RHOT1-mediated molecular mechanism of mitochondrial dysfunction and its phenotypic effects on gastric cancer cells

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Abstract. Mitophagy, a selective autophagy process that targets damaged mitochondria, plays a critical role in cellular homeostasis and disease progression, including tumorigenesis. Ras homolog family member T1 (RHOT1), a mitochondria-associated protein, has been reported to regulate mitochondrial dynamics and energy metabolism. However, its role in gastric cancer (GC) remains unclear. The present study aimed to investigate the function of RHOT1 in GC progression and its mechanistic link with mitochondrial quality control. To achieve this, RHOT1 was silenced in GC cells and its effect on the PINK1/Parkin pathway, mitochondrial homeostasis and cellular behavior examined. The study employed qPCR and western blotting to evaluate gene and protein expression, siRNA transfection to silence RHOT1 and flow cytometry, CCK-8 proliferation, wound-healing, and Transwell assays to investigate mitochondrial function and cellular phenotypes. Silencing RHOT1 reduced *PINK1* mRNA expression by 59.75% (P=0.025) and *Parkin* mRNA expression by 65.12% (P=0.0189), indicating suppressed mitophagy. This was accompanied by an 84.73% increase in reactive oxygen species (P<0.001) and a 36.94% decrease in mitochondrial membrane potential (P=0.0061). Silencing RHOT1 further caused G_0/G_1 phase arrest and increased apoptosis (P<0.05), thereby markedly inhibiting the proliferation, invasion and migration of GC cells. The present study revealed that RHOT1 drives the malignant phenotype of GC through regulation of mitochondrial quality control and induction of oxidative stress, providing a rationale for developing novel anti-tumor strategies by targeting mitochondrial function. RHOT1 may serve as a biomarker for prognostic assessment and individualized treatment of GC.

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Introduction

With an aging population and the continuation of organized cancer screening programs, gastric cancer (GC) has shown a consistent trend of increasing morbidity and mortality (1,2). GC has a significant demographic disparity, with male prevalence rates nearly twice that of females (1,3). Geographical variations are also evident, with disproportionately high incidence rates in East Asia (particularly Japan and Mongolia) and Eastern Europe, which together account for 87% of GC cases worldwide (1,3). GC has thus become a global public health challenge and even surgically treated patients, such as those with gastric stump cancer, still face a considerable disease burden and poor outcomes (4). The low detection rate of early-stage GC can be attributed to the lack of specific clinical symptoms (5). GC screening can detect precancerous lesions and early-stage GC in asymptomatic patients, reducing mortality and improving treatment efficacy (6). The majority (>70%) of patients are diagnosed at advanced stages, mainly due to the absence of clinical symptoms during early pathogenesis (7). This delay in diagnosis contributes to a poor prognosis and the clinical management of GC presents obvious challenges. Despite the availability of chemotherapy and radiotherapy, treatment outcomes for advanced gastrointestinal malignancies, such as

pancreatic and GC, remain unsatisfactory (8). Patients diagnosed at a late stage have a poor prognosis without targeted treatment (2). Traditional Chinese Medicine (TCM)-derived polysaccharides, such as *Rhizoma Coptidis* polysaccharides and *Anemarrhena asphodeloides* polysaccharides, have been explored as adjuncts in GC management (9,10). To address these clinical challenges, there is a pressing need to improve our understanding of the pathogenesis of GC. Therefore, it is necessary to elucidate the mechanisms associated with the pathogenesis and progression of GC and to explore potential targets for effective treatment. Such discoveries are essential for the development of more effective treatment strategies and improved survival outcomes.

Mitochondria are vital for cellular homeostasis concerning energy production, calcium regulation and apoptosis. Their dysfunction has been widely reported to be associated with neurodegenerative diseases, diabetes and chronic pulmonary conditions (11-14). Mitophagy, the selective degradation of defective mitochondria, is essential for maintaining cellular homeostasis and influencing various cancer-related pathways. In various types of cancer, including bladder and colorectal, mitophagy has been linked to survival and therapy resistance. The influence of mitophagy on tumor microenvironments and immune responses has the potential to facilitate tumor growth (15-17). Conversely, the progression of breast cancer is inhibited by Urolithin A, which activates transcription factor EB (TFEB)-mediated mitophagy in tumor macrophages (18). Esophageal cancer was associated with a reduction in mitophagy, which has been linked to an increased potential for metastasis (19). These results indicate that mitophagy inhibition may facilitate tumor spread. Consequently, the manipulation of mitophagy pathways may present a novel therapeutic strategy with the potential to enhance treatment outcomes in cancer management.

The Ras homolog family member T1 (*RHOT1*) gene encodes a mitochondrial GTPase that plays a crucial role in various processes within mitochondria, including mitochondrial transport, mitochondrial calcium buffering and mitophagy. Its role in disease pathogenesis, particularly neurodegenerative disorders and metabolic conditions, has been well-documented. For instance, the dysfunction of RHOT1 has been demonstrated to disrupt Parkin-mediated mitophagy, leading to the accumulation of damaged mitochondria and exacerbating neuronal loss in Parkinson's disease (20). Additionally, the dysfunction of RHOT1 has been observed to impede calcium handling and mitochondrial transport, thereby contributing to metabolic dysregulation in diabetes (11). *RHOT1* plays a pivotal role in maintaining mitochondrial quality control and has been identified as essential for preserving mitochondrial integrity (20). In GC, increasing research has identified mitochondrial dysfunction as a pivotal factor driving tumor progression, therapy resistance and metabolic reprogramming (21-24). Peng *et al* (25) investigated the correlation between the expression of RHOT1 and the clinicopathological features of tumor-node-metastasis staging and lymph node metastasis. RHOT1 has been thought to serve as a biomarker for GC. However, to date, no studies have reported the specific role of RHOT1 in the regulation of mitophagy in GC. Its conserved function in mitochondrial quality control suggests that aberrant RHOT1 activity may influence tumors by regulating mitophagy. Consequently, the investigation of RHOT1 in GC cells has the potential to reveal novel associations between mitochondrial dynamics and oncogenesis, thus providing potential therapeutic targets for diseases associated with mitochondrial dysfunction.

The present study hypothesized that RHOT1 may affect the behavior of GC cells through mitophagy. It aimed to investigate the role of RHOT1 in regulating mitochondrial quality control, energy metabolism and mitophagy-related signaling in GC. Furthermore, its effect on the proliferation, invasion and migration of GC cells *in vitro* was examined. These findings may provide a theoretical basis for considering RHOT1 as a potential alternative therapeutic target for GC.

