

Knockdown of ROS proto-oncogene 1 inhibits migration and invasion in gastric cancer cells by targeting the PI3K/Akt signaling pathway

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Objectives: Gastric cancer ranks the fourth most common cancer and the third leading cause of cancer mortality in the world. ROS proto-oncogene 1 (*ROS1*) is an oncogene and *ROS1* rearrangement has been reported in many cancers. Our study aimed to investigate the potential function and the precise mechanisms of *ROS1* in gastric cancer.

Methods: In our study, the analysis of *ROS1* expression and clinical pathologic factors of gastric cancer in gastric cancer using TCGA database demonstrated that *ROS1* expression was elevated in gastric cancer and related to T, N, M and TNM staging. High expression of *ROS1* predicted poor survival in patients with gastric cancer. Then, we measured *ROS1* expression in four human gastric cancer cell lines and knocked down *ROS1* expression in BGC-823 and SGC-7901 cells by specific shRNA transfection via Lipofectamine 2000. The effect of *ROS1* knockdown on cell proliferation, cell cycle distribution, cell apoptosis and metastasis in vitro was evaluated by MTT, colony formation, flow cytometric analysis, wound healing and Transwell invasion assays. The levels of apoptosis-related proteins, EMT markers and the PI3K/Akt signaling pathway members were measured by Western blotting.

Results: We demonstrated that sh*ROS1* transfection markedly downregulated *ROS1* expression in BGC-823 and SGC-7901 cells. Knockdown of *ROS1* inhibited cell survival, clonogenic growth, migration, invasion and epithelial-mesenchymal transition (EMT), as well as induced cell cycle arrest and apoptosis in gastric cancer cells. Furthermore, *ROS1* knockdown inhibited the phosphorylation of PI3K and Akt.

Conclusion: Collectively, our data suggest that *ROS1* may serve as a promising therapeutic target in gastric cancer treatment.

Keywords: gastric cancer, *ROS1*, shRNA, proliferation, apoptosis, metastasis, EMT

Introduction

Gastric cancer is the fourth common malignancies and the third leading cause of cancer death worldwide.^{1,2} Patients are diagnosed at the advanced stages of gastric cancer and 5-year survival rate is no more than 40% in China.³⁻⁵ Surgical resection, radiation therapy, chemotherapy and combination treatments are considered as therapeutic approaches for malignancies, including gastric cancer.⁴ Despite this, more than 80% of the cancer patients develop loco-regional or distant recurrence after curative resection.⁶ Peritoneal dissemination after surgery and lymph node or liver metastasis are still the main factors that contribute to the poor prognosis of gastric cancer.^{7,8}

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ROS1 is an oncogene and encodes a receptor tyrosine kinase (RTK) of insulin receptor family.^{9,10} *ROS1* shares 49% amino acid sequence homology with anaplastic lymphoma kinase (ALK) in tyrosine kinase domains.^{11,12} *ROS1* undergoes gene rearrangement and forms protein fusions to exhibit constitutive kinase activities in multiple cancers, such as colon cancer, glioblastoma multiforme, lung cancer and gastric cancer.^{13–15} Targeting *ROS1* with tyrosine kinase inhibitor has been approved by the FDA for the treatment of advance *ROS1*-positive NSCLC.¹⁶ Tiash S et al reported that *ROS1* knockdown enhanced the sensitivity of breast cancer cells to doxorubicin in vivo and in vitro.¹⁷ Deng G et al demonstrated that down-regulation of *ROS1* using shRNA inhibited cell proliferation, migration and invasion and induced cell apoptosis in intrahepatic cholangiocarcinoma cells.¹⁸ However, few studies have reported the effects of *ROS1* on gastric cancer and investigated the precise mechanisms.

In the present study, we knocked down *ROS1* expression in gastric cancer BGC-823 and SGC-7901 cells and further evaluated the effects of *ROS1* knockdown on gastric cancer cell proliferation, colony formation, apoptosis, migration, invasion and epithelial–mesenchymal transition (EMT).

Materials and methods

Analysis of The Cancer Genome Atlas (TCGA) database

RNA-Seq data of *ROS1* expression, related clinicopathologic factors and prognosis information of patients with gastric cancer included total 415 gastric cancer and 35 normal mucosa samples were obtained from TCGA (<https://portal.gdc.cancer.gov/>).

Cell culture

Human gastric cancer BGC-823, MGC-803, SGC-7901 and HGC-27 cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology (Shanghai, China). All the cells were cultured in RPMI-1640 containing 10% FBS and placed in a 5% CO₂ incubator at 37°C.

Construction of shRNA plasmid and cell transfection

The nucleotide sequences were used: sh*ROS1*-sense, 5'-GA TCCCGAGCCAATG TCATCCTGATTCAAGAGAATC AGG ATGACATTGGCTCTTTT-3' and antisense, 5'-AGC TAAAAAGAGCCAATGTCATCCTGATTCTCTTGAAT-CAGGATGACATTGGC TCGGG-3'; shCtr-sense, 5'-GATC

CCCTTCTCCGAACGTGTCACGTTTCAAGAGAAC GT GACACGTTCCGAGAATTTT-3' and antisense, 5'-AGCTAAAAAATTCTCCGAACG TGTCACGTTCTCTTGA AACGTGACACGTTCCGAGAAGGG-3'. The shRNAs were synthesized by GenePharma (Shanghai, China). The generated plasmid encoding sh*ROS1* or shCtr was named pRNA-H1.1-sh*ROS1* or pRNA-H1.1-shCtr. The recombinant plasmid was transfected into BGC-823 and SGC-7901 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Stable clones were selected in RPMI-1640 medium containing G418 for 5 days.

Western blotting

The cells were lysed in RIPA buffer (Beyotime, Haimen, China) containing 1% protease inhibitor PMSF (Beyotime) and centrifuged at 12,000 rpm for 10 min. The supernatant containing total proteins was aspirated and the protein concentration was determined. The total proteins were separated by SDS-PAGE (Beyotime) and then transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with primary antibodies against *ROS1* (1:500, Sangon Biotech, Shanghai, China), cleaved-caspase-3 (1:1000, Abcam, Cambridge Science Park, Cambridge, UK), Bcl-2 (1:400, BOSTER, Wuhan, China), Bax (1:400, BOSTER), cleaved-PARP (1:1000, Abcam), E-cadherin (1:400, BOSTER), Vimentin (1:500, BIOSS, Beijing, China), N-cadherin (1:400, BOSTER), p-PI3K (1:500, BIOSS), PI3K (1:400, BOSTER), p-Akt (1:200, Santa Cruz Biotechnology, Dallas, Texas, USA) and Akt (1:200, Santa Cruz Biotechnology) at 4°C overnight. After washing with TBS-Tween 20 buffer, the membranes were incubated with goat anti-rabbit IgG-HRP (Beyotime) at 37°C for 45 mins. The bands were developed using ECL solution (Beyotime).

