

# MiR-548d-3p Promotes Gastric Cancer by Targeting RSK4

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**Purpose:** Previous studies have demonstrated that RSK4 inhibits the proliferation of gastric cancer cells and the occurrence of tumors. However, to date, studies involving microRNAs (miRNAs) that target RSK4 have rarely been reported. Thus, this study aimed to investigate the miRNAs that target RSK4.

**Materials and Methods:** We screened miRNAs related to RSK4 in miRDB, microT-CDS, TargetScan, and miRDP databases and found 18 miRNAs. We chose miR-548d-3p for follow-up research, identified the interaction site in RSK4 by comparing the sequence, and mutated it. Thereafter, we used the dual-luciferase reporter system, real-time PCR (RT-PCR), and Western blotting to assess the effect of miR-548d-3p on RSK4. The proliferation, apoptosis, migration, and invasion of gastric cancer cells were evaluated using MTT assay, propidium iodide (PI), EdU, annexin V-FITC/PI apoptosis detection kit, wound healing assay, and transwell assay after overexpression of miR-548d-3p and RSK4. Finally, a nude mouse tumorigenesis experiment was conducted to explore the role of RSK4-targeting miR-548d-3p in tumorigenesis.

**Results:** miR-548d-3p negatively regulated the expression of RSK4, resulting in suppressed apoptosis, enhanced proliferation, migration, and invasion of gastric cancer cells, and accelerated tumor growth. In addition, an increase in miR-548d-3p expression enhanced the mRNA levels of CDK2, cyclin A1, cyclin D1, Bax, Bcl-2, N-cadherin, and Vimentin, and decreased E-cadherin mRNA levels by targeting RSK4.

**Conclusion:** miR-548d-3p promotes gastric cancer by lowering the expression of RSK4.

**Keywords:** microRNA, gastric cancer, RSK4, miR-548d-3p

## Introduction

Gastric cancer is the fifth most common malignant tumor worldwide.<sup>1</sup> Despite the decline in the incidence rate over the past century, its mortality rate in East Asia ranks third among cancer-related deaths.<sup>1-4</sup> The main reason for the poor prognosis of gastric cancer is the lack of early diagnostic methods.<sup>3</sup> The disease is usually diagnosed when tissue infiltration and distant metastasis have occurred, resulting in an average 5-year survival rate of less than 20% for patients in most parts of the world.<sup>1,3,5</sup> Therefore, gastric cancer is still a clinical challenge for which effective early diagnosis targets and new therapeutic targets must be detected.<sup>1</sup> Exploring the genes that regulate gastric cancer may assist in its early diagnosis and treatment.<sup>3,6,7</sup>

MicroRNA (miRNA) is a short single-stranded RNA molecule that can regulate the expression of genes and proteins.<sup>8-10</sup> miRNA binds to the 3'-untranslated region (UTR) of mRNA, increasing mRNA degradation and leading to downregulation of protein expression in the cytoplasm.<sup>8,11</sup> Therefore, the expression of certain

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miRNAs is highly correlated with cancer.<sup>8,12</sup> For example, miR-548d inhibits the progression of pancreatic cancer, miR-21 contributes to the occurrence of lung cancer, highly expressed miR-21 has been verified as important diagnostic markers for patients with breast and colon cancer, miR-200c and ovarian cancer are related, and the combination of hsa-miR-375 and hsa-miR-142-5p can be used to predict the risk of recurrence in patients with gastric cancer.<sup>8,13–17</sup>

RSK4 belongs to the p90 ribosomal S6 kinases (RSKs) family, and is known as RPS6KA6, which is involved in cell proliferation, invasion, and metastasis.<sup>1,18–23</sup> RSK4 has a tumor-suppressing effect in colon, breast, and kidney cancer.<sup>1,24–27</sup> Overexpressed RSK4 suppresses cell growth, invasion, and metastasis.<sup>1,28,29</sup> Moreover, some researchers recommend RSK4 as a marker for evaluating the prognosis of patients.<sup>1,30</sup> However, in some breast cancer tissues, the expression of RSK4 is higher than that in neighboring tissues, and whether RSK4 exerts a carcinogenic or a tumor suppressor effect depends on many factors.<sup>1,31,32</sup> Moreover, our previous study has shown that the expression of RSK4 has an inhibitory effect on the progression of gastric cancer.<sup>1</sup> However, reports on miRNAs that target RSK4 in gastric cancer are limited. The present study aimed to explore the biological functions of RSK4-targeting miRNAs in gastric cancer to provide a basis for its role in tumor therapy.

