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miR-218 Inhibits Proliferation, Migration, and EMT of Gastric Cancer Cells by Targeting WASF3

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MicroRNAs (miRNAs) play an important role in carcinogenesis. miR-218 is one of the most known miRNAs and has been demonstrated to inhibit progression in gastric cancer. However, the underlying molecular mechanism is not established. In this study, qRT-PCR and Western blot indicated that miR-218 was downregulated in gastric cancer cell lines SGC7901 and BGC823 compared to that in normal gastric epithelial cell line GES-1. MTT and wound scratch assays suggested that overexpression of miR-218 markedly suppressed cell proliferation, migration, and EMT of gastric cancer cells. Furthermore, we proved that WASF3 was a direct target of miR-218 by luciferase reporter assay, and restoration of WASF3 expression impairs miR-218-induced inhibition of proliferation, migration, and EMT in gastric cancer cells SGC7901. In summary, our results demonstrated that miR-218 functions as one of the tumor-suppressive miRNAs and inhibits gastric cancer tumorigenesis by targeting WASF3. miR-218 may serve as a potential therapeutic target for the treatment of gastric cancer.

Key words: miR-218; Proliferation; Migration; Epithelial–mesenchymal transition (EMT); WASF3; Gastric cancer (GC)

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies and has been the leading cause of cancer-related deaths all over the world[1]. Although tumor resection is the most effective therapeutic strategy, a large proportion of patients in China are often diagnosed at an advanced and unresectable clinical stage[2]. The majority of GC deaths are caused by cancer cell metastasis[3]. However, the underlying mechanisms of the proliferation, migration, and epithelial–mesenchymal transition (EMT) of GC in GC progression remain unclear, and further elucidation of the molecular mechanisms underlying these processes is urgently required in order to find new treatment strategies.

MicroRNAs (miRNAs) are a class of endogenous small RNAs that are naturally occurring, short, noncoding RNA molecules[4]. miRNAs regulate gene expression by inhibiting translation and/or decreasing the stability of target mRNAs, and they are differentially expressed in various tissues and cells, suggesting that miRNAs are involved in crucial biologic processes, including development, differentiation, apoptosis, and proliferation[5]. As reported previously, some miRNAs are reduced in malignancies and function as tumor suppressors[6–8]. miR-218 has been reported to be a tumor suppressor in several types of tumors, such as glioma, bladder cancer, and cervical cancer[9–11]. A previous study showed that the level of miR-218 was significantly lower in GC patients than that in healthy controls, and miR-218 was closely associated with the tumorigenesis or metastasis of GC[12]. However, the mechanisms by which miR-218 inhibits GC, especially suppressing EMT and inhibiting GC proliferation and migration, have not yet been found. During the progression of EMT, downregulation of adhesion molecules, such as E-cadherin, and upregulation of mesenchymal markers, such as N-cadherin, vimentin, and TWIST1, decrease epithelial cell–cell adhesion and promote cancer cell metastasis[13].

Wiskott–Aldrich syndrome protein family 3 (WASF3; also termed WAVE3) is a member of the Wiskott–Aldrich family of proteins that contain motifs at the C-terminal end and engage the ARP2/3 complex, which facilitates actin polymerization[14]. A new study has shown that WASF3 is integrally involved in cancer and plays a key role in cell proliferation and migration during the development and progression of cancer[15]. The *WASF3* gene is overexpressed in several types of tumors, such as breast cancer, osteosarcoma, etc.[16,17]. Loss of WASF3 expression results in the downregulation of metalloproteinases that control invasive properties[18]. However, how

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WASF3 participates in GC development is not clear at the moment.

To identify the potential target mRNAs of miR-218, the databases TargetScan, PicTar, and microcosm were used. We found that the 3′-UTR of WASF3 mRNA contains a sequence motif, which matched with the "seed sequence" of miR-218. All the information intensively suggested that WASF3 may be the target gene of miR-218.