Materials and methods

Cell lines and cell culture. The cell lines used were HGC-27, MKN-45, AGS, SNU-1 and GES-1 (human gastric cancer cell lines and a human gastric epithelial cell line, respectively) obtained from Wuhan Servicebio Technology Co., Ltd. (cell batch: IM-H084202303). They were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Clark Bioscience), 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Total RNA extraction and reverse transcription-quantitative (RT-q) PCR. RNA Easy Fast Tissue/Cell Kit (Tiangen Biotech Co., Ltd.) was prepared for total RNA extraction according to the manufacturer's instructions under RNase-free conditions. Total RNA was extracted from GC cell lines (HGC-27, MKN-45, AGS, SNU-1) and GES-1 after transfection or treatment, depending on the experimental group. PrimeScript RT Master Mix Kit (Takara Biotechnology Co., Ltd.) was used for reverse transcription and the SYBR Green Master Mix Kit (Takara Biotechnology Co., Ltd.) was employed to detect the relative expression of mRNAs. The reaction conditions were performed under the following conditions: 95˚C for 2 min, 95˚C for 15 sec, 60˚C for 1 min and 72˚C for 30 sec for 40 cycles. Primer sequences are presented in Table I. All primers were designed based on *Homo sapiens* RefSeq/NCBI sequences and targeted exon-exon junctions to ensure specificity for mRNA. GAPDH was used to normalize the gene being tested. All primers were synthesized by Sangon Biotech Co., Ltd. and validated by melt curve analysis and agarose gel electrophoresis to confirm specificity. Each sample was run in triplicate using the 7500 Fast (Applied Biosystems; Thermo Fisher Scientific, Inc.). Gene expression results were expressed as fold changes relative to GAPDH using the $2^{-\Delta\Delta Cq}$ method (26).

Short interfering (si)RNA transfection. Target-specific siRNA (si-RHOT1) (Table II) and a negative control siRNA (si-NC) were transfected into HGC-27 cells cultured in six-well plates. Cells were transfected with 20 μM siRNA using Lipo 6000 transfection reagent (Beyotime Institute of Biotechnology) and then cultured for 48 h at 37˚C. The silencing efficiency was evaluated by qPCR. The si-NC used in the present study was a non-targeting control siRNA purchased from Shanghai

Table I. Primer information.

Gene	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)	RefSeq/NCBI
RHOT1	CTGATTTCTGCAGGAAACACAA	GCAAAAACAGTAGCACCAAAAC	142	NM_001033566.3
PINK1	GTGGACCATCTGGTTCAACAGG	GCAGCCAAAATCTGCGATCACC	110	NM_032409.4
Parkin	CCAGAGGAAAGTCACCTGCGAA	CTGAGGCTTCAAATACGGCACTG	125	NM_004562.3
Tomm20	CGACCGCAAAAGACGAAGTGAC	GCTTCAGCATCTTTAAGGTCAGG	130	NM_007019.5
Timm23	ACACGAGGTGCAGAAGATGACC	CTGTCAGACCACCTCGTGCTAT	115	NM_006991.3
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG	101	NM_002046.7

Table II. siRNA sequences.

siRNA	Sense (5'-3')	Antisense (5'-3')
RHOT1-homo-393	CCAACACACAUUGUAGAUUTT	AAUCUACAAUGUGUGUUGGTT
RHOT1-homo-1384	GCUAUCUAGGCUAUUCAAUTT	AUUGAAUAGCCUAGAUAGCTT
RHOT1-homo-1804	GCUUAAUCGUAGCUGCAAATT	UUUGCAGCUACGAUUAAGCTT
RHOT1-homo-1174	CCUUUGACAAGCAUGAUUUTT	AAAUCAUGCUUGUCAAAGGTT

si-, short interfering; RHOT1, Ras homolog family member T1.

GenePharma Co., Ltd. and its sequence was not disclosed by the manufacturer.

CCK-8 proliferation assay. Cell counting kit 8 (CCK-8; MedChemExpress) was used for the detection of proliferation of HGC-27 cells. Cells (~2x10^3) were seeded in 100 μl of medium in a 96-well plate and incubated at 37˚C. CCK-8 reagent (10 μl) was added to each well at 0, 24, 48, 72 and 96 h. Incubation was performed at 37˚C for 2 h. A microplate reader (Molecular Devices, LLC) was used to read OD values at 450 nm. Blank control wells containing medium and CCK-8 reagent without cells were included and their absorbance values were subtracted from sample readings to eliminate background interference. Cell proliferation results were expressed as OD values at 450 nm, normalized to control wells.

Wound healing assay. A wound-healing assay was used to evaluate the migration ability of HGC-27 cells. Cells were cultured at 3x10^5/ml per well in a 12-well plate. After 6 h of incubation, 1 ml of cell medium was added to the 12-well plate. A 20 μl pipette tip was used to create a wound and PBS was used to wash the slide 2-3 times. Then serum-free medium was prepared and the images were captured under the microscope at 0, 24 and 48 h. The experiment was performed three times.

Transwell assay. For the Transwell migration assay, 200 μl of cell suspension was transferred to a Transwell upper chamber (Corning, Inc.) and 500 μl of medium with 20% FBS was transferred to a Transwell lower chamber and incubated with 5% CO_2 in a 37˚C incubator for 48 h. Finally, 50 μl of the diluted cell suspension was added to the upper chamber of the Transwell (Corning, Inc.) and incubated at 37˚C for 1 h. For the invasion assay, a layer of Matrigel matrix glue (Corning, Inc.; ratio of serum-free medium:matrix glue 8:1) was coated within the lower chamber, and the cells were incubated for 24 h at 37˚C. After incubation, a 4% paraformaldehyde solution was used to fix the cells in the Transwell. The Transwell was subsequently stained with 0.1% crystal violet for 20 min. Cell counts for migration and invasion were recorded by bright-field microscopy. Three biological replicates were performed. The migration and invasion abilities were expressed as the number of stained cells counted under the microscope.