## Materials and Methods

### Cell Lines

Stomach cancer cell lines, HGC-27 (IMMOCELL, catalog number: IM-H085, Xiamen, China) and MGC-803 (IMMOCELL, catalog number: IM-H086, Xiamen, China), and 293T (IMMOCELL, catalog number: IM-H086, Xiamen, China) were cultured in Dulbecco's modified eagle medium (DMEM, IMMOCELL, catalog number: IMC-201, Xiamen, China) supplemented with 10% fetal bovine serum (FBS, IMMOCELL, catalog number: IMC-101, Xiamen, China) and 1% penicillin-streptomycin (IMMOCELL, catalog number: IMC-601, Xiamen, China).

### Screening of miRNAs That Target RSK4

The miRNA sequences that are related with RSK4 were downloaded from microT-CDS, miRDB, TargetScan, and mirDIP databases, and analyzed the distribution of these miRNAs in these databases using the UpSetR package in

R software (version 3.5.1). Using survminer package (version 0.4.8) in R software, patients with gastric cancer from The Cancer Genome Atlas (TCGA) were divided into two groups: miR-548d-3p high expression and miR-548d-3p low expression. The Kaplan–Meier (KM) curve for the miR-548d-3p level and the disease-free survival was plotted using survival package (version 3.1–12) in R software.

### Plasmid Construction

pCDH-EF1 $\alpha$ -T2A-puro vector (Anti-HeLa BioTech, Xiamen, China) was used to prepare the plasmid expressing RSK4 (Gene ID: 27,330), named RSK4 plasmid, pCDH-EF1 $\alpha$ -T2A-blasticidin vector (Anti-HeLa BioTech, Xiamen, China) and miR-548d precursor were used to prepare the plasmid expressing miR-548d, named miR-548d plasmid, and pmriGLO vector (Anti-HeLa BioTech, Xiamen, China) was used to construct the plasmid expressing wild-type or mutant 3'UTR of RSK4, named 3'UTR WT and 3'UTR MUT, respectively. The primers were designed by DNAMAN 10.0 and are listed in Table 1.

### Dual-Luciferase Reporter Assay

HGC-27 and MGC-803 were used to performed dual-luciferase reporter assay through transfected 3'UTR WT or 3'UTR MUT with the negative control of miR-548d-3p mimic (mimic NC) or miR-548d-3p mimic for 48 h using Exfect 2000 Transfection Reagent (Vazyme, catalog number: T202-01, Nanjing, China) in accordance with the manufacturer's instructions. The dual Luciferase Reporter Assay Kit was bought from Vazyme (catalog number: DL101-01, Nanjing, China).

### Construction of Cell Lines Stably Overexpressing RSK4 or/and miR-548d

RSK4 plasmid, miR-548d plasmid, pCDH-EF1 $\alpha$ -T2A-puro vector or pCDH-EF1 $\alpha$ -T2A-blasticidin vector, and the suitable packaging plasmid (pMD2G, pspax2) were co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen, catalog number: 11,668,019, Waltham, Massachusetts, USA) to prepare lentiviral particles. After 48 h, the lentivirus-containing medium was collected, and the particles were termed RSK4 lentivirus, miR-548d lentivirus, pCDH-EF1 $\alpha$ -T2A-puro lentivirus, and pCDH-EF1 $\alpha$ -T2A-blasticidin lentivirus, respectively.

HGC-27 cells were classified into Vector, miR-548d, and miR-548d+RSK4 cells. Vector cells were infected

**Table 1** Primers for Plasmid Construction

Name	Sequence (5'-3')
3'UTR WT-F	GAGCTCGCTAGCCTCGAGTTGTATTACATTTCTAAAC
3'UTR WT-R	TGCATGCCTGCAGGTCGACCTGGGATTACAGGCATGAG
3'UTR MUT-F	GATTAGGAATGAAATATCTTTCTATGAAC
3'UTR MUT-R	AAGATATTTTCATTCTAATCAGTCACATTTTG
miR-548d-F	ATTCACGCGTGC GGCCGCCGACCCGGCCAGTATTAAG
miR-548d-R	TAGGGATCCGGGCCCCGGGAAGATTCACATAATGCTC
RSK4-F	ACTACAAAGACGATGACGACAAGATGCTACCATTGCTCCTC
RSK4-R	CTCAGCGGCCGCGGATCCCAGGCCAGTTGATGTTTCGC