The objective of the current study was to investigate whether miR-218 regulated EMT, cell proliferation, and migration of GC through directly repressing the expression of WASF3. First, we investigated the expression of miR-218 in GC cell lines. Second, we examined the cell growth, migration, and EMT following overexpression or downregulation of miR-218 in GC cell lines. Finally, we determined the target gene of miR-218 using the luciferase reporter assay and Western blot.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human GC cell lines SGC7901 and BGC823 [American Type Culture Collection (ATCC), Rockville, MA, USA] and normal gastric epithelial cell line GES-1 (ATCC) were cultured at 37°C in a 5% CO_2 humidified atmosphere. Cells were maintained in RPMI-1640 (Gibco, New York, NY, USA) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Chemical Reagents and Antibodies

The total RNA was prepared from cells using the Takara RNAiso Plus 9108 kit, and the cDNA for qRT-PCR was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara Company, Tokyo, Japan). Anti-E-cadherin, anti-N-cadherin, anti-vimentin, and anti-TWIST1 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-WASF3 antibody was purchased from GenePharma (Shanghai, P.R. China). Anti-β-actin antibody was purchased from Beyotime Biotechnology (Shanghai, P.R. China). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Transfection

miR-218 mimics, miR-218 inhibitor (anti-miR-218), and the scrambled miRNA control (miR-con) were synthesized by GenePharma. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative Real-Time RT-PCR Analysis

The expression level of the target gene was detected with SYBR Green Real-Time RT-PCR on an ABI Prism 7500 sequence detection system (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. The primers used for amplification of the target genes are listed in Table 1. The expression of the target gene normalized to GAPDH, used as the internal control, was defined as $\Delta CT = (CT_{target} - CT_{GAPDH})$, and the difference in the relative expression of the target gene between cell lines was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

The total protein was extracted using RIPA lysis buffer containing proteinase inhibitors. The homogenates were clarified by centrifugation at $12{,}000 \times g$ for 15 min at 4°C. Nuclear protein was extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology) according to the manufacturer's protocols. Next, the protein concentration was measured using the bicinchoninic acid method. A total of 40 μg of protein mixed with 4× SDS loading buffer was loaded into each lane and separated by 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), and the membranes were incubated in the above-described primary antibodies, followed by incubation with HRP-conjugated secondary antibody (Zhongshan Bio Corp., Beijing, P.R. China). Protein expression was visualized using the SuperSignal protein detection kit (Pierce, Rockford, IL, USA). β-Actin was used as an internal control.

Cell Proliferation Assay

A total of 1×10^4 SGC7901 cells or BGC823 cells per well were plated into 96-well plates and were cultured for

Table 1. Primers for Quantitative Real-Time PCR

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
WASF3	TGCCTTTAGTGAAGAGGAACA	CAGCCCATCCTTCTTGTCAT
miR-218	AAGACACCCTGGACGAAGCC	ACAACCAGAGTCCACCGGCG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

24 and 48 h. Cell proliferation was assessed using MTT assay as described previously[19].

Wound Scratch Assay

SGC7901 and BGC823 cells transfected with miR-218 mimics, miR-con, and anti-miR-218 were seeded in six-well plates. On the following day, when the cells were approximately ≥90%, each well was scraped with a 10-μl pipette tip to create two linear regions devoid of cells. Subsequently, the cells in each well were cultured with RPMI-1640 medium (Gibco) containing no fetal bovine serum in a humidified incubator. We monitored wound closure at 0 and 24 h after scraping. Five random non-overlapping images of each well were selected and quantified for statistical analysis.

Luciferase Reporter Assay

SGC7901 and BGC823 cells (3.5×10^4) were seeded in triplicate in 24-well plates and allowed to settle for 24 h; 100 ng of pGL3-WASF3-3′-UTR (WT/MUT) or control-luciferase plasmid plus 1 ng pRL-TK Renilla plasmid (Promega, Madison, WI, USA) was transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Luciferase and Renilla signals were measured 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol.

miRNA Target Prediction

miRNA target prediction and analysis were performed by employing online database TargetScan (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de), and miRanda (http://www.microrna.org).

Statistical Analyses

All experiments were performed at least in triplicate. For comparisons between two groups, statistical significance was determined using the Student's *t*-test. Comparisons among three or more groups were performed using least significant difference (LSD) following analysis of variance (ANOVA). Values of $p<0.05$ were considered statistically significant.