Flow cytometry. Flow cytometry was performed to evaluate the cell cycle and apoptosis of the cells. HGC-27 cells were stained with propidium iodide (PI; cat. Y267501; Beyotime Institute of Biotechnology) for the cell cycle assay and Annexin V and PI (Annexin V; cat. C1062M; Beyotime Institute of Biotechnology) were used for the apoptosis assay. Cells were stained with PI for cell cycle and Annexin V and PI for apoptosis at 37˚C for 30 min. FACSCalibur and FACSCelesta (BD Biosciences) were used for cell cycle and apoptosis detection, respectively. The flow cytometer was operated with a 488 nm excitation laser at standard power. For cell cycle analysis, gating was based on PI fluorescence intensity to distinguish populations with different DNA content, corresponding to G_0/G_1, S and G2/M phases. For apoptosis analysis, gating was performed on Annexin V/PI two-parameter dot plots to classify cells as viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), or late apoptotic/necrotic (Annexin V+/PI+). Three biological replicates were performed. Cell cycle distribution was expressed as the percentage of cells in G_0/G_1, S and G_2/M phases and apoptosis results were expressed as the percentage of apoptotic cells.

Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). ROS were detected using the

Reactive Oxygen Specific Test Kit (Beyotime Institute of Biotechnology). ROS levels were measured using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). HGC-27 cells ($1x10^6$) were divided equally. In the positive control group, 1 μl of Rosup stimulating drug was added and incubated at 37˚C for 30 min. The treatment group was directly treated with 10 μM DCFH-DA dissolved in serum-free culture medium (1 ml) and incubated at 37˚C for 30 min. Fluorescence was detected by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm and changes in MMP were detected using the Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology). The HGC-27 cells were harvested by centrifugation at 1,000 x g for 5 min at 37˚C and resuspended in 50 mM phosphate-buffered saline (pH 7.0). A total of $5x10^4$ cells collected by centrifugation were resuspended in 188 μl Annexin V-FITC conjugate. Then 2 μl Mito-Tracker Red CMXRos was added and incubated at 25˚C for 30 min and the cells placed in an ice bath. Fluorescence was detected by flow cytometry using an excitation wavelength of 579 nm and an emission wavelength of 599 nm. ROS levels were expressed as mean fluorescence intensity and MMP was expressed as fluorescence intensity. The experiment was performed three times.

Bioinformatics analysis. The String website (https://cn.string-db.org/) was used to retrieve target genes for analysis of protein-protein interactions (PPI). The species was limited to *Homo sapiens* and the parameters were set as follows: Choice of significance term for network edges; evidence: Choice of active interaction source; experimental: Choice of minimum required interaction score; confidence: 0.2.

After downloading the enriched gene set, de-duplication of the gene set was carried out to obtain the set of interacting genes. The final PPI network map was calculated by selecting the top 10 pivotal genes in the Hubba node using the Cytoscape (https://cytoscape.org/; version 3.8.0) plugin cytoHubba. R software (https://www.r-project.org/; version 3.6.3) was used to analyze the RHOT1 mRNA expression levels of STAD RNA-seq based on The Cancer Genome Atlas (TCGA) database and a single-gene co-expression heatmap was constructed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of selected data were performed using the clusterProfiler package (https://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html; version 3.14.3) and visualized using the ggplot2 package (https://cran.r-project.org/web/packages/ggplot2/index.html; version 3.3.3). The intersection of mitophagy genes obtained from the Reactome database (https://curator.reactome.org; version 87) with those obtained from the KEGG database was taken and plotted as a Venn diagram. The expression matrix of 20 mitophagy pathway core genes was screened based on the intersection of mitophagy pathways from KEGG and Reactome. Principal component analysis (PCA) was performed (after normalization) using Omicstudio (https://www.omicstudio.cn/tool) and the correlation heatmap of these core genes was plotted.

Western blotting. Total proteins from transfected HGC-27 cells were isolated by RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) and then separated by 8-11% SDS-PAGE. Protein concentration was determined using the BCA protein assay kit (cat. no. WLA019; Wanleibio Co., Ltd.). The 20 μg protein samples were dissolved and transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes (cat. no. IPVH00010; MilliporeSigma). The PVDF membranes were blocked with 5% skimmed milk powder for 2 h at room temperature and then incubated with primary antibodies at 4˚C overnight. The antibodies were RHOT1 (cat. no. A5838; 1:500; ABclonal Biotech Co., Ltd.), PINK1 (cat. no. WL04963; 1:500; Wanleibio Co., Ltd.), Parkin (cat. no. WL02512; 1:500; Wanleibio Co., Ltd.), GAPDH (cat. no. 5174, 1:1,000; Cell Signaling Technology, Inc.). The PVDF membranes were then washed with tris-buffered saline-Tween 20 (0.1% TBST-20; cat. GC204002; Wuhan Servicebio Technology Co., Ltd.) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. WLA023; 1:5,000; Wanleibio Co., Ltd.) for 1.5 h at room temperature. The membranes were scanned and analyzed using the Gel-Pro Analyzer 4.5 program for Windows (Media Cybernetics, Inc.). Three biological replicates were performed. Protein expression levels were expressed as band intensities normalized to GAPDH.

Statistical analysis. The data were presented as the mean ± standard deviation. The findings from each experiment were corroborated through independent replicates. Normality of data distribution was assessed using the Shapiro-Wilk test prior to parametric analyses. For comparisons between two groups, a two-tailed unpaired Student's t-test was applied. For comparisons among multiple groups with a single variable, one-way ANOVA followed by the Tukey-Kramer post hoc test was used. For datasets involving more than one variable, two-way ANOVA with appropriate post-hoc analyses was performed. SPSS (version 29.0; IBM Corp.) and GraphPad Prism (version 9.5.0; Dotmatics) were used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RHOT1 is involved in mitophagy via PINK1/Parkin pathway. To investigate the function of RHOT1, the interacting proteins of RHOT1 were investigated from the STRING search sets. Analysis of RHOT1-interacting proteins identified *RHOT2, LRRK2, GRID2IP, PLEKHA4* and *CIT* as hub genes (Fig. 1A). Single-gene co-expression analysis using TCGA-STAD RNA-seq data showed that *RHOT2, LRRK2, GRID2IP* and *CIT* were significantly co-expressed with RHOT1 ($P<0.001$; Fig. 1B). GO and KEGG enrichment analysis indicated involvement in mitochondrion organization, mitophagy, microtubule binding and pathways including Parkinson's disease and neurodegeneration (Fig. 1C and D).