Quantitative real-time PCR

RNA extraction was performed using Total RNA Extraction Kit (BioTeke, Beijing, China). Total RNAs were reverse transcribed into cDNAs and real-time PCR analysis was performed on Exicycler™ 96 Thermal Block (Bioneer, Daejeon, Republic of Korea). The real-time PCR protocols were at 95°C for 10 mins, followed by 40 amplification cycles (at 95°C for 10 s, at 60°C for 20 s and at 72°C for 30 s). β -actin was used as an internal control. The results were analyzed using 2- $\Delta\Delta$ Ct method. The primers were synthesized by Sangon Biotech (Shanghai, China) and the primer sequences were: *ROS1*-forward, 5'-CAGTCCACGGATAGTGAAGA

TT-3' and reverse, 5'-GGATAAGGCTGATGACC AAG-3'; β -actin-forward, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3' and reverse, 5'-CTGTACCTTCACCGTTCCAGTTT-3'. Then, the transcription factor Snail, Slug and Twist were also be detected by Real-time PCR. The primer sequences are seen in Table 1.

MTT assay

After knockdown of *ROS1*, the cells were plated in 96-well plates and cultured in a CO₂ incubator at 37°C. Then, the cells were treated with 5 mg/mL MTT solution (Sigma-Aldrich, St. Louis, MO, USA) for 4 hrs at 37°C. The supernatant was removed and DMSO was added to dissolve the formazan crystals. OD values at 490 nm were measured using a microplate reader (BitoTEK, Winooski, VT, USA).

Colony formation assay

The harvested cells were re-suspended in RPMI-1640 and plated in 35 mm petri dishes. After culturing at 37°C, the colonies were washed with PBS and paraformaldehyde (4%) fixed for 20 mins. After washing, the cells were stained with Wright-Giemsa solution (Jiancheng Bioengineering Institute, Nanjing, China) for 5 mins and photographed. Colonies containing more than 50 cells were counted.

Cell cycle analysis by flow cytometry

The cells were harvested (550×g, 5 mins) and ethanol fixed at 4°C for 2 hrs. Then, the ethanol-fixed cells were washed and stained with 25 μ L propidium iodide (PI) (Beyotime) for 30 mins at 37°C. Cell cycle distribution was analyzed by BD flow cytometer (Franklin Lakes, NJ, USA).

Cell apoptosis by flow cytometry

The cell culture was centrifuged at 309×g for 5 mins. After washing with PBS, the cells were re-suspended in Binding

buffer. The cells were gently mixed with 5 μ L Annexin V-FITC and 10 μ L PI (KeyGEN, Nanjing, China) for 15 mins at room temperature. Cell apoptosis was analyzed by BD flow cytometer (Franklin Lakes, NJ, USA).

Wound healing assay

After transfection sh*ROS1*, the supernatant was removed using pipettes and the monolayer was wounded by 200 μ L sterile pipette tips. The monolayer was washed with serum-free medium and photographed (0 hr). Then, the cells were cultured in serum-free medium for 12 and 24 hrs to test the migratory capabilities of shRNA-transfected gastric cancer cells.

Transwell invasion assay

The cells (2×10^4 cells) were added into the upper chamber of the Matrigel-coated Corning Incorporated Transwell chamber (Corning, NY, USA) and RPMI-1640 containing 30% FBS was added into the lower chamber. After culturing for 24 hrs, the cells were fixed and stained with 0.5% crystal violet. The cells in five random fields were counted under an inverted microscope.

Statistical analysis

Data are presented as mean \pm SD and analyzed by one-way ANOVA and Bonferroni's multiple comparison test using GraphPad Prism, version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The differences in clinicopathological parameters between gastric cancer patients with high or low *ROS1* expression were compared using the Chi-squared test. The difference between the survival curves was analyzed by Log-rank test. A value of $P < 0.05$ was considered a statistically significant difference.

Table 1 Primer sequences

Name	Sequences	Primer length	Tm	Products length
Twist F	GGAGTCCGCGAGTCTTACGA	19	55.7	162
Twist R	CCAGCTTGAGGGTCTGAATC	20	56.9	
Slug-F	GTTGCCATTGTTGAACTAAAGCC	23	60.9	168
Slug-R	CTCCTCCCCAAGGCACATACT	22	64.2	
Snail-F	GCCCCACAGGACTTTGATGA	20	60.4	214
Snail-R	AGTGAGTCTGTCAGCCTTTGTC	22	55.9	
β -actin F	CTTAGTTGCGTTACACCCTTTCTTG	25	62	156
β -actin R	CTGTACCTTCACCGTTCCAGTTT	24	64.4	

Results

Analysis of *ROS1* expression, clinicopathologic factors and prognosis of gastric cancer using TCGA database

To validate the mRNA expression of *ROS1* and clinicopathologic factors of gastric cancer, the data was obtained from TCGA (<https://portal.gdc.cancer.gov/>). The *ROS1* mRNA expression in gastric cancers was significantly elevated compared to 35 normal mucosa samples ($P<0.0001$, Figure 1A). The *ROS1* mRNA expression in patients with gastric cancer showed four significantly relative relationship with depth of invasion,

lymph node metastasis, distant metastasis and TNM staging ($P<0.0001$, Table 2). The P -value of tumor grade is 0.014, but it may be because the samples were different. Its P -value was larger than others. Additionally, results revealed that the median survival time in *ROS1*-high group was 22.170 ± 2.823 , shorter than that in *ROS1*-low group (55.390 ± 11.846). Higher *ROS1* mRNA expression was associated with worse prognosis compared with the low-expression ($P=0.003$, Figure 1B). These data suggested that *ROS1* might act as an oncogenic gene in gastric cancer and predict poor prognosis.