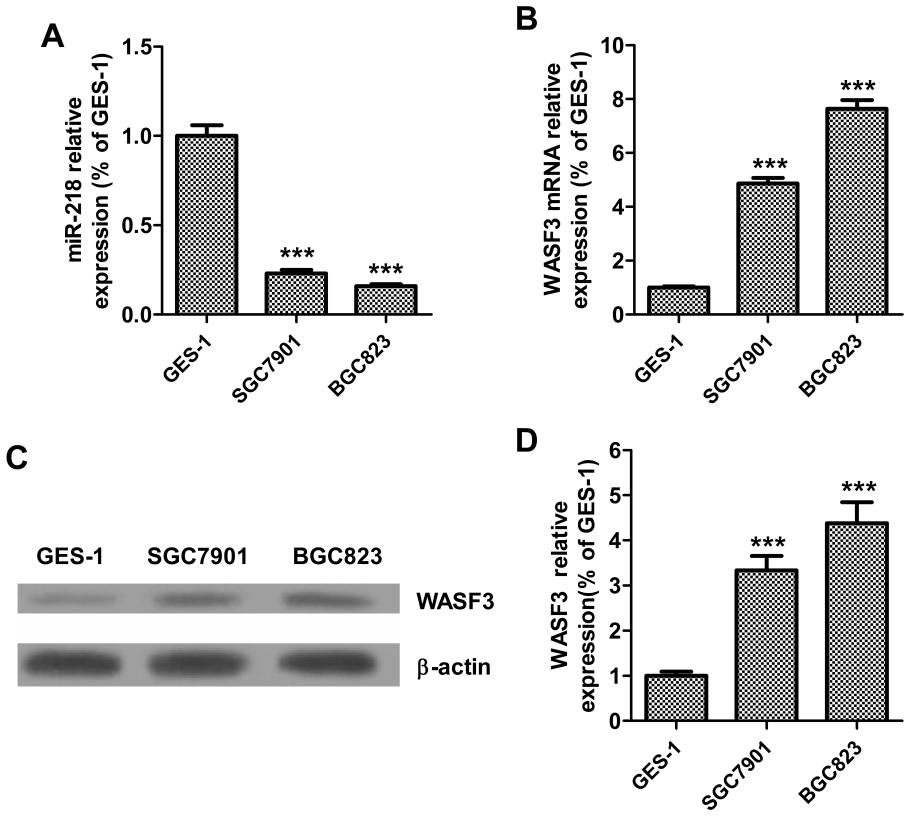
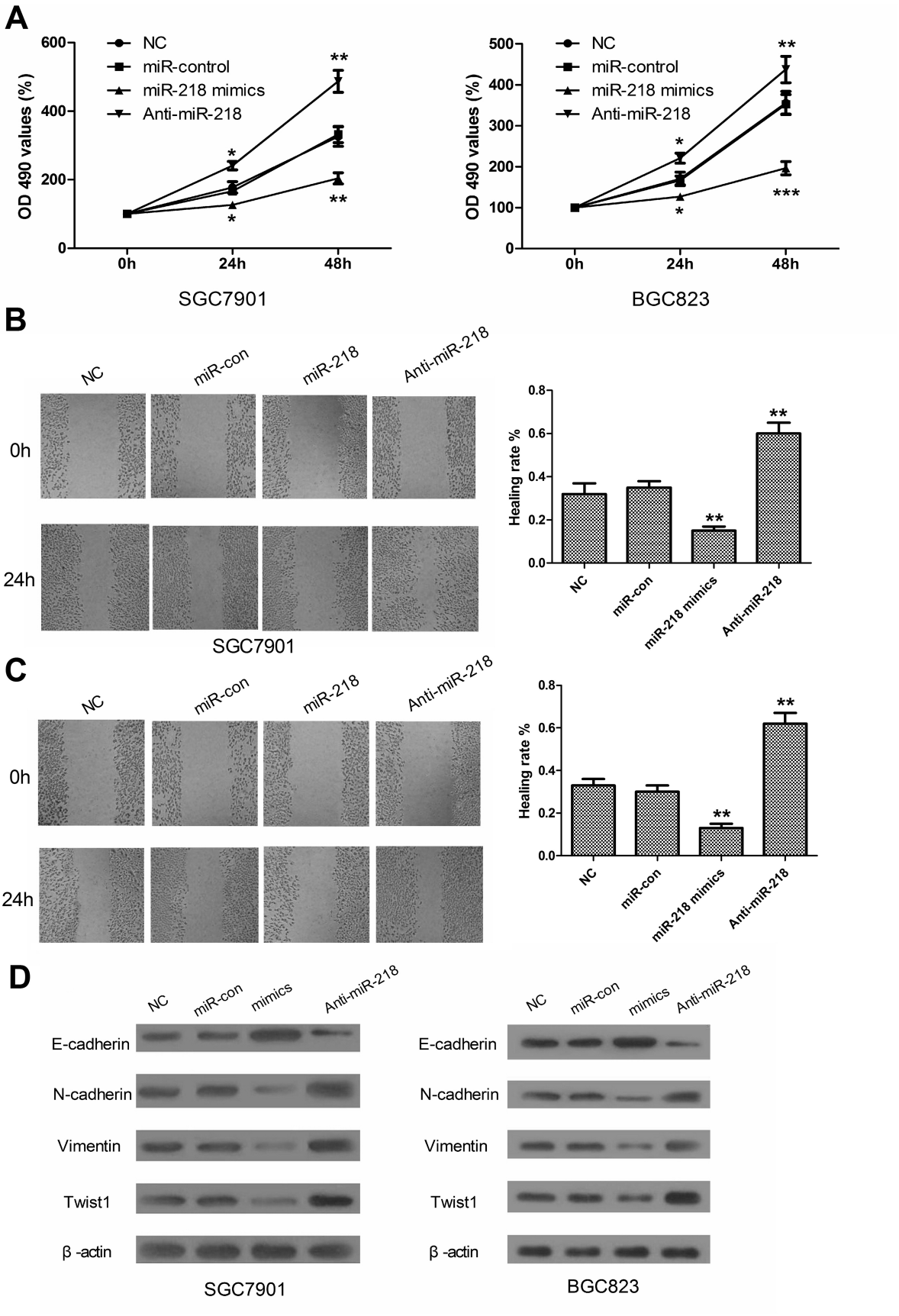