A Venn diagram identified 20 mitophagy-related genes from Reactome and KEGG databases (Fig. 1E) and PCA showed significant differences between tumor and normal groups (Fig. 1F). Next, a correlation heatmap was constructed from the 20 mitophagy genes (Fig. 1G). The correlation heatmap displayed the genes with a statistically significant difference $P<0.01$ (Fig. 1H). Intersection of *RHOT1*-interacting genes and mitophagy genes revealed *PINK1* and *Parkin* (Fig. 1I). *RHOT1* expression was upregulated in GC cell lines HGC-27, MKN-45, AGS and SNU-1 compared with GES-1, with HGC-27 showing

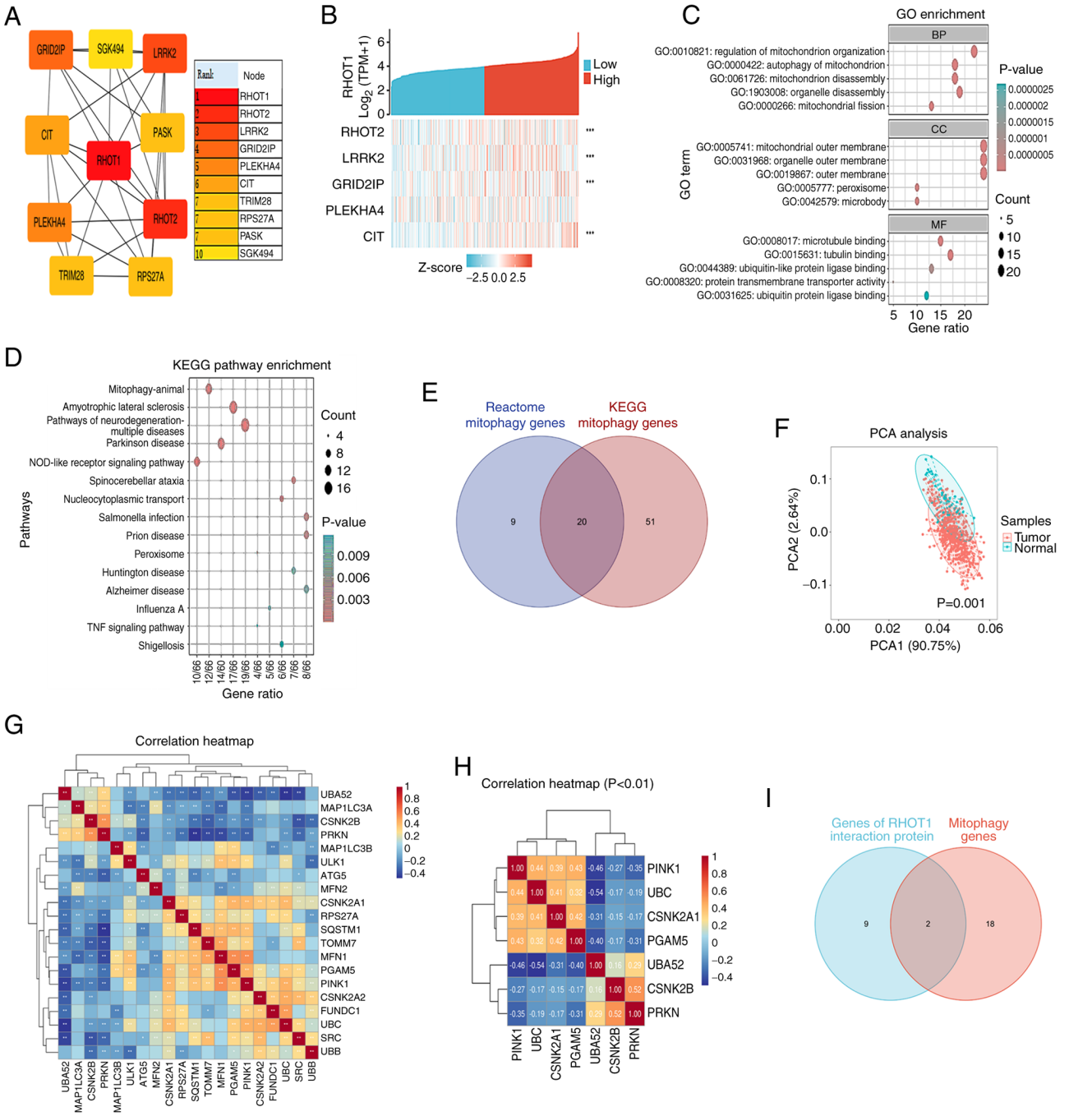

Figure 1. Bioinformatics analysis of RHOT1 involvement in the mitophagy pathway. (A) The interacting proteins of RHOT1. (B) The heatmap of the interacting genes co-expressed with RHOT1. (C) GO analysis of RHOT1. (D) KEGG analysis of RHOT1. (E) Venn diagram analysis of mitophagy genes from different databases. (F) PCA analysis was conducted on mitophagy genes. (G) Heatmap and (H) correlation analysis of mitophagy genes. (I) Venn diagram analysis of STRING interaction genes, taking the intersection with mitophagy genes. ***P<0.001. RHOT1, Ras homolog family member T1; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

the highest expression (Fig. 2A). Among four siRHOT1 constructs, siRNA RHOT1-homo-393 achieved the highest silencing efficiency (Fig. 2B). Subsequently, the mRNA expression levels of the genes were examined. The result showed that the mRNA expression of *PINK1* and *Parkin* were both downregulated after silencing RHOT1, with *PINK1* decreased by 59.75% (P=0.025) and *Parkin* decreased by 65.12% compared with the si-NC group (P=0.0189) (Fig. 2C and D). Additionally, the mRNA expression of mitochondrial membrane-related genes *TOMM20* (translocase of the outer mitochondrial membrane 20) and *TIMM23* (translocase of the inner mitochondrial membrane 23) were increased after silencing RHOT1 (Fig. 2E and F). The protein expression of PINK1 and Parkin were downregulated after silencing RHOT1 (Fig. 2G).

These results indicated that silencing RHOT1 leads to decreased PINK1 and Parkin expression and inhibition of mitophagy.