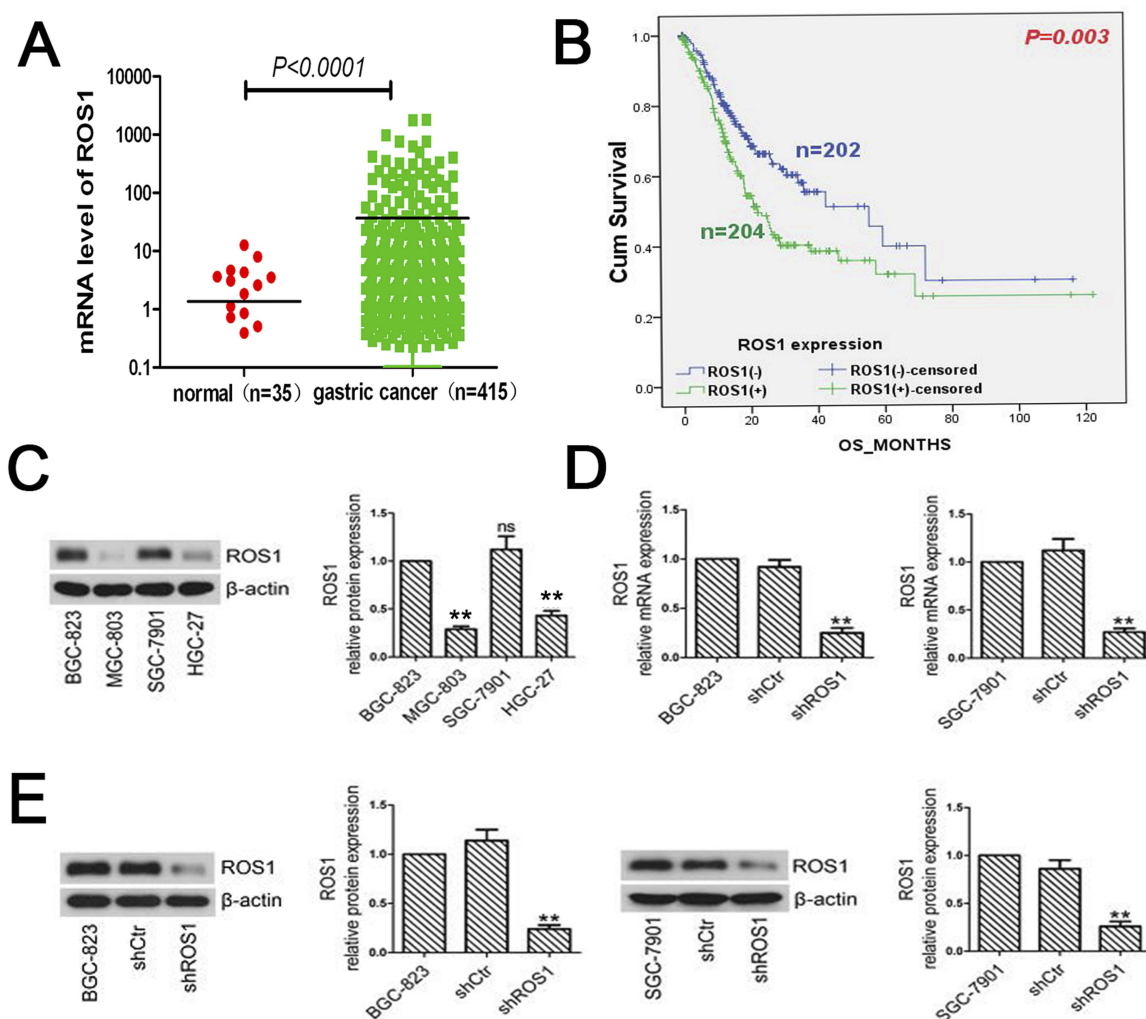


Figure 1 *ROS1* expression in gastric cancer tissue and gastric cancer cell lines. **(A)** The *ROS1* mRNA expression was significantly elevated in gastric cancer compared to normal mucosa samples according to the data from TCGA ($P<0.0001$). **(B)** Patients with high expression of *ROS1* predicted a worse prognosis than that with low-expressed *ROS1* ($P=0.003$). **(C)** Total proteins were extracted from BGC-823, MGC-803, SGC-7901 and HGC-27 cells. The expression of *ROS1* was determined by Western blotting. **(D)** BGC-823 and SGC-7901 cells were transfected with shROS1 or shCtrl. Real-time PCR was performed to examine *ROS1* expression in shRNA-transfected cells. The results were normalized to internal control (β -actin). **(E)** After shRNA transfection, we measured the changes of *ROS1* expression in BGC-823 and SGC-7901 cells by Western blotting. The results were shown as mean \pm SD. ** $P<0.01$ versus the shCtrl group.

Abbreviation: ns, not significant.

Table 2 Correlation between *ROS1* expression and clinicopathological features in gastric cancer

Variable	n	ROS1 expression		χ^2	P-value
		Low	High, n (%)		
Age (year)	409			2.093	0.148
≤60	132	59	73 (55.3)		
>60	277	145	132 (47.65)		
Gender	414			0.095	0.758
Male	267	132	135 (50.56)		
Female	147	75	72 (48.398)		
Grade	405			10.566	0.014
G1	12	8	4 (33.33)		
G2	147	87	60 (40.82)		
G3	246	107	139 (56.50)		
Depth of invasion	405			112.960	0.000
T1	22	22	0 (0.00)		
T2	87	74	13 (14.94)		
T3	181	81	100 (55.25)		
T4	115	21	94 (81.74)		
Lymph node metastasis	395			212.770	0.000
N0	122	116	6 (4.92)		
N1	112	59	53 (47.32)		
N2	79	13	66 (83.54)		
N3	82	1	81 (98.78)		
Distant metastasis	384			24.312	0.000
M0	367	194	173 (47.14)		
M1	27	1	26 (96.30)		
TNM staging	389			377.164	0.000
I	56	56	0 (0.00)		
II	123	123	0 (0.00)		
III	169	3	166 (98.22)		
IV	41	0	41 (100.00)		

Expression of *ROS1* in human gastric cancer cells

We measured the expression of *ROS1* in four gastric cancer cell lines (BGC-823, MGC-803, SGC-7901 and HGC-27) using Western blotting. The results showed that BGC-823 and SGC-7901 cells expressed higher levels of *ROS1* than the other two cell lines (MGC-803 and HGC-27) (Figure 1C).