Figure 1. miR-218 is downregulated in gastric cancer cells, and WASF3 is overexpressed in gastric cancer cells. (A) Relative miR-218 levels analyzed by qRT-PCR were normalized with U6 snRNA in two gastric cancer cell lines (SGC7901 and BGC823) and a human normal gastric epithelial cell line (GES-1). (B) Relative levels of WASF3 mRNA were quantified by qRT-PCR and were normalized with GAPDH. (C, D) Expression status of WASF3 protein in two gastric cancer cell lines (SGC7901 and BGC823) and a human normal gastric epithelial cell line (GES-1). All data are presented as mean±SD. ***$p<0.001$.


A
NC
miR-control
miR-218 mimics
Anti-miR-218
OD 490 values (%)
0h
24h
48h
SGC7901
BGC823
B
NC
miR-con
miR-218
Anti-miR-218
0h
24h
SGC7901
Healing rate %
miR-218 mimics
C
D
mimics
E-cadherin
N-cadherin
Vimentin
Twist1
β -actin

RESULTS

The Expression Status of miR-218 and WASF3 in Gastric Cancer Cells

miR-218 is usually downregulated[20], and WASF3 is usually upregulated in GC[21]. To confirm the expression levels of miR218 and WASF3 in GC cells, we determined the expression of miR-218 and WASF3 in both SGC7901 and BGC823 cells compared with the normal gastric epithelial cell line GES-1. The qRT-PCR results showed that both SGC7901 and BGC823 cells had significantly lower miR-218 expression levels and higher WASF3 mRNA expression levels than the normal gastric epithelial cell line GES-1 (Fig. 1A and B). Western blot analysis results showed that compared with the normal gastric epithelial cell line GES-1, WASF3 expression levels were significantly increased in both SGC7901 and BGC823 cells (Fig. 1C and D).

miR-218 Inhibits Proliferation, Migration, and EMT of Gastric Cancer

To explore the role of miR-218 in the development of GC, SGC7901 and BGC823 cells were transfected with miR-218 mimics or inhibitor (anti-miR-218). The enforced expression of miR-218 led to a significant decrease in the proliferation of both SGC7901 and BGC823 cells (Fig. 2A). In contrast, anti-miR-218 significantly increased the proliferation of both SGC7901 and BGC823 cells. Cells treated with miR-218 mimics were distinctively less migratory in comparison to the scrambled miR-con or untreated cells (NC) 24 h after scratching (Fig. 2B and C). Cells transfected with the miR-218 mimics showed an increase in E-cadherin levels and a decrease in N-cadherin, vimentin, and TWIST1 levels compared to the miR-con or NC, but miR-218 inhibitor decreased the levels of E-cadherin and increased the levels of N-cadherin, vimentin, and TWIST1 compared to the miR-con or NC. These findings suggest that miR-218 inhibits the proliferation, migration, and EMT of GC cells.