**Abbreviations:** F, forward primer; R, reverse primer.

with pCDH-EF1 $\alpha$ -T2A-blasticidin lentivirus, whereas miR-548d and miR-548d+RSK4 cells were infected with miR-548d lentivirus. After 48 h, the medium was replaced with fresh medium containing 15.0  $\mu$ g/mL blasticidin S (Gibco, catalog number: A1113902, Shanghai, China). After 2 weeks, the cells were collected for analysis of miR-548d levels. When the levels of miR-548d in miR-548d and miR-548d+RSK4 cells were significantly higher than those in Vector cells, miR-548d and Vector cells were infected with pCDH-EF1 $\alpha$ -T2A-puro lentivirus, and miR-548d+RSK4 cells were infected with RSK4 lentivirus. After 48 h, the medium was replaced with fresh medium containing 2.5  $\mu$ g/mL puromycin (Gibco, catalog number: A1113803, Shanghai, China). After 1 week, cells were collected for miR-548d and RSK4 level analysis.

### Real-Time PCR (RT-PCR)

RNA isolation Total RNA Extraction Reagent (Vazyme, catalog number: R401-01, Nanjing, China) was used to extract total RNA from cells or tissues. After reverse transcription of RNA into cDNA, RT-PCR was performed using cDNA as described previously.<sup>33</sup> Primers for RT-PCR, designed using DNAMAN 10.0, are presented in Table 2.

### Western Blotting

Ice-cold RIPA buffer (Cell Signaling Technology, Catalog No.: 9806, Shanghai, China) was used to extract proteins from cells or tissues. After protein quantification with BCA Protein Quantification Kit (Vazyme, Catalog No.: E112-01, Nanjing, China), Western blot analysis was performed as previously described.<sup>33</sup> Anti-RSK4 antibody

(Abcam, catalog number: ab76117, 1:1000, Shanghai, China) and anti-GAPDH antibody (Abcam, catalog number: ab8245, 1:2000, Shanghai, China) were used as primary antibodies. Goat anti-mouse IgG (Abcam, catalog number: ab6789, 1:2000, Shanghai, China) and goat anti-rabbit IgG (Abcam, catalog number: ab6721, 1:2000, Shanghai, China) were used as secondary antibodies conjugated with horseradish peroxidase.

### Cell Proliferation Analysis

MTT, EdU, and cell cycle assays were used to analyze the cell proliferation. After HGC-27 and MGC-803 cell lines were transfected mimic NC or miR-548d-3p mimic with RSK4 plasmid or the negative control for 24 h (for EdU assay and cell cycle assay) or different time points: 24, 48, and 72 h (for MTT assay). MTT (Beyotime, catalog number: ST316, Shanghai, China), EdU (Beyotime, catalog number: ST067, Shanghai, China), and propidium iodide (PI) (Beyotime, catalog number: ST511, Shanghai, China) were used to detect cell proliferation as previously described.<sup>33</sup>

### Apoptosis Assay

Cells treated as described above were used to perform apoptosis assay with annexin V-FITC/PI apoptosis detection kit purchased from Beyotime (catalog number: C1062M, Shanghai, China) as previously described.<sup>33</sup>

### Cell Migration and Invasiveness Analysis

After cell treatment, wound healing assay, Matrigel-free transwell plates, transwell plates with Matrigel, and

**Table 2** Primers for RT-PCR

Name	Sequence (5'–3')	Gene ID
miR-548d-3p	CAAAAACCACAGUUUCUUUUGC	693,131
miR-548d-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACGCAAAA	
miR-548d-3p-QF	CGCGCAAAAACCACAGTTTC	
miR-548d-3p-QR	AGTGCAGGGTCCGAGGTATT	
RNA18SN5-QF	ACCCGTTGAACCCCATTCGTGA	100,008,588
RNA18SN5-QR	GCCTCACTAAACCATCCAATCGG	
RSK4-QF	CCTCCTTTCAAACCTGCTTCTGG	27,330
RSK4-QR	GCTGATGAGCATTTGCACTGGC	
U6-QF	CGCTTCGGCAGCACATATAC	26,827
U6-QR	AAATATGGAACGCTTCACGA	
CDK2-QF	ATGGATGCCTCTGCTCTCACTG	1017
CDK2-QR	CCCGATGAGAATGGCAGAAAGC	
Cyclin A1-QF	GCACACTCAAGTCAGACCTGCA	8900
Cyclin A1-QR	ATCACATCTGTGCCAAGACTGGA	
Cyclin D1-QF	TCTACACCGACAACCTCCATCCG	595
Cyclin D1-QR	TCTGGCATTCTTGAGAGGAAGTG	
Bax-QF	TCAGGATGCGTCCACCAAGAAG	581
Bax-QR	TGTGTCCACGGCGGCAATCATC	
Bcl-2-QF	ATCGCCCTGTGGATGACTGAGT	596
Bcl-2-QR	GCCAGGAGAAATCAAACAGAGGC	
E-cadherin-QF	GCCTCCTGAAAAGAGAGTGGAAG	999
E-cadherin-QR	TGGCAGTGTCTCTCCAAATCCG	
N-cadherin-QF	CCTCCAGAGTTTACTGCCATGAC	1000
N-cadherin-QR	GTAGGATCTCCGCCACTGATTC	
Vimentin-QF	AGGCAAAGCAGGAGTCCACTGA	7431
Vimentin-QR	ATCTGGCGTTCCAGGGACTCAT	

