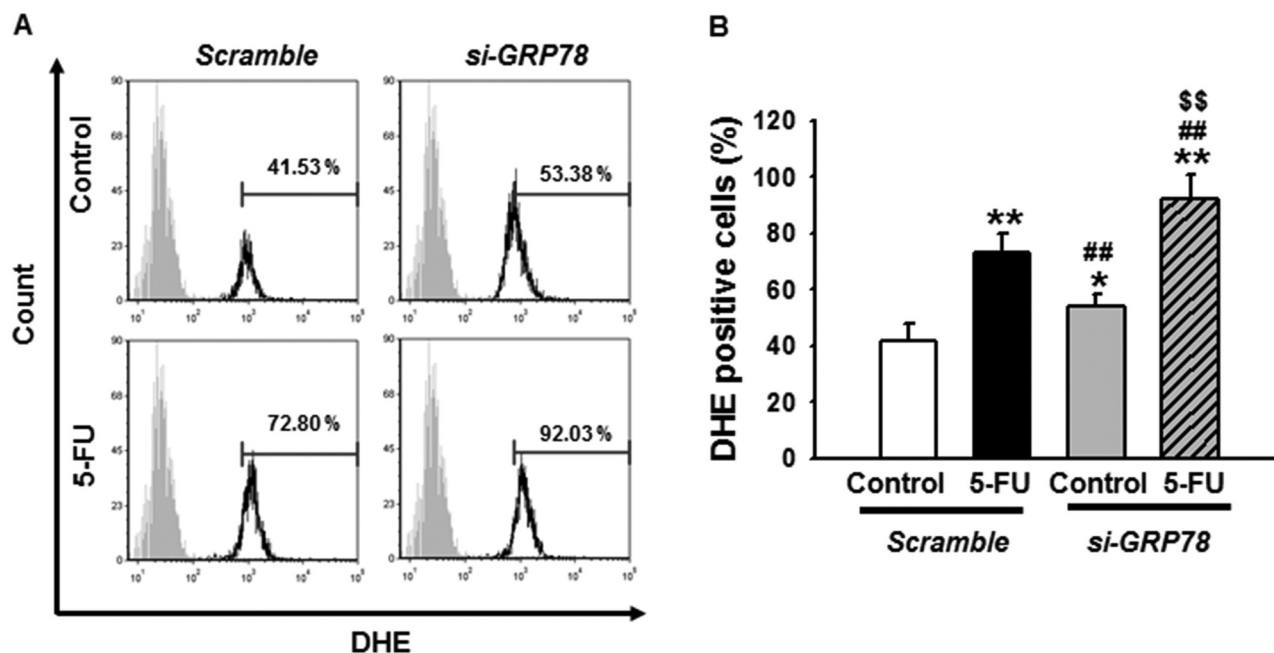


Figure 2. Glucose-regulated protein (*GRP78*) silencing enhanced 5-fluorouracil-induced reactive oxygen species accumulation in LoVo colon cancer cells. LoVo cells were pre-transfected with *GRP78* siRNA (*si-GRP78*) or non-targeting scrambled siRNA 48 h before the treatment with 25 μ M 5-fluorouracil (5-FU) for 48 h. Reactive oxygen species accumulation was measured using dihydroethidium (DHE) staining. A: Representative flow cytometry image. B: Quantitative analysis of the percentage of DHE-positive cells by flow cytometry. Data are expressed as the mean \pm SEM. Statistical significance is indicated as follows: * p <0.05 and ** p <0.01 vs. control, ## p <0.01 vs. scrambled siRNA +5-FU, \$\$ p <0.01 vs. *si-GRP78*+5-FU.

exposure to 5-FU and *GRP78* silencing have synergistic effects on the inhibition of LoVo colon cancer cell growth mediated by the induction of ER stress-dependent apoptosis.