WASF3 Is a Direct Target of miR-218 in Gastric Cancer Cells

WASF3 was a binding target of miR-218 predicted by the online databases, TargetScan, PicTar, and microcosm (Fig. 3A). To confirm that WASF3 was a direct target of miR-218, dual-luciferase reporter assays were performed, and the results showed that luciferase activity of wild-type 3′-UTR, but not mutant 3′-UTR, was significantly decreased by miR-218 (Fig. 3B and C). This regulation was dependent on specific sequences, as shown in Figure 3A. We performed qRT-PCR and Western blot to observe the expression of WASF3 in SGC7901 and BGC823 cells transfected with miR-218 mimics or inhibitor. qRT-PCR results showed that the expression levels of WASF3 were not affected by the changes of miR-218 levels (Fig. 3D). The protein level of WASF3 was remarkably decreased after upregulation of miR-218, but evidently increased after downregulation of miR-218 (Fig. 3E and F). Collectively, these results indicate that miR-218 reversely regulates WASF3 expression by directly targeting its 3′-UTR in GC cells.

Overexpression of WASF3 Impaired miR-218-Induced Inhibition of Proliferation, Migration, and EMT of Gastric Cancer Cells

We used rescue experiments to further validate that WASF3 targeting is involved in miR-218-induced antitumor properties in SGC7901 cells. WASF3 expression vector pcDNA-WASF3 was used to restore WASF3 expression. Overexpression of miR-218 decreased the levels of WASF3 protein, but the protein levels of WASF3 increased when cells were cotransfected with miR-218 mimics and pcDNA-WASF3 for 24 h compared with the miR-218 group or the miR-218 + pcDNA group (Fig. 4A). Overexpression of miR-218 inhibited the proliferation of SGC7901 cells, but this effect could be reversed by WASF3 upregulation 24 and 48 h after transfection (Fig. 4B). Overexpression of miR-218 inhibited the migration of cells, but reintroduction of WASF3

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Figure 2. Overexpression of miR-218 inhibits the proliferation, migration, and EMT of gastric cancer cells SGC7901 and BGC823. (A) miR-218 inhibited the viability of gastric cancer cells. The viability of SGC7901 and BGC823 cells transfected with miR-218 mimics and anti-miR-218 was measured using MTT assay 24 and 48 h after transfection. The data were analyzed by using the LSD test and shown as mean ± SEM. $*p<0.05$, $**p<0.01$, and $***p<0.001$ versus miR-con. (B, C) The relative ratio of wound closure per field was measured using wound healing assays, and the result showed that miR-218 attenuated the migration of SGC7901 and BGC823 cells. The data were analyzed using the LSD test and shown as mean ± SD. $**p<0.01$ versus miR-con. (D) Western blot analysis was used to determine the expression levels of E-cadherin, N-cadherin, vimentin, and TWIST1 in both SGC7901 and BGC823 cells transfected with miR-218 mimics, anti-miR-218, scramble, or no transfection, and the results showed that miR-218 increased the expression levels of E-cadherin and decreased the levels of N-cadherin, vimentin, and TWIST1 in gastric cancer cells. β-Actin was used as a loading control.