**Abbreviations:** RT, reverse transcription; QF, forward primer for RT-PCR; QR, reverse primer for RT-PCR.

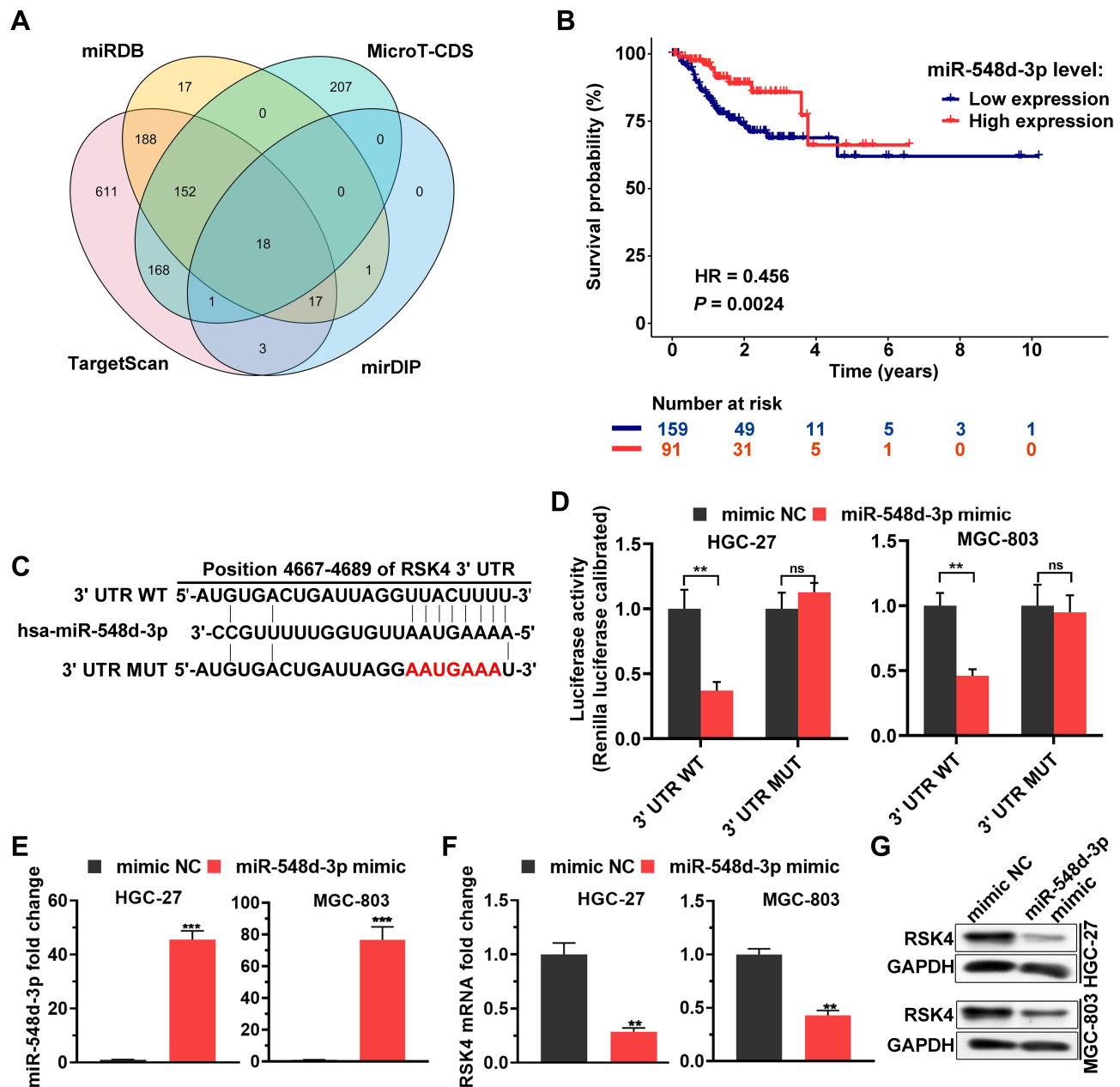
transwell assay were performed for cell migration and invasiveness analyses.<sup>33</sup>