After lung and liver cancer, CRC is the third most prevalent form of malignant cancer and the fourth leading contributor to cancer-related deaths in the world (24). It has been estimated that approximately 1.2 million new cases and 600,000 deaths per year occur as a result of CRC (24). Currently, the only viable treatment available to treat patients with CRC is surgical resection followed by chemotherapy or radiotherapy (24). Specifically, the most widely utilized chemotherapeutic drug to treat CRC is 5-FU. It is a pyrimidine-based analog that is frequently used for treatment of various malignancies, including gastrointestinal, breast, head and neck, ovarian cancer, in addition to CRC (15). However, the extent of 5-FU-induced cancer cell apoptosis is limited due to the developing resistance to the drug and its use is restricted because of harmful side-effects (25). In particular, it has been suggested that *GRP78* counteracts the cytotoxic action of chemical therapy with 5-FU by protecting cells from 5-FU-induced apoptosis and promoting resistance to this drug (10, 26). As a result, there has been an increasing interest to synergistic combination therapies, whereby multiple targets would be simultaneously affected to enhance

effects of anticancer drugs. In our study, we aimed to discover a potential synergistic therapeutic intervention that would comprise a cytotoxic treatment (5-FU) together with the modulation at a genetic level (*GRP78* silencing) to stop proliferation of human colon cancer cells. Our hypothesis was that because 5-FU is a potent anticancer drug, and *GRP78* overexpression renders cancer cells resistant to 5-FU, down-regulation of *GRP78* would enhance pro-apoptotic, anticancer effects of 5-FU.

Given the relationship between *GRP78* and 5-FU, we postulated that silencing *GRP78* in cancer cells may potentiate the activity of drug-induced pro-apoptotic pathway, that would potentially lead to cancer cell death due to increased susceptibility to apoptosis. Thus, our study aimed to test whether silencing *GRP78* expression was able to enhance 5-FU-induced inhibition of cancer cell proliferation in LoVo cells. The results obtained supported our initial hypothesis, as we demonstrate that a combination of 5-FU and *GRP78* silencing led to a higher level of CRC cell death than that observed when either 5-FU or *GRP78* silencing were applied separately (Figure 1). Statistical analysis suggested an additive effect of *GRP78* gene silencing to the cytotoxic action of 5-FU chemical treatment (Figure 1). Nevertheless, further mechanistic studies will be