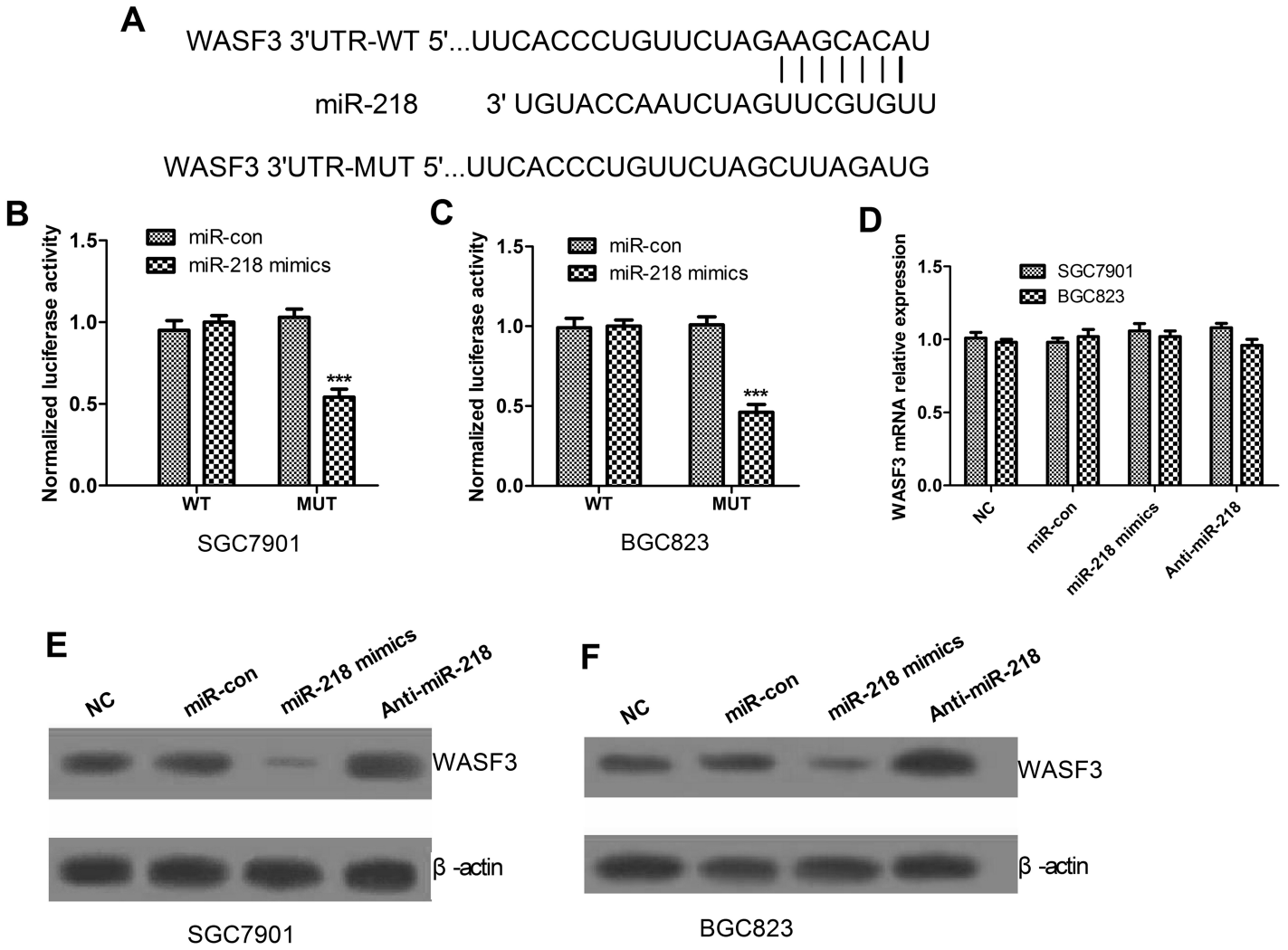

Figure 3. miR-218 targets WASF3 in gastric cancer cells. (A) The putative miR-218 binding sequence in the 3′-UTR of the WASF3 mRNA. WASF3 3′-UTR-WT represents the reporter constructs containing the wild-type 3′-UTR sequences of WASF3. WASF3 3′-UTR-MUT represents the reporter constructs containing mutated nucleotides. (B, C) Luciferase reporter assay showed that miR-218 markedly repressed the luciferase activity of SGC7901 and BGC823 cells transfected with the wild-type WASF3 compared to the negative control but did not inhibit the luciferase activity of the mutant luciferase reporter. The error bars are derived from triplicate experiments. (D) qRT-PCR analysis of WASF3 mRNA expression in both SGC7901 and BGC823 cells after transfection with miR-218 mimics, anti-miR-218, scramble, or no transfection. (E, F) Western blot analysis of WASF3 expression in both SGC7901 and BGC823 cells transfected with miR-218 mimics, anti-miR-218, scramble, or no transfection. β-Actin was detected as a loading control. The data were analyzed by using the LSD test and shown as mean±SD. ***$p<0.001$ versus miR-con.