ROS1 knockdown inhibits cell proliferation and colony formation in gastric cancer cells

To evaluate the function of *ROS1* in human gastric cancer cells, we transfected BGC-823 and SGC-7901

cells with sh*ROS1* or shCtr and examined *ROS1* expression using quantitative real-time PCR and Western blotting. The results showed that *ROS1* expression was significantly downregulated in both BGC-823 and SGC-7901 cells after sh*ROS1* transfection (Figure 1D and E). We then measured cell viabilities for 5 consecutive days using MTT assay. The results showed that the viable cell numbers in the sh*ROS1* group were lower than those in the shCtr group on day 2, 3, 4 and 5 (Figure 2A). Colony formation assay showed that clonogenic growth ability of the cells that transfected with sh*ROS1* was reduced compared with that of shCtr-transfected cells (Figure 2B). Cell cycle progression was analyzed by flow cytometry. We found that the cell percentages of G1 phase and G2 phase in the sh*ROS1*

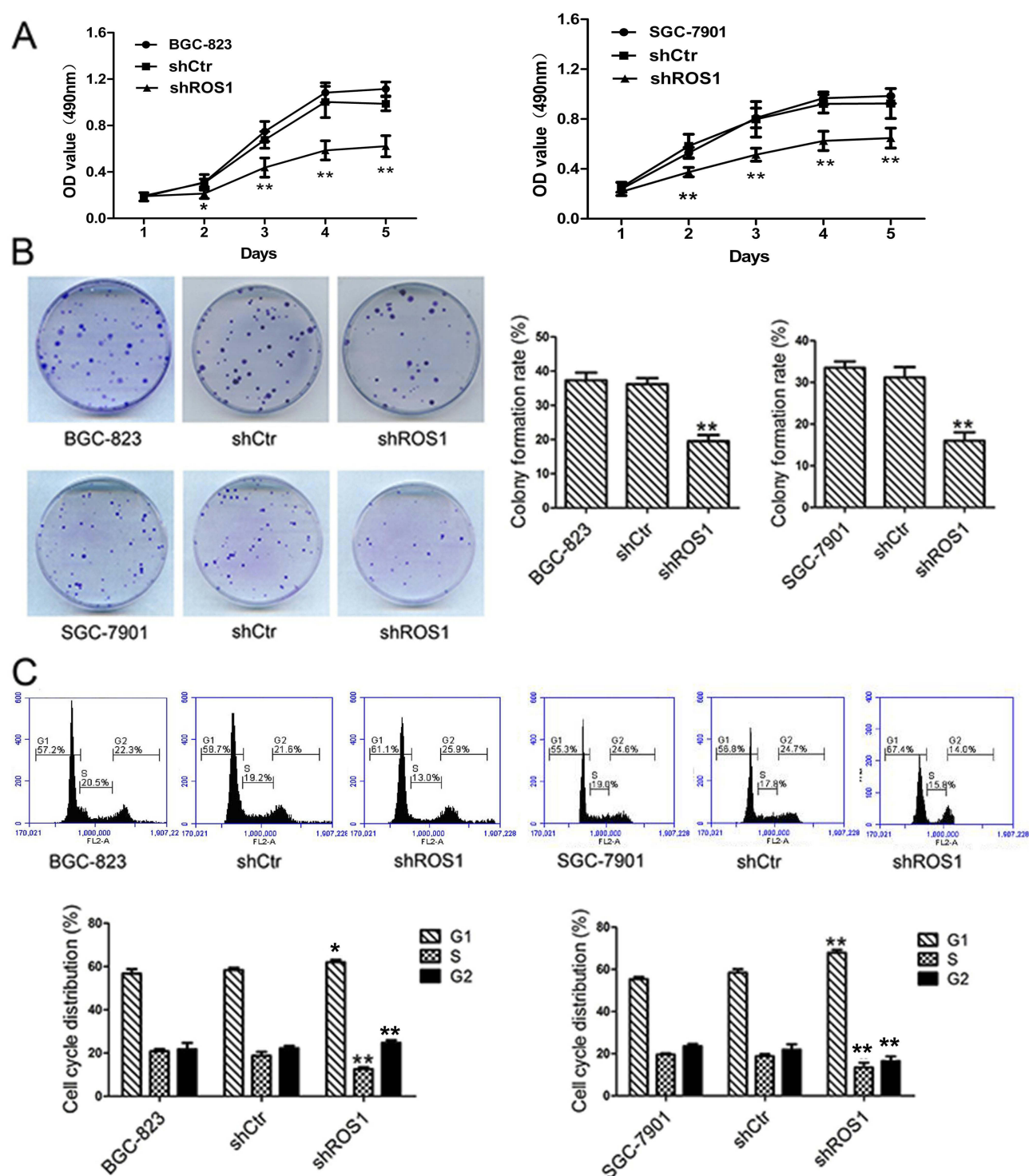


Figure 2 *ROS1* knockdown reduced cell viability and suppresses colony formation of gastric cancer cells. (A) Cell viability was examined by MTT assay on day 1, 2, 3, 4 and 5. (B) Three groups of cells were seeded in 35 mm petri dishes for assessment the colony formation capacity of BGC-823 and SGC-7901 cells. (C) The cells were subjected to flow cytometric analysis for assessment of cell cycle progression. The results were shown as mean±SD. * $P < 0.05$, ** $P < 0.01$ versus the shCtr group.

group in BGC-823 cells were higher than those in the shCtr group. The fraction of sh*ROS1*-transfected BGC-823 cells in the S phase was significantly lower than that of the shCtr-transfected cells. In SGC-7901 cells,

the cell percentage of G1 phase was markedly increased while the cell percentages of S and G2 phases were decreased in the sh*ROS1* group compared with those in the shCtr group (Figure 2C).

***ROS1* knockdown promotes cell apoptosis in gastric cancer cells**

We further investigated the effect of *ROS1* knockdown on cell apoptosis by flow cytometry. Flow cytometric analyses showed that the *ROS1* knockdown significantly increased the cell apoptosis rate when compared with the shCtr group (Figure 3A). Western blotting was performed to quantify the expression of cleaved-caspase-3, cleaved-PARP, Bcl-2 and Bax. We found that sh*ROS1*-transfected cells expressed increased expression levels of cleaved-caspase-3, cleaved-PARP and Bax and a decreased expression level of Bcl-2 compared with the shCtr-transfected cells (Figure 3B).

***ROS1* knockdown inhibits cell migration, invasion and cell EMT in gastric cancer cells**

The migration and invasion capabilities of shRNA-transfected gastric cancer cells were examined by wound healing and Transwell assays. As shown in Figure 4, *ROS1* knockdown inhibited the migration and invasion of both BGC-823 and SGC-7901 cells as compared with the shCtr group. We then investigated whether *ROS1* knockdown influenced EMT progression in gastric cancer in vitro. We found that transfection of sh*ROS1* into BGC-823 and SGC-7901 cells resulted in upregulated levels of E-cadherin and downregulated levels of Vimentin and N-cadherin (Figure 5). At the same time, gene expression of the transcription factor Twist Snail and Slug associated with EMT was also downregulated when *ROS1* was knocked down in BGC-823 and SGC-7901 cells (Figure 6).