## Nude Mouse Tumorigenicity Assay

HGC-27 cells stably overexpressing miR-548d or both miR-548d and RSK4 were used for tumor formation experiments in nude mice. Fifteen nude mice purchased from Shanghai Experimental Animal Research Center (Shanghai, China) were divided equally into three groups:

Vector, miR-548d, and miR-548d+RSK4. Nude mice in the Vector group were injected subcutaneously with negative control HGC-27 cells (Vector cells), nude mice in the miR-548d group were injected subcutaneously with HGC-27 cells stably and highly expressing miR-548d (miR-548d cells), and nude mice in the miR-548d+RSK4 group were injected subcutaneously with HGC-27 cells stably and highly expressing of miR-548d and RSK4 (miR-548d +RSK4 cells). The tumor size was measured on the 18th





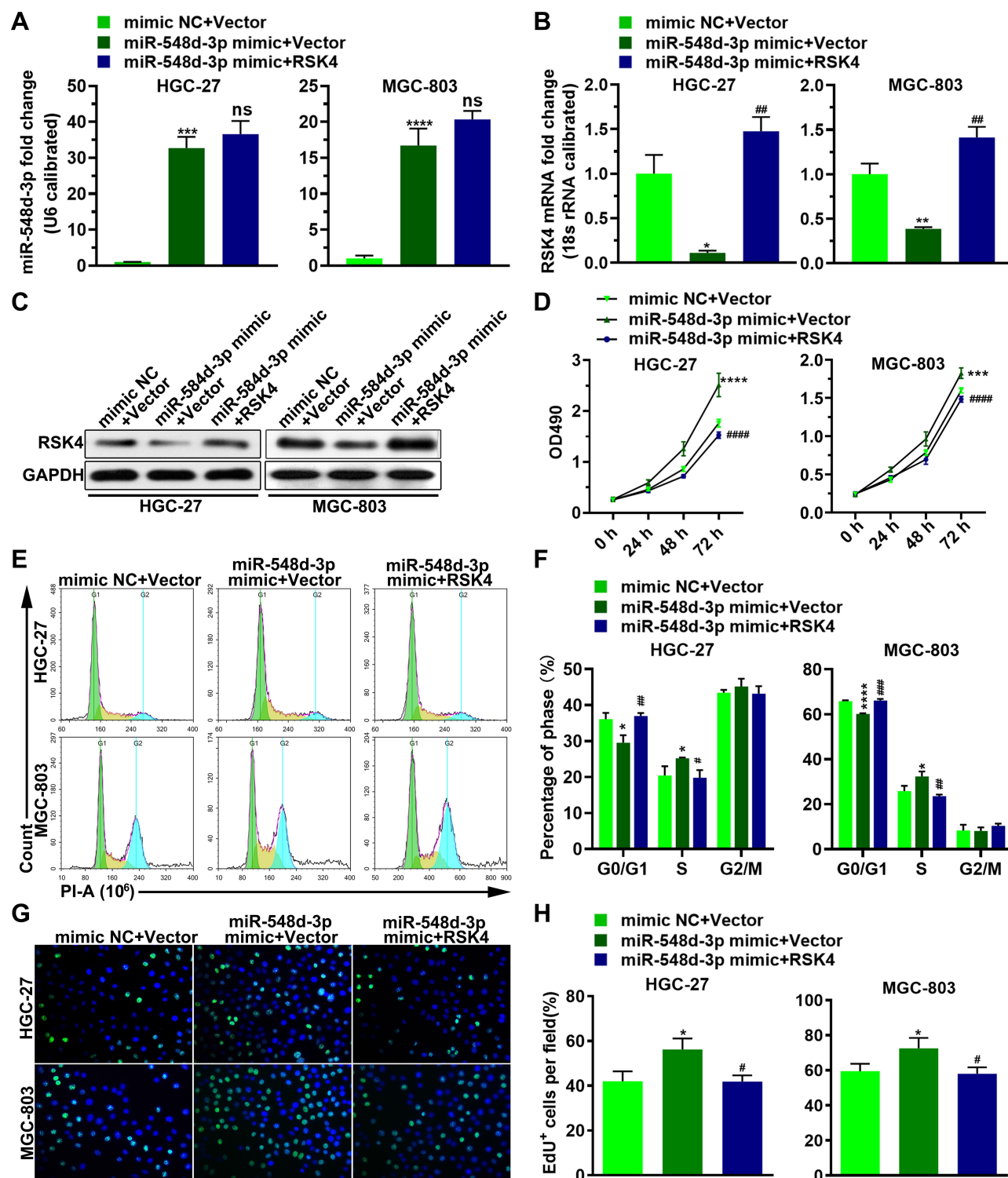
**Figure 1** miR-548d-3p negatively regulates RSK4. (A) The distribution of miRNAs related to RSK4 in microT-CDS, miRDB, TargetScan, and mirDIP databases. (B) KM curve was used to analyze the relationship between the miR-548d-3p level and disease-free survival in gastric cancer patients. (C) Schematic diagram of RSK4 3'UTR mutation site. (D) The dual-luciferase report assay verified the regulation of RSK4 by miR-548d-3p and the sites where miR-548d-3p targets RSK4. (E–G) After miR-548d-3p mimic and mimic NC were separately transfected into HGC-27 or MGC-803 cells, the RNA level of miR-548d-3p and RSK4 (E and F), and the protein level of RSK4 (G) were detected by RT-PCR and Western blotting, respectively. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