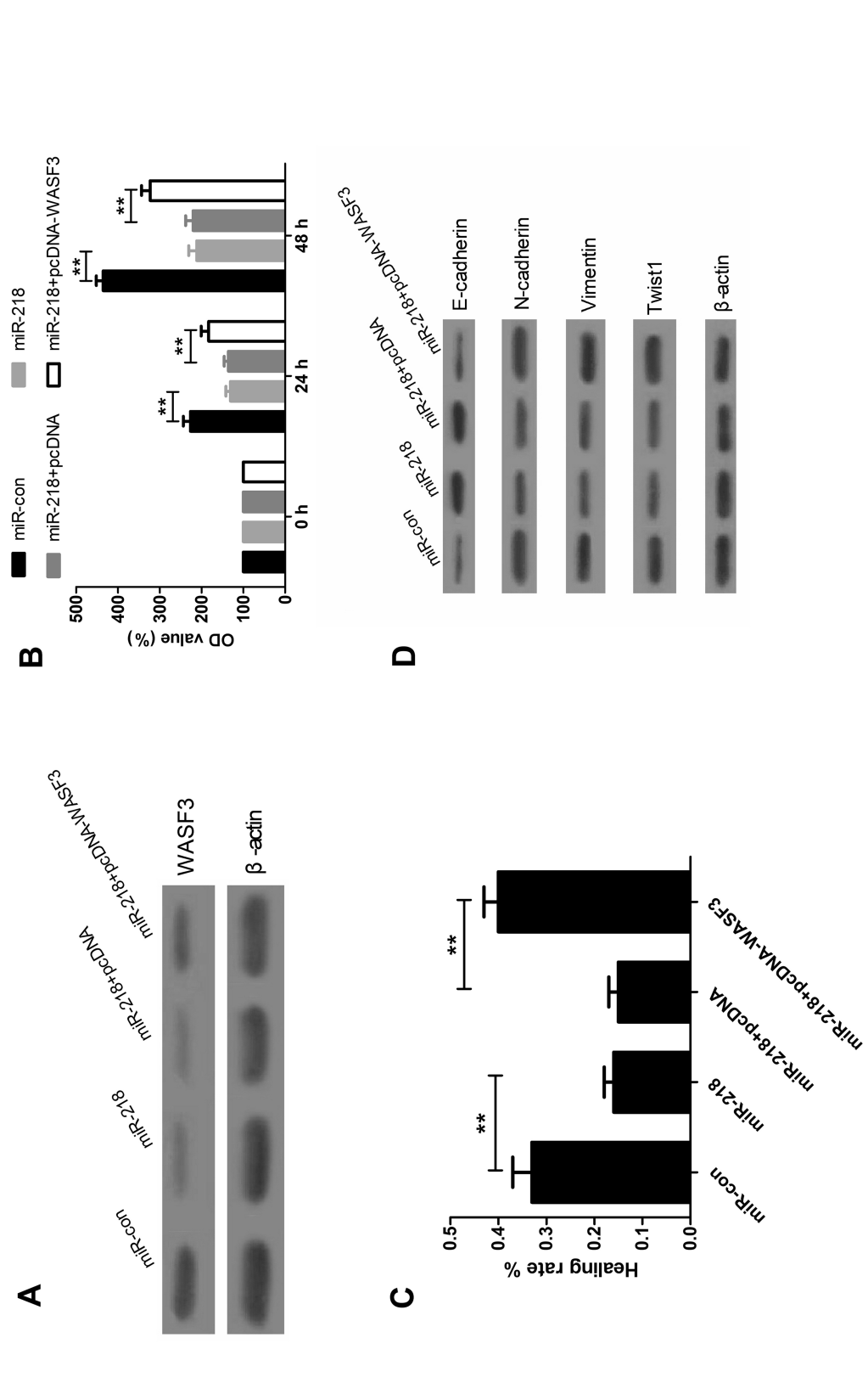

Figure 4. Restoration of WASF3 expression impairs miR-218-induced inhibition of proliferation, migration, and EMT in SGC7901 cells. (A) WASF3 expression status in SGC7901 cells transfected with miR-218 mimic or/and pcDNA-WASF3. (B) Cell growth curves in SGC7901 cells transfected with different combinations. Cell viability was assessed using MTT assay. The data were analyzed by using the LSD test and are shown as mean ± SEM. $^{**}p<0.01$. (C) Wound healing assays of SGC7901 cells after treatment with different combinations. The data were analyzed using the LSD test and are shown as mean ± SD. $^{**}p<0.01$. (D) Western blot analysis showed expression levels of E-cadherin, N-cadherin, vimentin, and TWISE1 in SGC7901 cells transfected with miR-218 mimics or/and pcDNA-WASF3. β-Actin was detected as a loading control.

resisted this effect (Fig. 4C). The level of E-cadherin was decreased, and the levels of N-cadherin, vimentin, and TWIST1 increased markedly when we restored the WASF3 expression using pcDNA-WASF3 (Fig. 4D). In total, these findings reveal that WASF3 is involved in miR-218-modulated proliferation, migration, and EMT of SGC7901 cells.

DISCUSSION

miRNAs are important regulators involved in different biological processes, such as cell proliferation, metastasis, differentiation, and tumorigenesis[5]. Recently, evidence has demonstrated the role of miRNAs in tumorigenesis and progression of GC, and miRNAs and their target genes have been proven to represent potential novel therapeutic biomarkers for GC[20,22–24]. miR-218 is one of the most known miRNAs. Several investigations have demonstrated that miR-218 acts as a tumor suppressor in GC, colon cancer, breast cancer, and some other cancers[25–27]. Although the tumor-suppressive effects in GC of miR-218 have been reported, the molecular mechanisms underlying the protective effects against GC are still not clear.