Silencing RHOT1 affects ROS and MMP in GC cells. Based on the aforementioned results for PINK1, Parkin

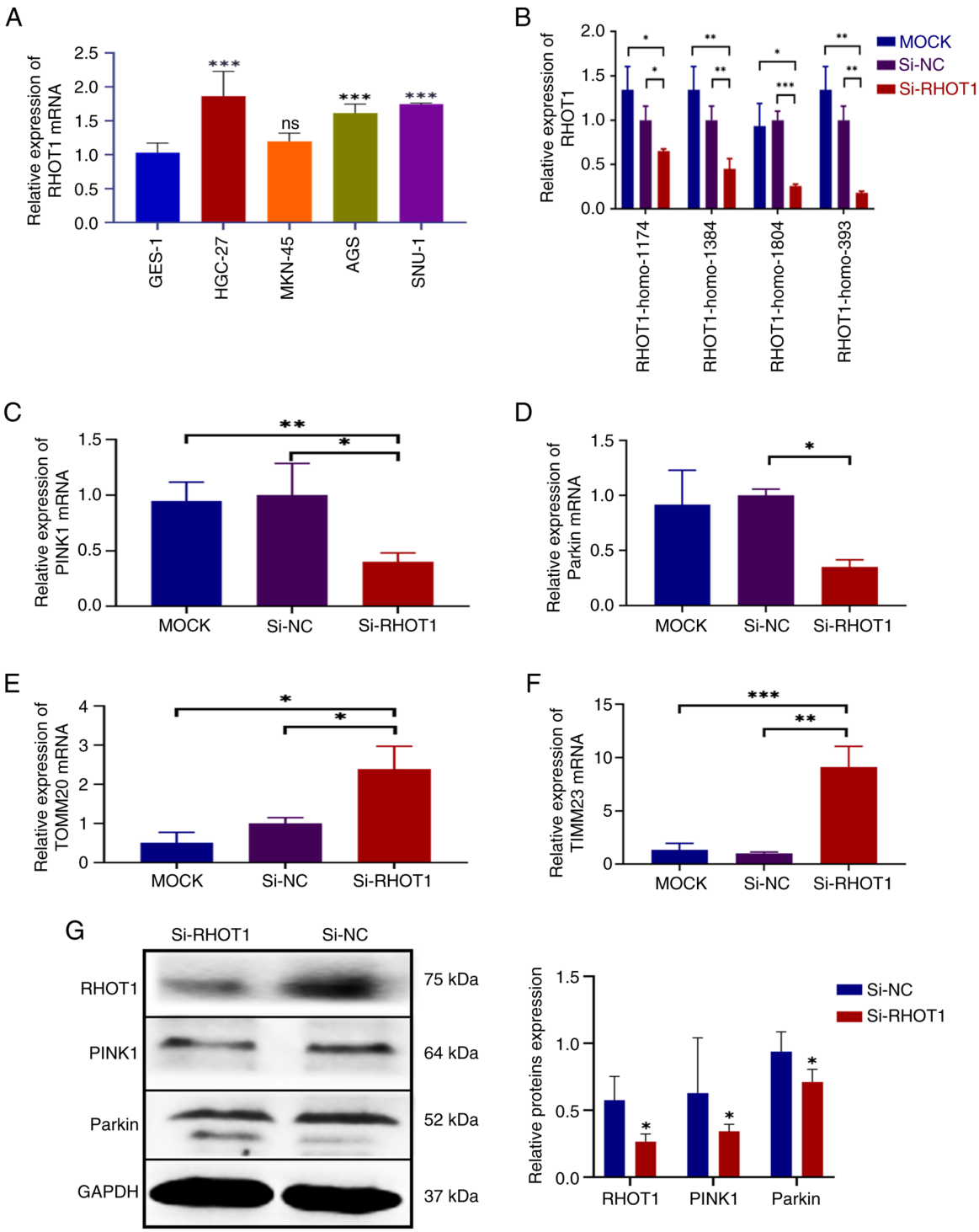

Figure 2. Expression analysis of mitophagy-related genes. (A) The relative expression of RHOT1 in GC cell lines. (B) The expression of RHOT1 transfected with different specific si-RHOT1. The relative expression of (C) PINK1 and (D) Parkin after silencing RHOT1. The relative expression of (E) TOMM20 and (F) TIMM23 after silencing RHOT1. (G) Protein expression changes of PINK1 and Parkin after silencing RHOT1. *P<0.05, **P<0.01, ***P<0.001, ns, not significant. RHOT1, Ras homolog family member T1; GC, gastric cancer; si-, short interfering; NC, negative control.

and mitochondrial membrane-related genes, mitochondrial function was further examined to assess mitophagy. Flow cytometry analysis showed that the average ROS fluorescence intensity in HGC-27 cells was increased by 84.73% (P<0.001) after silencing RHOT1 compared with the negative control, while it remained lower than the positive control (Fig. 3A). MMP assessment indicated that silencing RHOT1 caused a 36.94% decrease in MMP compared with the negative control (P=0.0061), reflecting mitochondrial depolarization (Fig. 3B).

These results demonstrate that silencing RHOT1 elevates ROS levels and depolarizes the mitochondrial membrane, indicating mitochondrial damage and dysfunction in HGC-27 cells.

Silencing RHOT1 inhibits the proliferation of GC cells arrests the cell cycle and promotes cell apoptosis. Mitochondria were