***ROS1* knockdown inhibits PI3K/Akt signaling pathway activation**

To explore whether PI3K/Akt signaling pathway was involved in the inhibitory effects of *ROS1* knockdown on clonogenic growth and metastasis of gastric cancer cells in vitro, we examined p-PI3K, PI3K, p-Akt and Akt protein levels by Western blotting and then calculated the ratios of p-PI3K/PI3K and p-Akt/Akt. The results showed that shRNA-mediated knockdown of *ROS1* reduced the levels of p-PI3K and p-Akt in both BGC-823 and SGC-7901 cells (Figure 7).

Discussion

ROS1 rearrangement has been reported in multiple cancers, such as gastric adenocarcinoma, non-small cell lung cancer (NSCLC), ovarian cancer, glioblastoma and

colorectal cancer.¹⁹ However, no study has clarified the role of *ROS1* in gastric cancer. In the present study, TCGA database was applied to analyze the mRNA expression of *ROS1* in gastric cancer. The results showed that *ROS1* was overexpressed in gastric cancer compared to normal mucosa samples. Higher level of *ROS1* was related to depth of invasion, lymph node metastasis, distant metastasis and TNM staging. In addition, higher expression predicted worse prognosis of patients with gastric cancer. Then, we knocked down *ROS1* expression in gastric cancer BGC-823 and SGC-7901 cells. Davies KD et al have demonstrated that *ROS1* inhibition mediated by crizotinib and NVP-TAE684 (both are ALK inhibitors with activities against *ROS1*) inhibits HCC78 cell proliferation.²⁰ We found that *ROS1* knockdown mediated by specific shRNA transfection significantly reduced cell viability and inhibited colony formation of gastric cancer cells (BGC-823 and SGC-7901). Deng G et al have observed similar results in intrahepatic cholangiocarcinoma cells,¹⁸ which are consistent with our studies. Cell cycle is a highly ordered process that tightly regulated by multiple cell cycle-related regulators.²¹ Cell cycle dysregulation is the hallmark of cancer.²² Cell cycle arrest can lead to inhibition of cell growth.²³ Therefore, in our study, we further confirmed whether cell growth inhibition in gastric cancer cells was associated with cell cycle arrest. Flow cytometric analysis showed that *ROS1* knockdown arrested the cell cycle at G1 phase. The observations indicate that *ROS1* knockdown may inhibit proliferation and clonogenic growth of gastric cancer cells in vitro by inducing cell cycle arrest.

Apoptosis, which is also called Type 1 programmed cell death, is characterized by shrinkage of cells, DNA fragmentation, nuclear condensation and the formation of apoptotic bodies.^{24,25} Flow cytometric analysis showed that knockdown of *ROS1* in gastric cancer cells significantly induced cell apoptosis compared with the shCtr group. Caspase, a family of cysteine proteases, regulates the initiation and execution of apoptosis.²⁶ Poly ADP-ribose protein (PARP) is the substrate of caspase-3 (a crucial executioner in apoptosis) and can be cleaved into two fragments during apoptosis.²⁷ Our results showed that *ROS1* knockdown markedly increased cleaved-caspase-3 and cleaved-PARP levels in gastric cancer cells. Anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are two members of Bcl-2 family.²⁸ Bax and Bcl-2 imbalance is key to the regulation of cell apoptosis.²⁹ Next, we

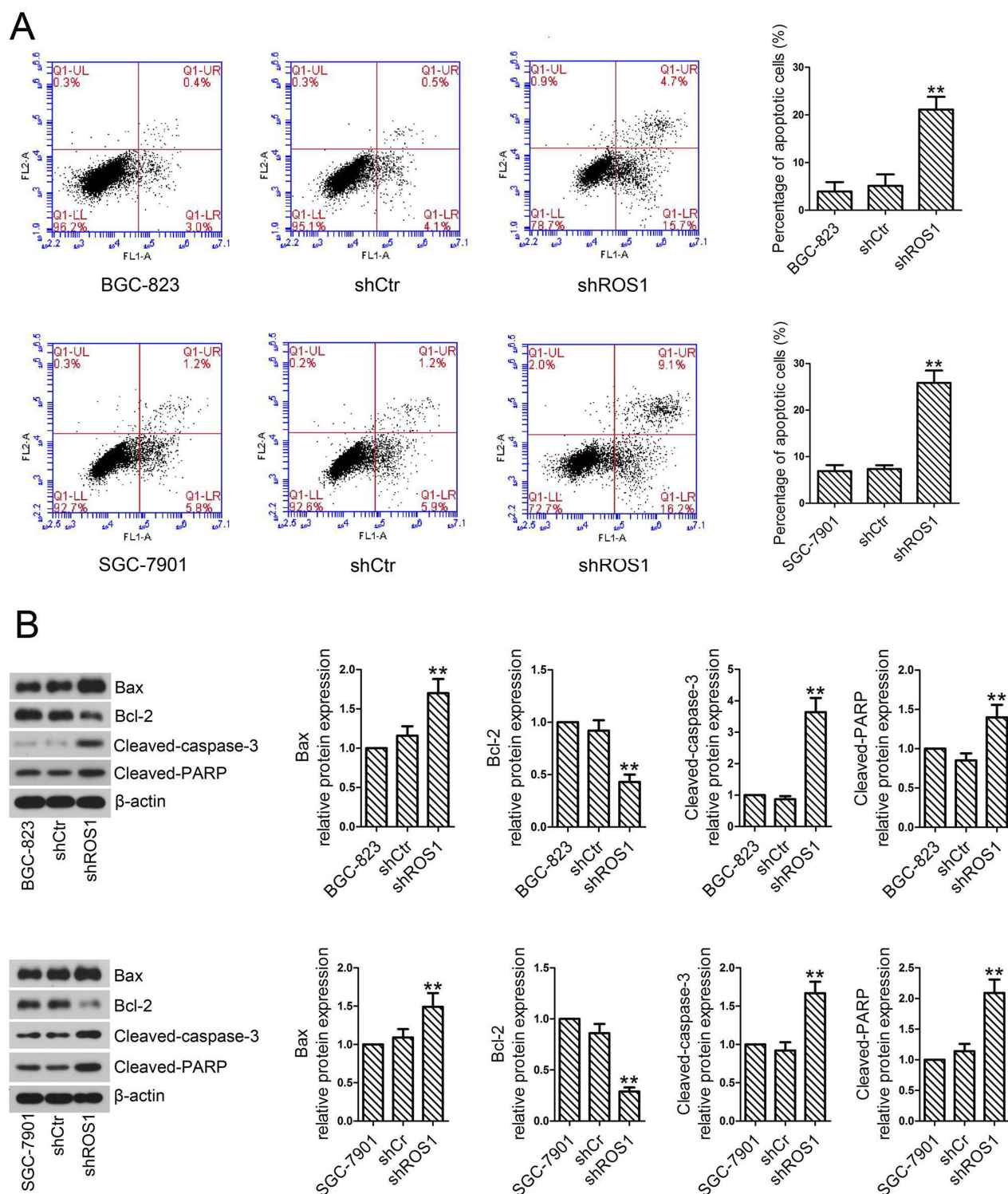


Figure 3 *ROS1* knockdown promotes apoptosis of gastric cancer cells. **(A)** Cell apoptosis rate of gastric cancer cells transfected with sh*ROS1* or shCtrl was determined by Annexin V/PI staining assay. **(B)** Protein levels of cleaved-Caspase-3, cleaved-PARP, Bcl-2 and Bax were quantified by Western blotting. The results were shown as mean \pm SD. ** $P < 0.01$ versus the shCtrl group.