In this study, we analyzed the expression of miR-218 in the normal gastric epithelial cell line GES-1 and two GC cells SGC7901 and BGC823; we found that the level of miR-218 was lower in the GC cells SGC7901 and BGC823 compared to the normal gastric epithelial cell line GES-1. Furthermore, we proved that overexpression of miR-218 inhibited cell proliferation and migration. On the other hand, many studies have demonstrated that EMT plays a crucial role in the initial stage of metastatic progression of cancer cells[28–30]. In the EMT process, epithelial cells lose their characteristics, gain mesenchymal features, and become motile and invasive. Loss of epithelial marker E-cadherin and gain of mesenchymal markers N-cadherin, vimentin, and TWIST1 are regarded as the most important molecular markers of EMT[31]. Emerging evidence has demonstrated that many miRNAs play a crucial role in the EMT of cancer cells. For example, miR-23a regulated TGF-β-induced EMT in lung cancer cells[32], miR-194 repressed the EMT of endometrial cancer cells[33], and miR-134 inhibits EMT in NSCLC cells[34]. Therefore, we investigated the effects of miR-218 on the EMT of GC cells, and the results showed that miR-218 partly reversed the expression levels of epithelial marker and mesenchymal markers in GC cells SGC7901 and BGC823 and inhibited the EMT process of GC. These findings further proved that miR-218 functions as an important modulator of EMT in SGC7901 and BGC823 cells and plays a crucial role in the EMT of GC.

In addition, we investigated the effects of miR-218 on the proliferation and migration abilities of GC cells and found that miR-218 repressed cell proliferation and migration of GC cells SGC7901 and BGC823. Moreover, we identified that WASF3 was a direct target of miR-218. Identification of cancer-specific miRNAs and their targets is pivotal for understanding their roles in tumorigenesis. WASF3 was predicted to be one of the targets of miR-218 through employing online databases TargetScan, PicTar, and miRanda analysis. Furthermore, overexpression of miR-218 led to a significant reduction in WASF3 protein levels, and luciferase reporter assay proved that overexpression of miR-218 suppressed luciferase activity in cells with wild-type 3′-UTR of WASF3, and this effect was abolished by mutations of the miR-218 seed-binding sites. The function of WASF3 was further supported by the observations that overexpression or downregulation of WASF3 could enhance or inhibit cell proliferation and migration of GC cells. Cell proliferation induced by overexpression of miR-218 was significantly attenuated by reintroduction of WASF3. These results indicated that miR-218 might function as a tumor suppressor by directly repressing WASF3 expression in GC cells.

The WASF3 gene is a member of the Wiskott–Aldrich syndrome family of proteins (WASP), which contained verprolin–cofilin–acidic domains at their C-terminal ends[35]. These domains were thought to coordinate the recruitment of monomeric actin and the ARP2/3 complex of proteins to facilitate actin polymerization, which was essential for cell movement and migration. It has been shown that inactivation of the WASF3 gene in prostate cancer cells led to suppression of tumorigenicity and metastases[36]. Moreover, activation of WASF3 was required for metastasis and lamellipodia formation in some cancer cells, such as breast cancer[37]. WASF3 was previously shown to be required for metastasis in different cancer cell types, and knockdown of WASF3 led to suppression of metastasis and invasion[36,38]. In our study, we confirmed that WASF3 was upregulated in GC, and overexpression of WASF3 induced GC cell proliferation and migration and resulted in the loss of epithelial marker E-cadherin and gain of mesenchymal markers N-cadherin, vimentin, and TWIST1. However, the probable mechanisms were still not clearly confirmed. The ability of miR-218 to target WASF3 might provide one possible mechanism.

In this study, we found that restoration of miR-218 significantly inhibited cell proliferation and migration and repressed the cell EMT process in GC cell lines. We also indicated that downregulation of miR-218 significantly promoted cell proliferation, migration, and EMT ability in GC cell lines. On the basis of the association between deregulated miRNA expression and cancers, therapeutic strategies based on miRNAs were developing either by restoring the expression or reducing miRNA expression in cancer or, conversely, abrogating overexpressed miRNA. Consequently, our findings provided a molecular basis for the role of miR-218/WASF3 in the progression of human

GC and suggested that miR-218 could be a potential target for the treatment of GC in the future. In conclusion, our results suggested that miR-218 acted as a tumor suppressor, of which downregulation may contribute to the progression of GC by targeting WASF3.

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