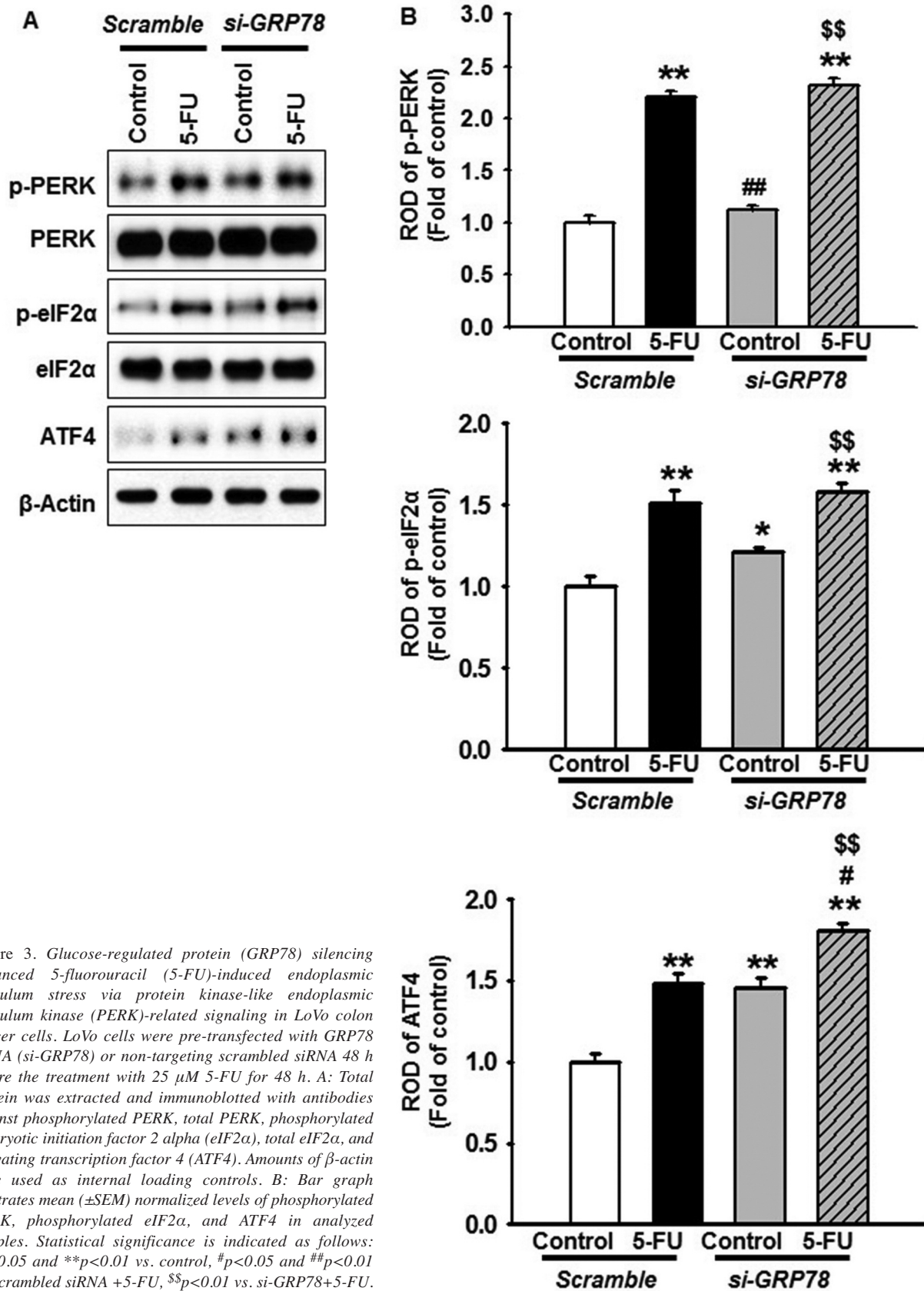


Figure 3. Glucose-regulated protein (GRP78) silencing enhanced 5-fluorouracil (5-FU)-induced endoplasmic reticulum stress via protein kinase-like endoplasmic reticulum kinase (PERK)-related signaling in LoVo colon cancer cells. LoVo cells were pre-transfected with GRP78 siRNA (si-GRP78) or non-targeting scrambled siRNA 48 h before the treatment with 25 μ M 5-FU for 48 h. A: Total protein was extracted and immunoblotted with antibodies against phosphorylated PERK, total PERK, phosphorylated eukaryotic initiation factor 2 alpha (eIF2 α), total eIF2 α , and activating transcription factor 4 (ATF4). Amounts of β -actin were used as internal loading controls. B: Bar graph illustrates mean (\pm SEM) normalized levels of phosphorylated PERK, phosphorylated eIF2 α , and ATF4 in analyzed samples. Statistical significance is indicated as follows: * p <0.05 and ** p <0.01 vs. control, # p <0.05 and ## p <0.01 vs. scrambled siRNA +5-FU, \$\$ p <0.01 vs. si-GRP78+5-FU.