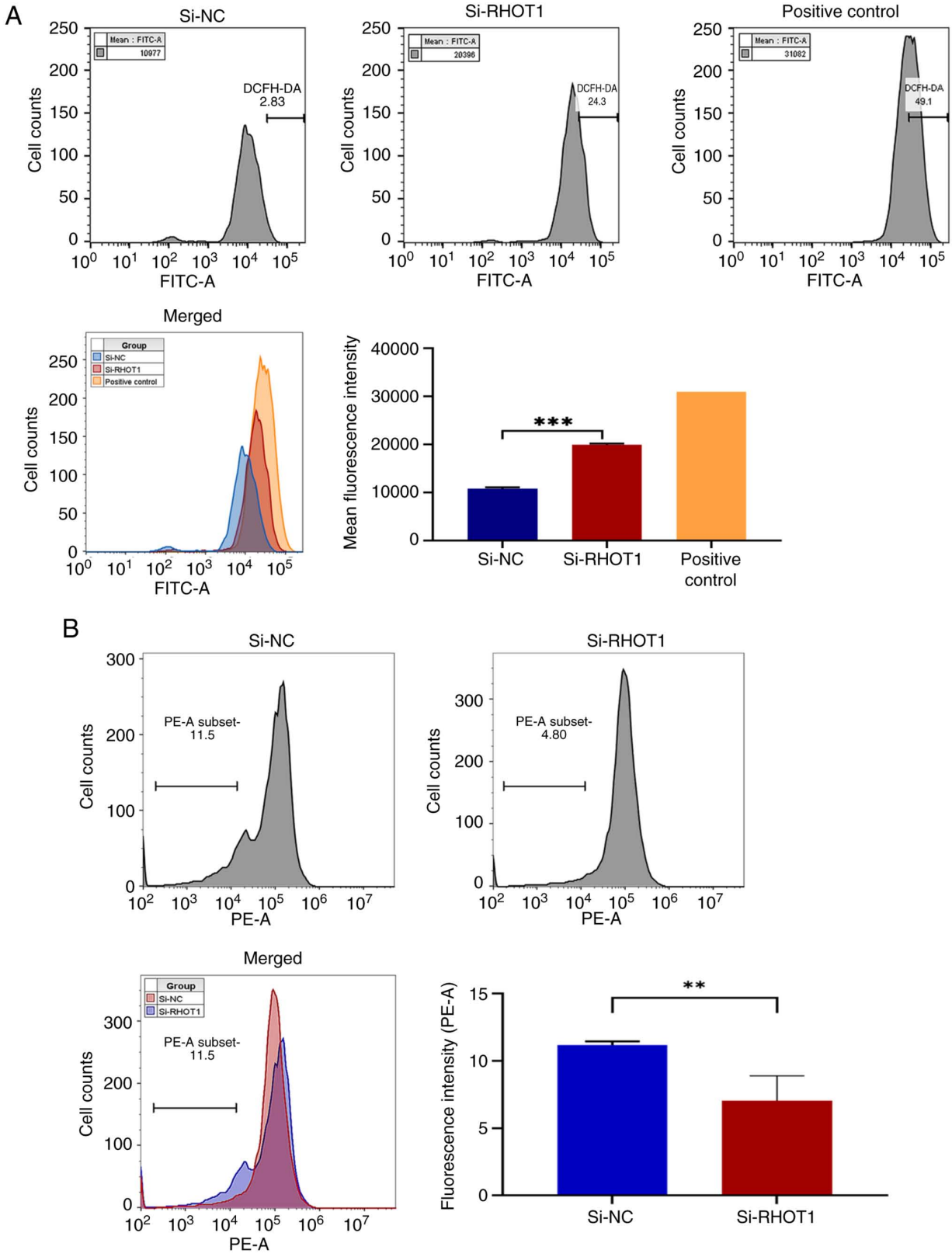

Figure 3. Changes in the level of cellular ROS and MMP after silencing RHOT1. (A) Changes in ROS levels after silencing RHOT1 in HGC-27 cells. (B) Changes in MMP after silencing RHOT1 in HGC-27 cells. **P<0.01, ***P<0.001, ROS, reactive oxygen species; MMP, mitochondrial membrane potential; RHOT1, Ras homolog family member T1; si-, short interfering; NC, negative control.

damaged and dysfunctional, which would cause abnormal cellular energy metabolism and affect cell growth. The effect of silencing RHOT1 on the growth state and apoptosis of GC cells was assessed in HGC-27 cells. CCK-8 assay showed that silencing RHOT1 markedly slowed HGC-27 cell proliferation from 48 h to 96 h compared with the si-NC group (Fig. 4A). Flow cytometry analysis revealed that silencing RHOT1 increased the proportion of cells in G_0/G_1 phase, causing cell