measured the expression levels of Bax and Bcl-2. Western blotting results showed that a significant up-regulation of Bax level was detected in cells after *ROS1* knockdown, whereas Bcl-2 was down-regulated. Our observations

suggest that *ROS1* knockdown may promote gastric cancer cell apoptosis by inducing the imbalance between Bax and Bcl-2 and activating caspase-3 and its downstream substrate PARP.

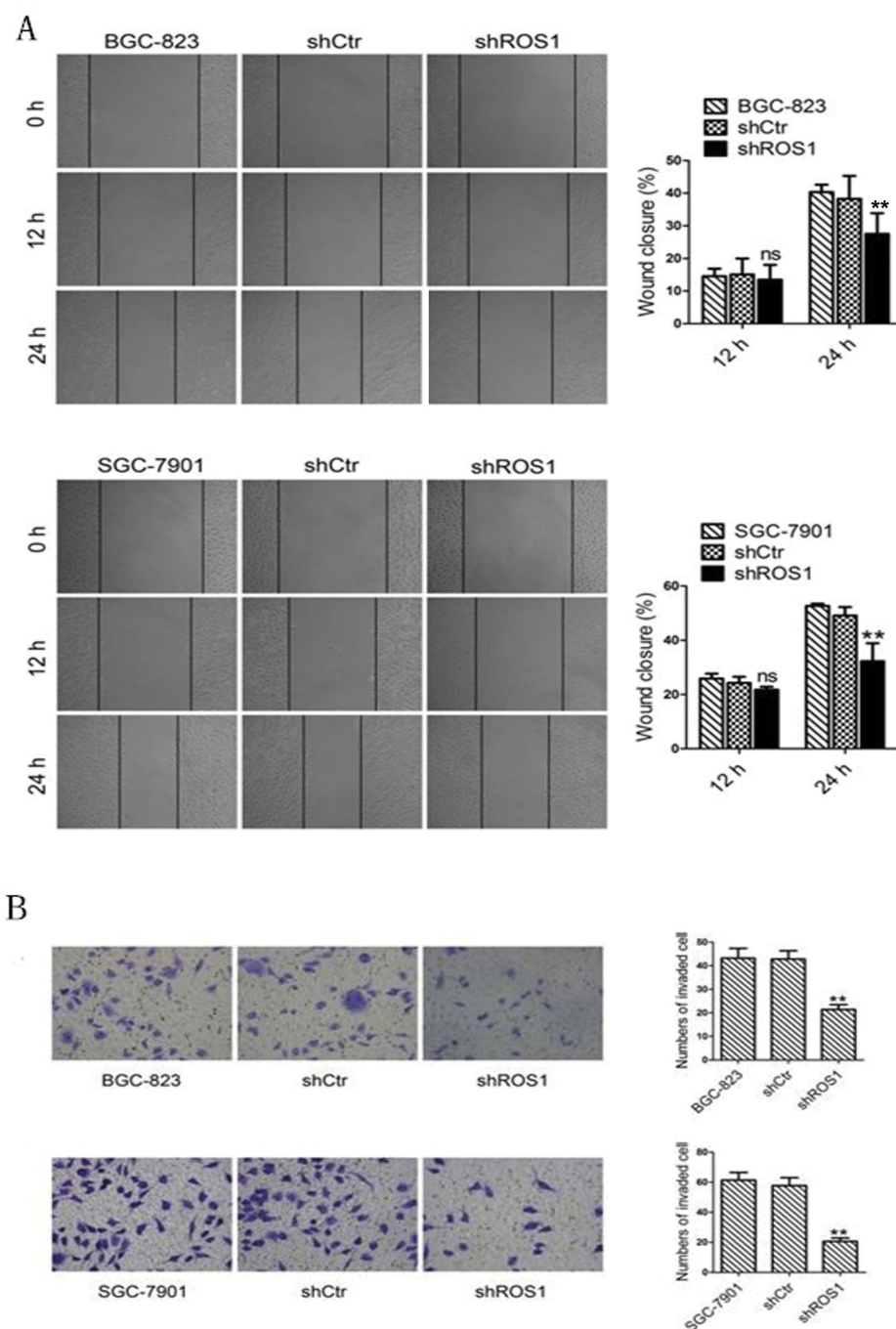


Figure 4 (A) *ROS1* knockdown suppresses migration of gastric cancer cells. Migration abilities of gastric cancer cells transfected with shRNA were determined by wound healing assay. **(B)** Invasion abilities of the cells in the three groups were assessed by Transwell assay. The results were shown as mean \pm SD. ** P <0.01 versus the shCtr group.

Abbreviation: ns, not significant.

Cancer metastasis, which involves many processes, such as cell migration and invasion, intravasation, metastasis through blood and lymph, extravasation and tumor formation in new tissues or organs, is the leading cause of treatment failure in cancer patients.^{30,31} Previous evidences have shown that the invasion of breast cancer cells and lung cancer cells is inhibited

by miRNA-33a and curcumin, accompanied by the decrease of *ROS1*.^{32,33} Deng G et al have demonstrated that *ROS1* shRNA transfection inhibits the migration and invasion of intrahepatic cholangiocarcinoma cells.¹⁸ Consistently, we found that *ROS1* knockdown significantly reduced the migratory and invasive capabilities of gastric cancer cells. EMT has been demonstrated to be a