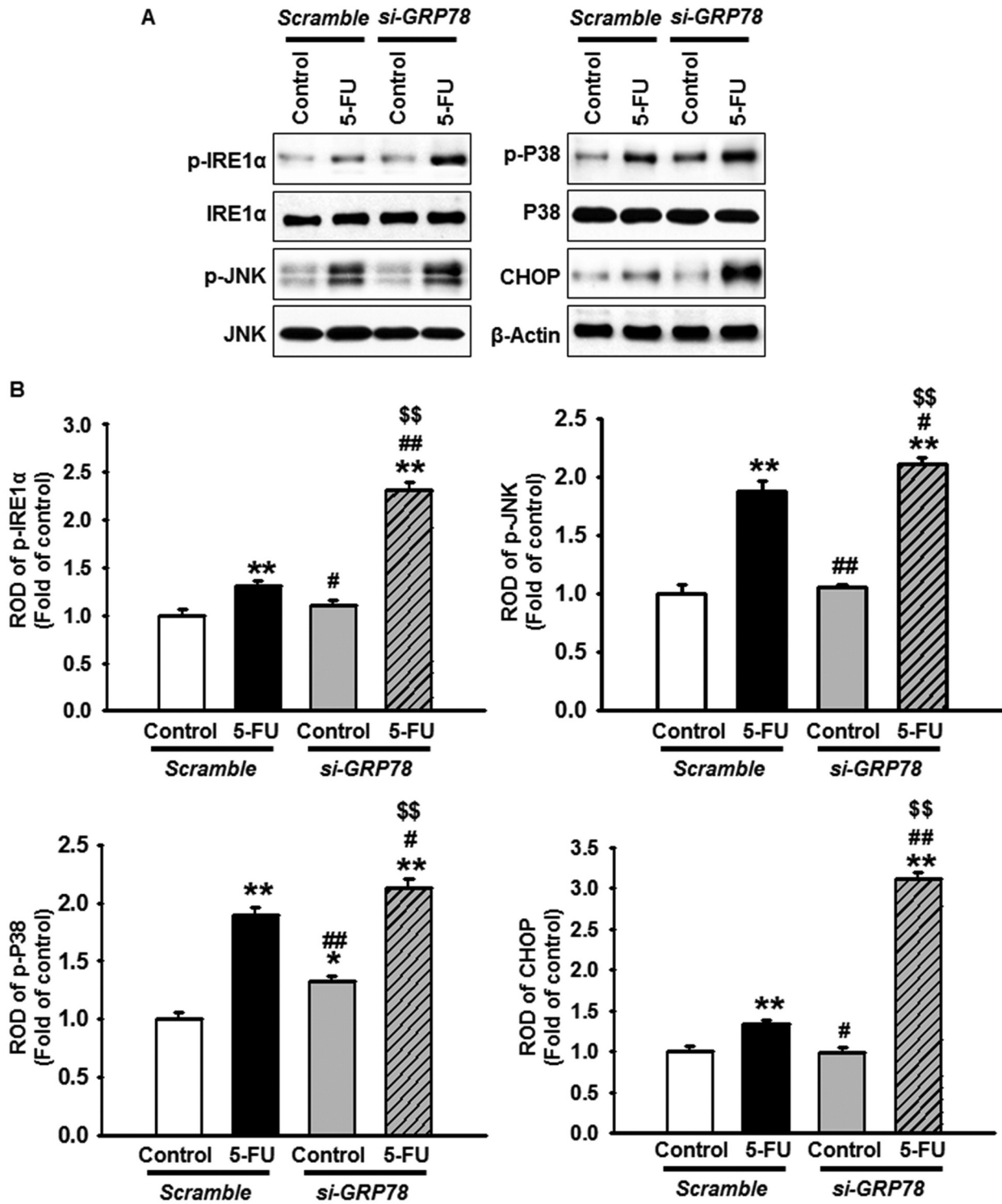


Figure 4. Glucose-regulated protein (GRP78) silencing enhanced 5-fluorouracil (5-FU)-induced endoplasmic reticulum stress via inositol-requiring enzyme 1 alpha (IRE1α)-related signaling in LoVo colon cancer cells. LoVo cells were pre-transfected with GRP78 siRNA (si-GRP78) or non-targeting scrambled siRNA 48 h before the treatment with 25 μM 5-FU for 48 h. A: Total protein was extracted and immunoblotted with antibodies against phosphorylated IRE1α, total IRE1α, phosphorylated p38, total p38, and C/EBP homologous protein (CHOP). Amounts of β-actin were used as internal loading controls. B: Bar graph illustrates mean (±SEM) normalized levels of phosphorylated IRE1α, phosphorylated p38, and CHOP in analyzed samples. Statistical significance is indicated as follows: **p*<0.05 and ***p*<0.01 vs. control, #*p*<0.05 and ##*p*<0.01 vs. scrambled siRNA +5-FU, \$\$*p*<0.01 vs. si-GRP78+5-FU.