**Abbreviation:** Ns, not significant.

day after the nude mice were injected with the cells, thereafter, tumor size was measured every 4 days. On the 38th day after the injection of the cells, the nude mice were euthanized in a euthanasia chamber with carbon dioxide at a flow rate of 30–70% chamber volume per minute. Subsequently, the tumor was removed and weighed, and the levels of miR-548d and RSK4 were measured.

## Statistical Analysis

Student's *t*-test (unpaired) and analysis of variance (ANOVA) were used to perform comparisons between two groups and comparisons between multiple groups, respectively, using SPSS software (version 22.0).  $P < 0.05$  indicates a significant difference. GraphPad Prism software (version 8.2.1) was used to draw the graph.



**Figure 2** miR-548d-3p promotes cell proliferation by decreasing the expression of RSK4. HGC-27 and MGC-803 cell lines were transfected with mimic NC or miR-548d-3p mimic with RSK4 plasmid or the RSK4 plasmid negative control for 24 h. (A–C) The RNA level of miR-548d-3p and RSK4 (A and B), and the protein level of RSK4 (C) were determined by RT-PCR and Western blotting, respectively. (D–H) Cell proliferation was analyzed by MTT assay (D), cell cycle assay (E and F), and EdU assay (G and H). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs mimic NC + Vector. # $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$ , ##### $p < 0.0001$  vs miR-548d-3p mimic + Vector. Abbreviation: ns, not significant; PI, propidium iodide.

## Results

### MiR-548d-3p Negatively Regulates RSK4

To screen for miRNAs that target RSK4, miRNAs related to RSK4 were downloaded from the microT-CDS, miRDB, TargetScan, and mirDIP databases. Eighteen miRNAs associated with RSK4 were present in all four databases, including miR-548d-3p (Figure 1A). Furthermore, KM curve analysis suggested that the level of miR-548d-3p is associated with disease-free survival of patients with gastric cancer (Figure 1B). Subsequently, we identified the site of RSK4 targeted miR-548d-3p and mutated it (Figure 1C). The site was verified by dual-luciferase reporter assay, and it was found that miR-548d-3p suppressed RSK4 transcription (Figure 1D). RT-PCR and Western blot analysis showed that miR-548d-3p also reduced RSK4 mRNA and protein levels (Figure 1E–G). These findings indicate that miR-548d-3p inhibits RSK4 expression.

### MiR-548d-3p Promotes the Proliferation of Gastric Cancer Cells by Decreasing the Expression of RSK4

To investigate the effect of RSK4-targeting miR-548d-3p on the biological process of gastric cancer cells, we co-transfected miR-548d-3p mimic and RSK4 plasmids into HGC-27 and MGC-803. The results showed that miR-548d-3p attenuated the level of RSK4, and after supplementing RSK4, the level of RSK4 was restored (Figure 2A–C). MTT, EdU and cell cycle analysis revealed that overexpression of miR-548d-3p increased cell proliferation and accelerated mitosis, while reparative RSK4 suppressed the cell proliferation induced by miR-548d-3p (Figure 2D–H). These results indicate that miR-548d-3p enhances proliferation by targeting RSK4 in gastric cancer cells.