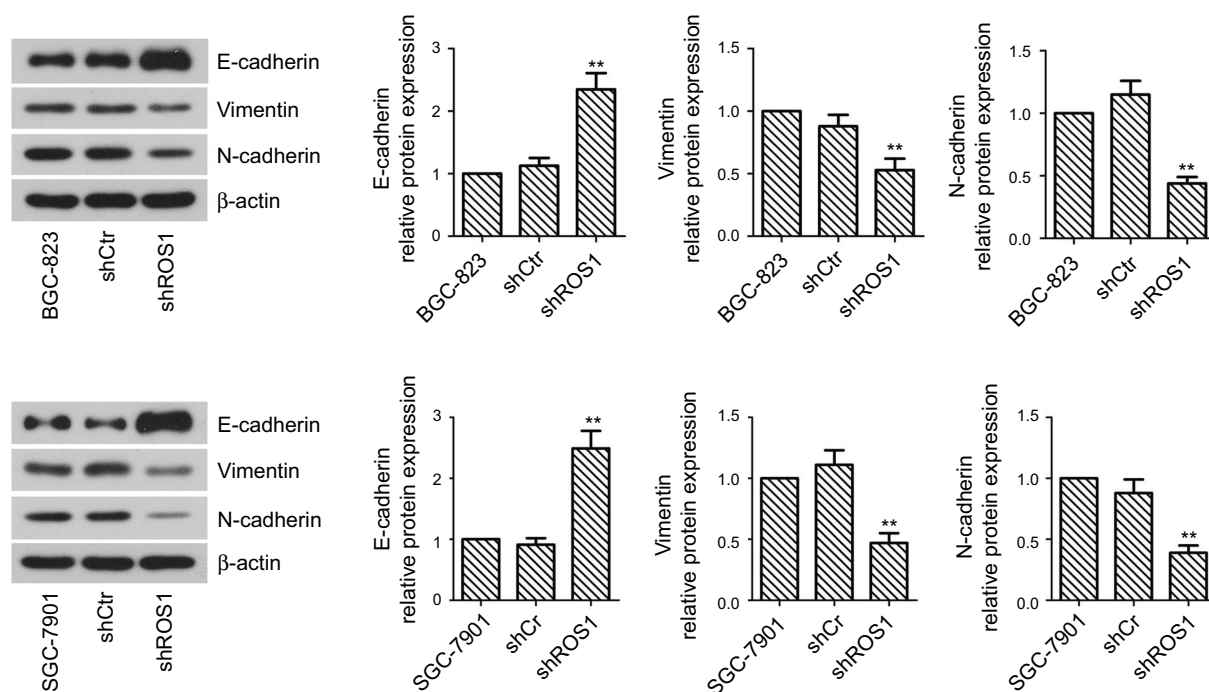


Figure 5 *ROS1* knockdown suppresses invasion and EMT of gastric cancer cells. The cells in the three groups were subjected to total protein extraction. The expression of EMT-related markers, including E-cadherin, N-cadherin and Vimentin, was measured by Western blotting. The results were shown as mean±SD. ** $P < 0.01$ versus the shCtrl group.

major cause of cell migration and invasion. EMT is a process during which cancer cells lose epithelial characteristics and acquire mesenchymal features, which is accompanied by the downregulation of E-cadherin and the upregulation of N-cadherin, Snail and Vimentin.³⁴ EMT has been demonstrated to be a crucial step in the metastasis of cancer.³⁵ Our results showed that knock-down of *ROS1* greatly increased E-cadherin expression and decreased N-cadherin and Vimentin levels in gastric cancer cells. Twist, Slug and Snail were all upstream transcription factor of EMT.

Twist is a member of the basic helix loop helix transcription factor (bHLH) family of transcription factors, including Twist1 and Twist2. It has the ability to regulate embryonic tissue development and the ability to migrate cells during embryonic development. Studies have shown that in the cells that expressed Twist, E-cadherin and beta-catenin and other adhesion protein expression was reduced, at the same time invasion ability of the cells also declined.³⁶ Snail gene is a transcription factor with zinc finger structure, which is the first transcription factor to induce EMT. During embryonic development, Snail can bind to E-cadherin on the proximal promoter of E-box, inhibit the transcription of E-cadherin gene, decrease the level of E-cadherin protein in cells and promote the

process of cell EMT. Studies have shown that ectopic expression of Snail in epithelial cells of the phenotype of fibroblasts and the ability to obtain tumor invasion.³⁷ Slug, also known as Snail2, is another transcription factor in the Snail family. It is the sign of the migration ability of mesoderm and neural crest cells in embryonic development. Like Snail, it is also combined with E-cadherin on the proximal promoter of E-box to inhibit the transcription of E-cadherin gene, decrease the level of intracellular E-cadherin protein and promote the process of EMT cell.³⁸ TGF can upregulate the expression of Snail and Slug, and promote the occurrence and development of EMT.³⁹ In this study, the expression of Twist, Slug and Snail in gastric cancer cells, was all downregulated after the *ROS1* was knocked down. So *ROS1* can induce cancer cells EMT through activating Twist, Slug and Snail. All these results suggest that *ROS1* knockdown may impair the migration and invasion of gastric cancer cells by reversing EMT.

Accumulating studies have reported that PI3K/Akt signaling pathway activation plays a critical role in cancer progression by regulating the malignant phenotypes of cancer cells, including gastric cancer.^{40–42} Moreover, inhibition of PI3K/Akt signaling pathway has been used as a therapeutic strategy to treat gastric