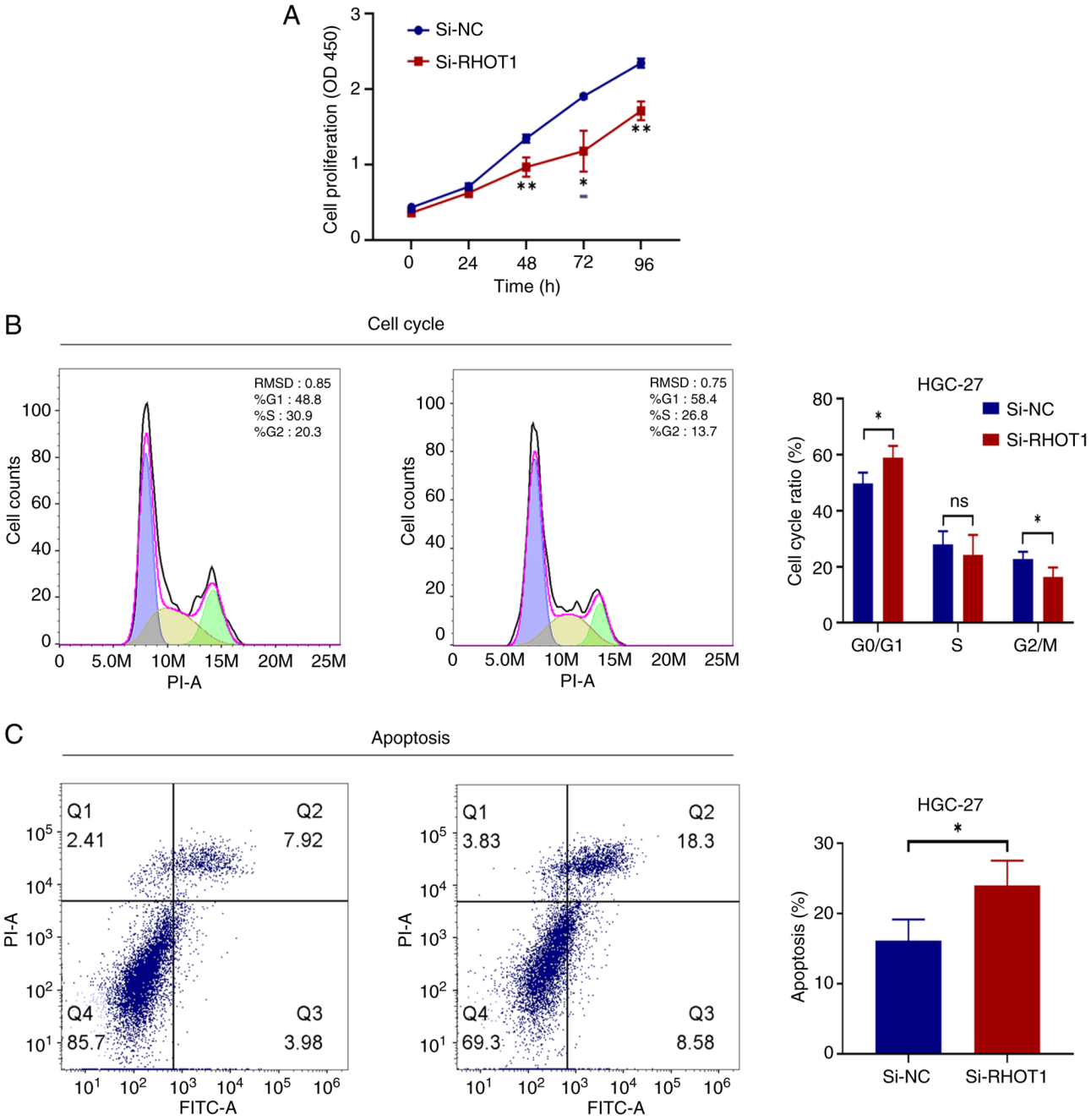

Figure 4. RHOT1 expression affected proliferation, cell cycle and apoptosis. (A) The CCK-8 assay assessed the changes in the proliferation of HGC-27 cells with silenced RHOT1. (B) Flow cytometry was performed to detect the cell cycle of HGC-27 cells. (C) Flow cytometry was used to detect the level of apoptosis in HGC-27 cells. The data is presented as mean ± SD. *P<0.05, **P<0.01, ns, not significant. RHOT1, Ras homolog family member T1; si-, short interfering; NC, negative control.

cycle arrest. Specifically, the G_0/G_1 phase proportion was markedly higher than the si-NC group (P<0.05) (Fig. 4B). Apoptosis assessment demonstrated that silencing RHOT1 elevated the percentage of apoptotic cells compared with the si-NC group (P<0.05) (Fig. 4C).

These results indicated that silencing RHOT1 affected mitophagy to reduce the proliferation of GC cells, arrest the cell cycle in the G_0/G_1 phase and promote cell apoptosis.

Silencing RHOT1 inhibits the migration and invasion of GC cells. The present results showed that the expression of *PINK1* and *Parkin* was downregulated after silencing RHOT1, so RHOT1 may affect mitophagy, leading to the accumulation of damaged mitochondria, which further increased ROS and depleted energy in the cells and consequently affected the invasion and migration phenotypes of the cells. The effects of RHOT1 on the invasion and migration of GC cells were subsequently investigated. Wound healing and Transwell migration assays were employed to evaluate the migration ability of HGC-27 cells. Wound healing assay showed that silencing RHOT1 markedly reduced the migration of HGC-27 cells compared with the si-NC group (Fig. 5A); Transwell migration assay confirmed that the number of migrating cells in the si-RHOT1 group was markedly lower than in the si-NC

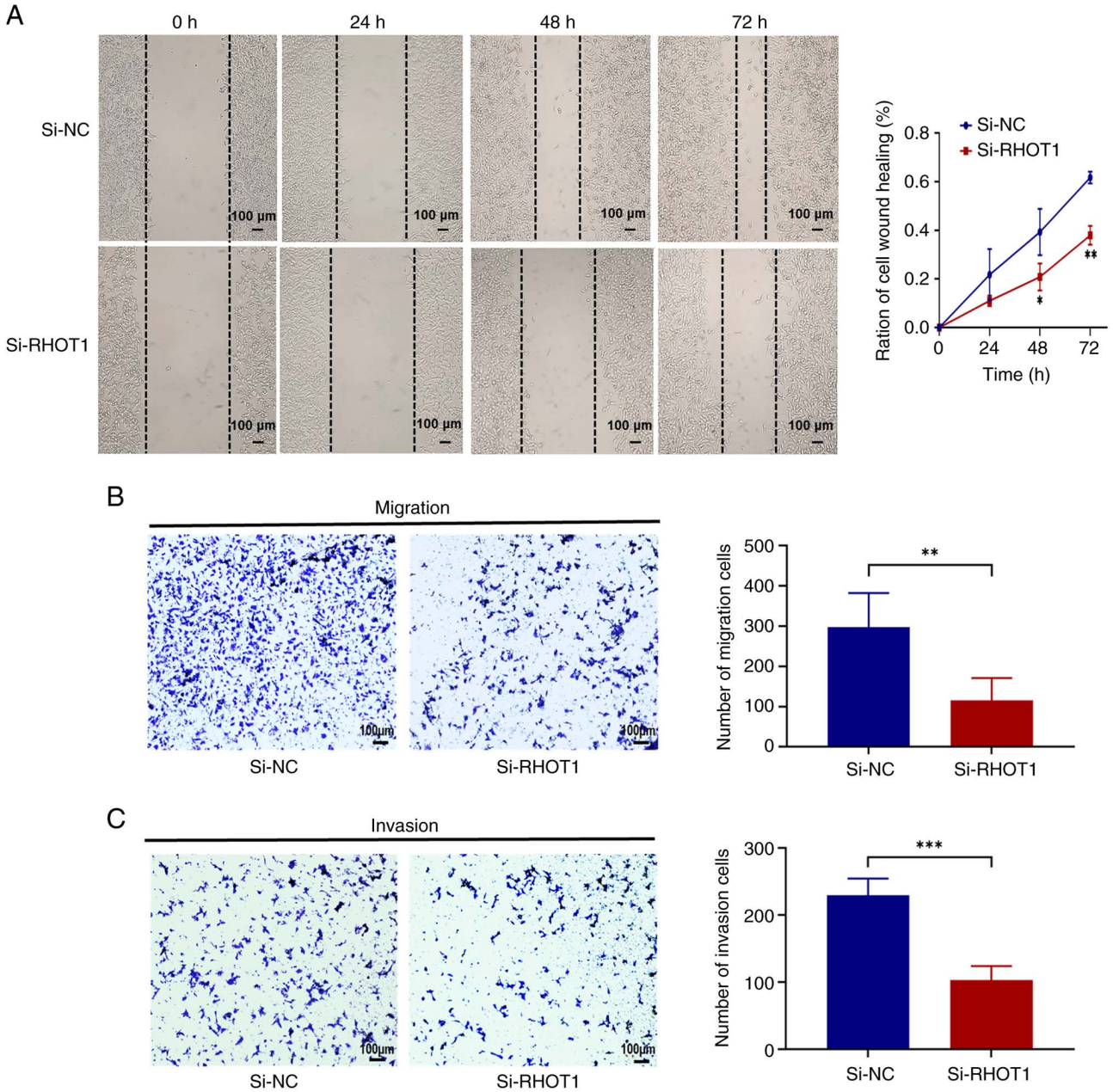

Figure 5. The RHOT1 expression affects cell migration and invasion. (A) The wound healing assay to detect the migration ability of HGC-27 cells with silenced RHOT1. (B) The Transwell migration assay was used to assess the migration ability of HGC-27 cells with silenced RHOT1. (C) The Transwell invasion assay was performed to assess the invasion of HGC-27 cells with silenced RHOT1. The data is presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. RHOT1, Ras homolog family member T1; si-, short interfering; NC, negative control.

group (P<0.05) (Fig. 5B). Transwell invasion assay further demonstrated that silencing RHOT1 markedly reduced the number of invasive HGC-27 cells through Matrigel compared with the control group (P<0.05; Fig. 5C).