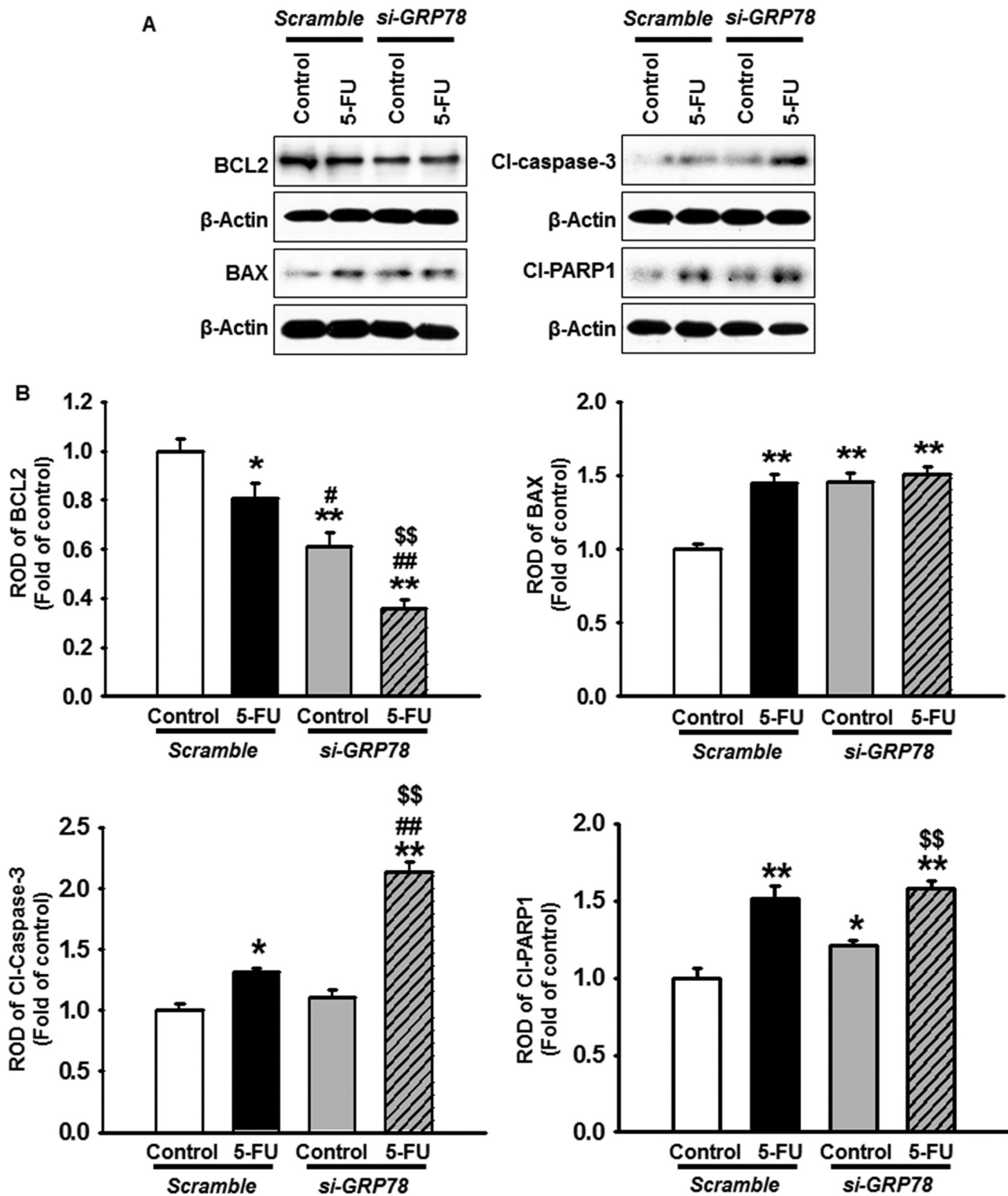


Figure 5. Effect of glucose-regulated protein (GRP78) silencing on 5-fluorouracil (5-FU)-induced LoVo colon cancer cell apoptosis. LoVo cells were pre-transfected with GRP78 siRNA (si-GRP78) 48 h before the treatment with 25 μ M 5-FU. A: Total protein was extracted and immunoblotted with antibodies against B-cell lymphoma 2 (BCL2), BCL2-associated X protein (BAX), cleaved (Cl) caspase-3, and cleaved poly (ADP-ribose) polymerase 1 (Cl-PARP1). The amount of β -actin was used as internal loading control. B: Bar graph illustrates the mean (\pm SEM) normalized levels of BCL2, BAX, cleaved caspase-3, and PARP1 in analyzed samples. Statistical significance is indicated as follows: * p <0.05 and ** p <0.01 vs. control, # p <0.05 and ## p <0.01 vs. scrambled siRNA +5-FU, \$\$ p <0.01 vs. si-GRP78+5-FU.

critical to establish the precise pathway(s) that link the effects of treatment with 5-FU and *GRP78* silencing. Several studies have demonstrated that 5-FU induces cancer cell death by increasing ROS generation, thereby producing ER stress and promoting apoptosis in cancer cells (4, 16, 27). ROS generation could be an important tool of cancer therapy due to its potential ability to inflict oxidative damage to vital biological molecules, including DNA, lipids, and proteins, altering cellular integrity and initiating apoptosis (28-31). In LoVo colon cancer cells, 5-FU negatively affected CRC growth by increasing ROS generation, causing ER stress, which, in turn, induced cell apoptosis. In addition, higher levels of *GRP78* production in cancer cells is known to produce ER-stress tolerance because *GRP78* is a calcium-binding molecular chaperone located in the ER that works to maintain