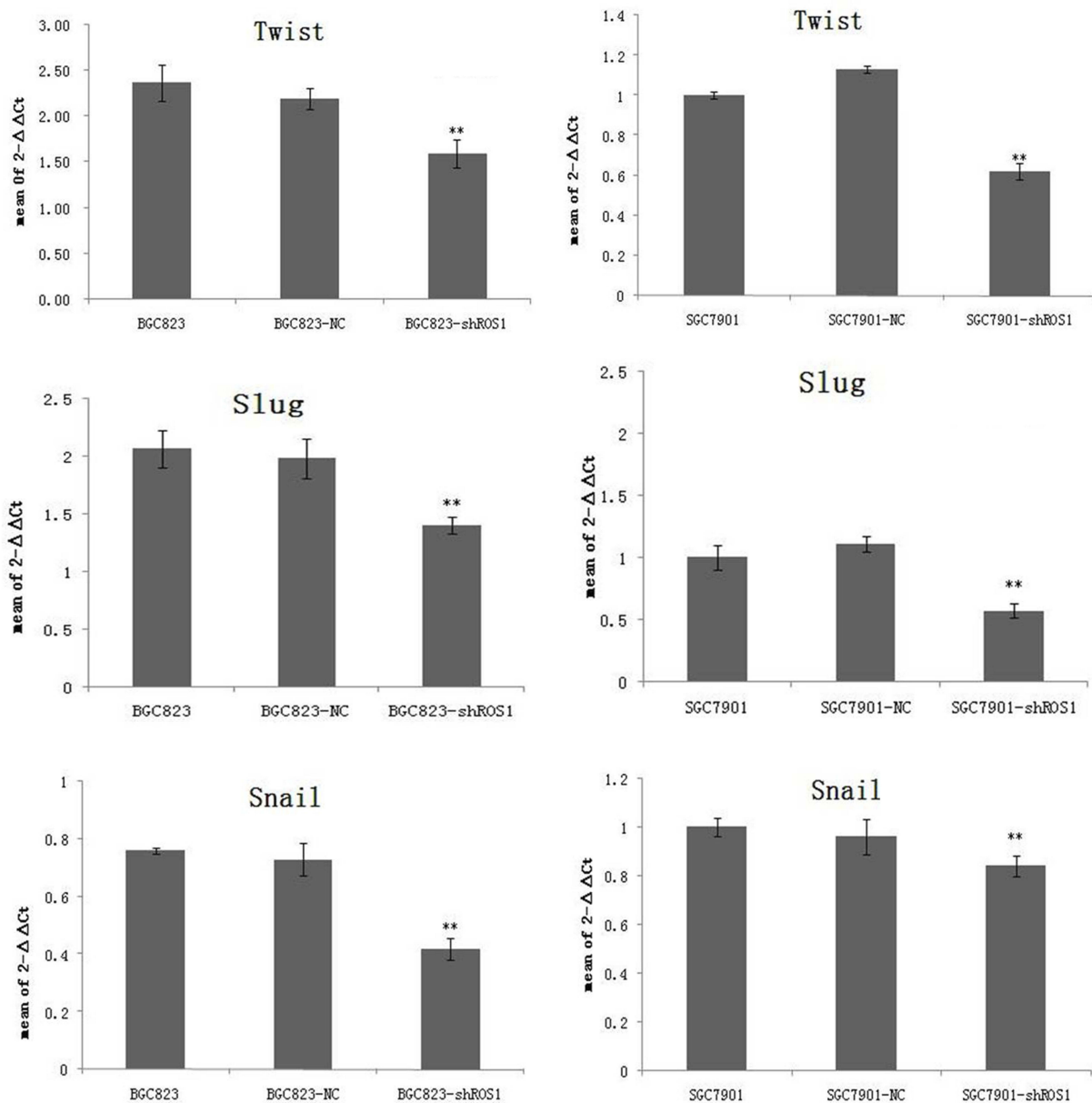


Figure 6 *ROS1* knockdown suppresses expression of transcription factor Twist, Slug and Snail. The results were shown as mean±SD. ** $P < 0.01$ versus the shCtr group.

cancer patients.⁴³ Evidences of *ROS1* targeted PI3K/Akt signaling pathway existed in some aspects. *ROS1* was reported to be involved in BMSC fate switching between osteogenesis and adipogenesis, mediated via PI3K/AKT/mTORC1 signaling.⁴⁴ Silencing of *ROS1* gene could sensitize mouse breast cancer cells both in vitro and in vivo, preventing activation of the survival pathway protein AKT1.¹⁷ *ROS1* also involved in cell proliferation, colony formation, cell cycle progression, migration and invasion of intrahepatic cholangiocarcinoma through AKT signaling pathway.¹⁸ Our results

showed that the levels of p-PI3K and p-Akt downgraded in both BGC-823 and SGC-7901 cells, which indicated PI3K/Akt signaling pathway was significantly inhibited after *ROS1* knockdown in gastric cancer cells. Above evidences may suggest that *ROS1* knockdown may inhibit the development of gastric cancer via the PI3K/Akt signaling pathway.

Conclusion

Taken together, our results demonstrated for the first time that *ROS1* was upregulated in gastric cancer. *ROS1*

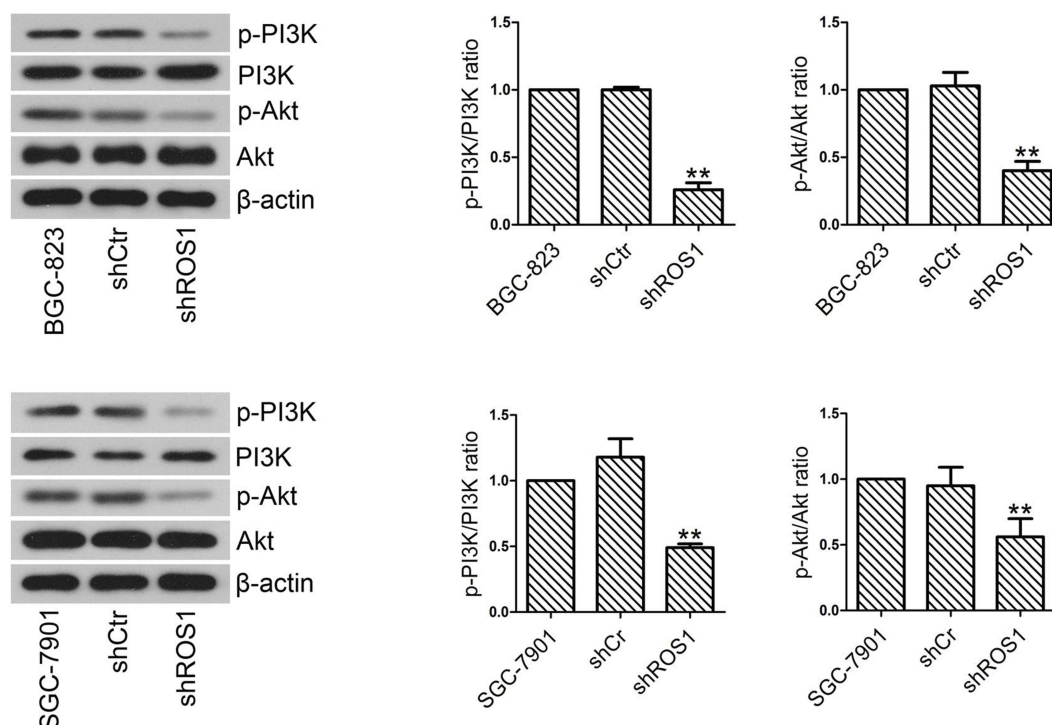


Figure 7 The levels of PI3K, p-PI3K, Akt and p-Akt were quantified by Western blotting. The ratios of p-PI3K/PI3K and p-Akt/Akt were calculated. The results were shown as mean±SD. ** $P < 0.01$ versus the shCtrl group.

knockdown significantly inhibited cell growth, induced cell apoptosis, inhibited EMT and metastasis in gastric cancer by inhibiting the PI3K/Akt signaling pathway. Our study provides a new therapeutic strategy for the treatment of gastric cancer.

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Author contributions

The study was conceived and designed by Yan Xin. The experiments and manuscript were performed and prepared by Jingjing Qiao and Dan Sun. Wenhui Li analyzed the data. Man Li supervised the study progression. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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