These results indicated that silencing RHOT1 affected mitophagy to reduce the migration and invasion of GC cells.

Discussion

GC is the fifth most common cause of cancer-related death worldwide, contributing to 8.8% of all such deaths (27). Screening programs have reduced mortality in some cancers by detecting early-stage and precancerous lesions in asymptomatic patients, but organized GC screening has only been implemented in a few high-prevalence countries (1). The etiology of GC is complex and multifactorial, with numerous molecular alterations influencing tumorigenesis and progression through aberrant gene expression or protein dysfunction (28). At present, there remains a necessity to explore treatment targets and effective biomarkers.

Mitochondrial health is critical for cellular function and mitophagy, the selective degradation of damaged mitochondria, is central to maintaining mitochondrial quality (29,30). Mitophagy exerts dual roles in cancer, either prompting or suppressing carcinogenesis (31). RHOT1, an outer mitochondrial membrane protein, serves as a molecular switch in the mitochondrial dynamics and mitophagy (11,20,32). Mutations in RHOT1 disrupt mitochondria transport, impair quality

control and have been linked to neurodegenerative diseases such as Parkinson's (11,33-35). RHOT1 is upregulated in several cancers: Jiang *et al* (36) predicted its prognostic value in pancreatic cancer and Li *et al* (37) found that silencing RHOT1 inhibited pancreatic cancer cell proliferation and migration. In mice, *RHOT1* knockdown reduced mitochondrial motility and impaired quality control (38). It was also found that RHOT1 interacted with LRRK2, consistent with findings that LRRK2 knockdown or mutation impaired basal mitophagy (39). Collectively, these data position RHOT1 as a critical regulator of mitochondrial dynamics and mitophagy. Database analyses revealed strong associations between RHOT1 and PINK1/Parkin, the canonical ubiquitin-dependent mitophagy pathway (40). In the present study, silencing RHOT1 altered PINK1 and Parkin expression, suggesting that RHOT1 modulates mitophagy via this axis. GO and KEGG enrichment of RHOT1-interacting genes further supported predominant effects on mitochondrial function and the involvement of the PINK1/Parkin pathway.

Silencing RHOT1 also induced mitochondrial dysfunction, characterized by increased ROS production and MMP depolarization. ROS are central regulators of proliferation, differentiation and metabolism, but excessive ROS promotes oxidative stress, mtDNA mutations and mitochondrial damage (41,42). RHOT1 has been implicated in peroxisomal transport, which contributes to ROS clearance (43,44). Thus, the present findings of ROS accumulation and MMP depolarization aligned with reports that RHOT1 deficiency aggravates oxidative stress and compromises mitochondrial homeostasis. Consequently, dysfunctional mitochondria accumulate, reinforcing oxidative stress and energy imbalance.

In addition, silencing RHOT1 upregulated TOMM20 and TIMM23, proteins essential for mitochondrial protein import. This upregulation probably reflects a compensatory response to mitochondrial stress (45-47), consistent with previous studies showing increased import machinery and UPRmt components to preserve organelle integrity (48-50). Thus, RHOT1 deficiency appears to couple impaired mitophagy with a UPRmt-like compensatory mechanism, which will be a subgoal for future research.

Mitochondrial dysfunction also influenced cell cycle progression and apoptosis. Oxidative stress stabilizes p53, upregulates p21, inhibits CDK2/Cyclin E and blocks G_1-S transition (48,51-53). Disruption of MMP triggers cytochrome *c* release, caspase activation and PARP cleavage, promoting apoptosis (54-56). In agreement, silencing RHOT1 in GC cells induced G_0/G_1 arrest and apoptosis, linking mitochondrial quality control to cell-cycle regulation and survival. Furthermore, RHOT1 loss impaired migration and invasion. Reduced ATP production and ROS accumulation inhibit cytoskeletal remodeling and pseudopodia formation (57,58), while ROS modulates EMT gene expression via NF-κB and HIF-1α signaling (59). Myc has been shown to transcriptionally regulate RHOT1, coordinating mitochondrial trafficking with cytoskeletal dynamics (60). Dysregulated mitophagy and accumulation of damaged mitochondria further promote tumor progression (61).

In summary, RHOT1 deficiency in GC cells perturbs mitophagy, exacerbates oxidative stress, disrupts mitochondrial homeostasis and impairs key cellular processes, including cell cycle progression, apoptosis and invasion. These findings highlighted RHOT1 as a potential therapeutic target in GC. A potential limitation of the present study is that functional assays were mainly conducted in the HGC-27 cell line, which showed the highest RHOT1 expression among the GC cell lines examined. While this strengthens the rationale for its selection as the primary model, further validation in additional GC subtypes would help to generalize the findings. Addressing this limitation will be an important focus of future research. In addition, the results remained correlative, as no additional experiments were designed to directly validate the causal relationship. This limitation was acknowledged and the findings were therefore interpreted with caution. Future studies will be required to provide more definitive evidence of causality.

Silencing RHOT1 induced mitochondrial dynamics disorder, resulting in the inhibition of the PINK1/Parkin mitophagy pathway, which impaired mitophagy and caused the accumulation of intracellular ROS and depolarization of MMP. The aforementioned multiple ways triggered cell cycle arrest and promoted apoptosis, resulting in decreased proliferation of GC cells and weakened invasion and migration ability. Therefore, suppression of RHOT1 may become a new strategy for the treatment of GC and provide a new direction for the study of targeted treatment and drug resistance mechanisms in GC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YQP and XC conceived and designed the research; YQP, XC, FK, JZ, SY were responsible for data analysis. RF, JW and YYP were provided supervision and project administration. XC provided supervision. YQP wrote the first draft and YYP reviewed and edited the manuscript. FK, JZ and YYP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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