ER homeostasis and suppresses stress-induced apoptosis (32, 33). Thus, our results demonstrated that in combination with 5-FU, down-regulation of *GRP78* in LoVo cancer cells led to higher generation of ROS and ER stress level (Figures 2-4). Specifically, the expression of ER stress-related proteins, such as PERK, IRE1a, and ATF became higher. Pro-apoptotic proteins BAX, cleaved caspase-3, and cleaved-PARP1 were increased in cells treated at both genetic and protein levels, suggesting that silencing of *GRP78* enhances 5-FU-induced cell apoptosis. Therefore, our results strongly suggest that a combination of 5-FU treatment and down-regulation of *GRP78* successfully enhances ROS generation, ER stress, and eventual apoptosis of LoVo cancer cell. Further studies are needed to ascertain whether alterations in apoptosis caused by 5-FU are mediated by known cell senescence mechanistic pathways specifically in LoVo cells.

Taken together, the results of our study revealed a potentially better approach for chemotherapy by establishing knockdown of *GRP78* enhances cancer cell apoptosis, ROS generation, and activation of the ER stress signaling pathway by 5-FU. Given that cells may become resistant to increased ROS levels, the induction of ER stress in human colon cancer cells by a novel combination therapy that diminishes *GRP78* expression and up-regulates ROS generation by 5-FU may not only have implications for understanding individual anticancer effects of these two treatments, but also pave the way to more effective cancer cure and better patient outcomes.

Conflicts of Interest

The Authors have no conflicts of interest to declare with regards to this study.

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