### RSK4-Targeting miR-548d-3p Weakens Apoptosis and Strengthens Cell Migration and Invasion

Next, we investigated the role of miR-548d-3p in apoptosis, migration, and invasion. By using the annexin V-FITC/PI apoptosis detection kit, wound healing assay, and transwell assay, it was found that overexpression of miR-548d-3p reduced the apoptosis rate and increased the number of migrating and invading cells, while supplementing the RSK4 heightened apoptosis rate and led to a decline in

the number of migrating and invading cells (Figures 3 and 4). These data suggest that miR-548d-3p suppresses RSK4 expression, weakening apoptosis and intensifying the ability of cell migration and invasion.

### RSK4-Targeting miR-548d-3p Regulates the mRNA Levels of Molecules Related to Cell Cycle, Apoptosis, and Epithelial-Mesenchymal Transition

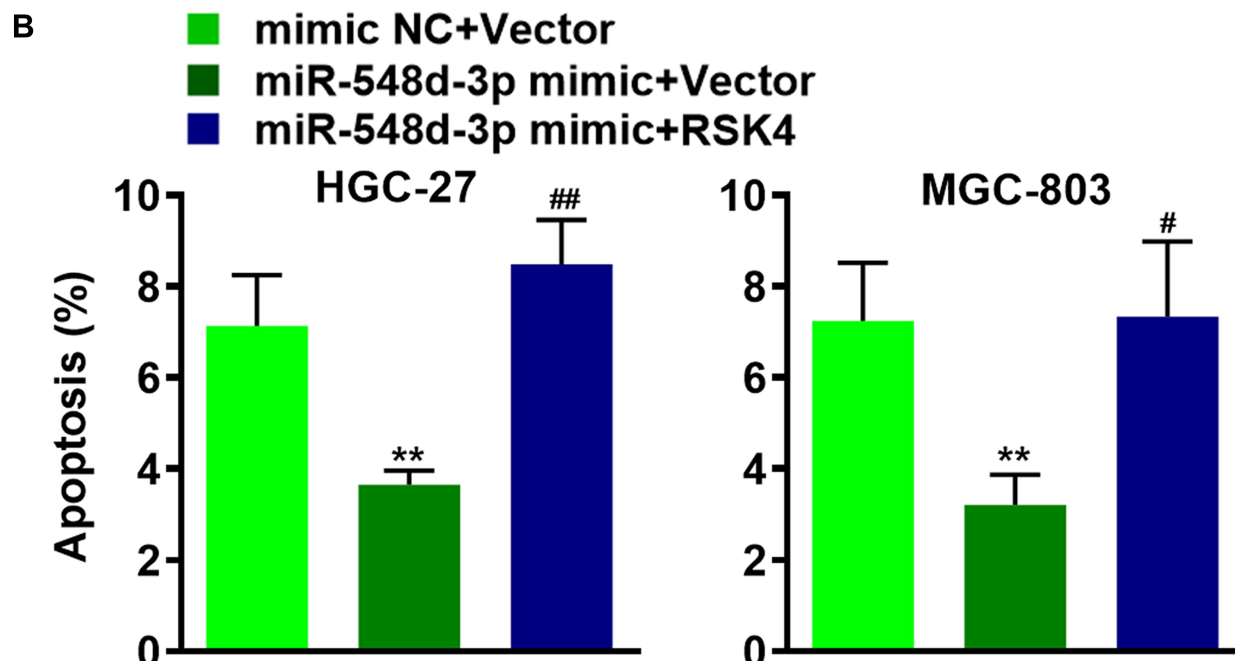
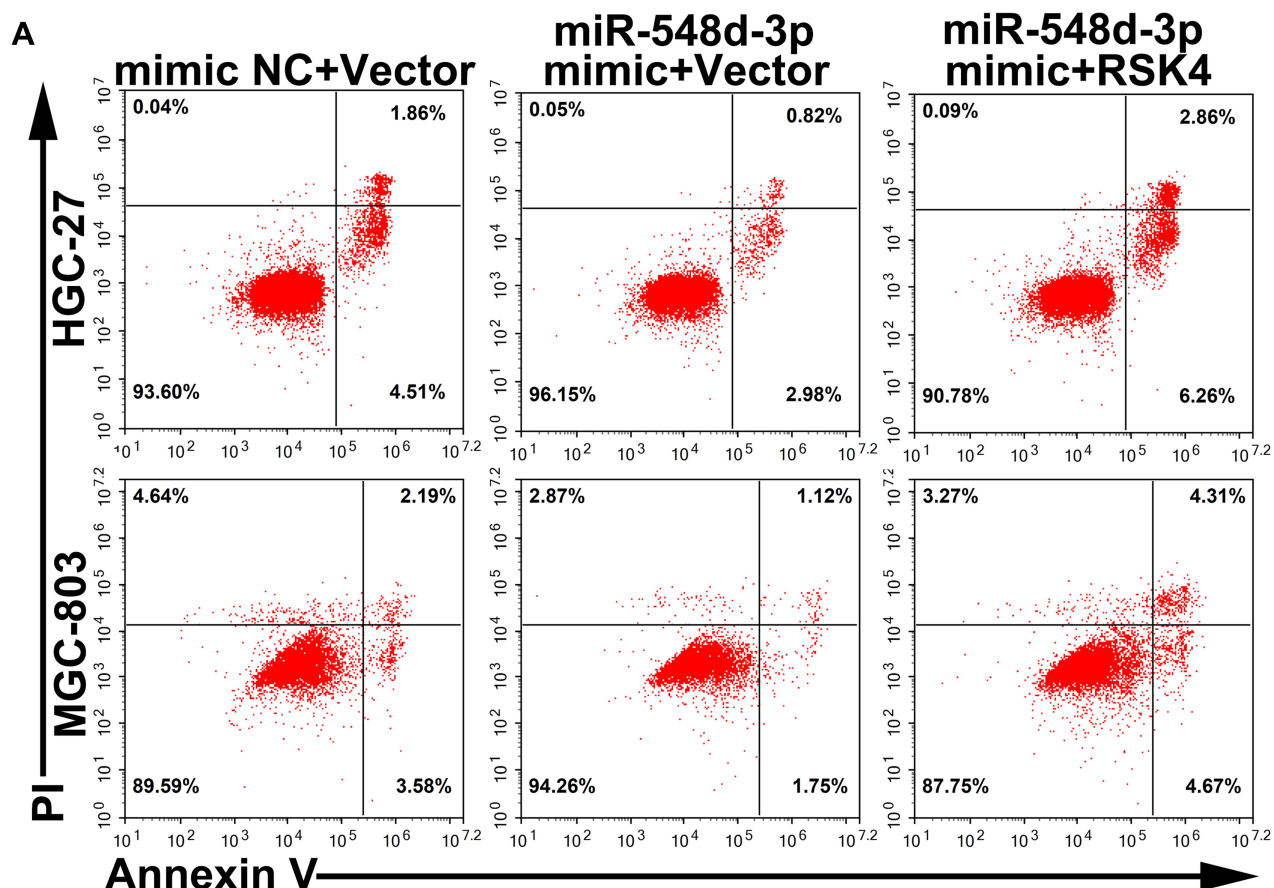
We further verified the regulatory effects of miR-548d-3p on molecules related to cell cycle, apoptosis, and epithelial-mesenchymal transition. Overexpression of miR-548d-3p increased the mRNA levels of CDK2, cyclin A1, cyclin D1, Bax, Bcl-2, N-cadherin, and Vimentin, and decreased the mRNA level of E-cadherin. After supplementing RSK4, these effects of miR-548d-3p were alleviated (Figure 5), thus indicating that miR-548d-3p regulates the expression of other molecules by inhibiting the expression of RSK4, thereby regulating the cell cycle, cell apoptosis and cell epithelial-mesenchymal transition.

### MiR-548d Promotes Tumor Growth by Targeting RSK4

To investigate the RSK4-regulating role of miR-548d in tumors, we constructed cell lines with stable overexpression of miR-548d or stable overexpression of both miR-548d and RSK4 (Figure 6A and B). The results showed that the tumors formed in the miR-548d group were larger than those in the negative control and the miR-548d+RSK4 groups (Figure 6C) and had the fastest growth rate (Figure 6D). The weight of tumors formed in the miR-548d group was also higher than that in the other two groups (Figure 6E). The RT-PCR and Western blot experiments proved that in the formed tumors, the miR-548d levels of the miR-548d and the miR-548d+RSK4 groups were similar, while the RSK4 mRNA and protein levels of the miR-548d group were significantly lower than those of miR-548d+RSK4 group (Figure 6F–I). These results indicate that miR-548d negatively regulates RSK4 to accelerate tumor growth.

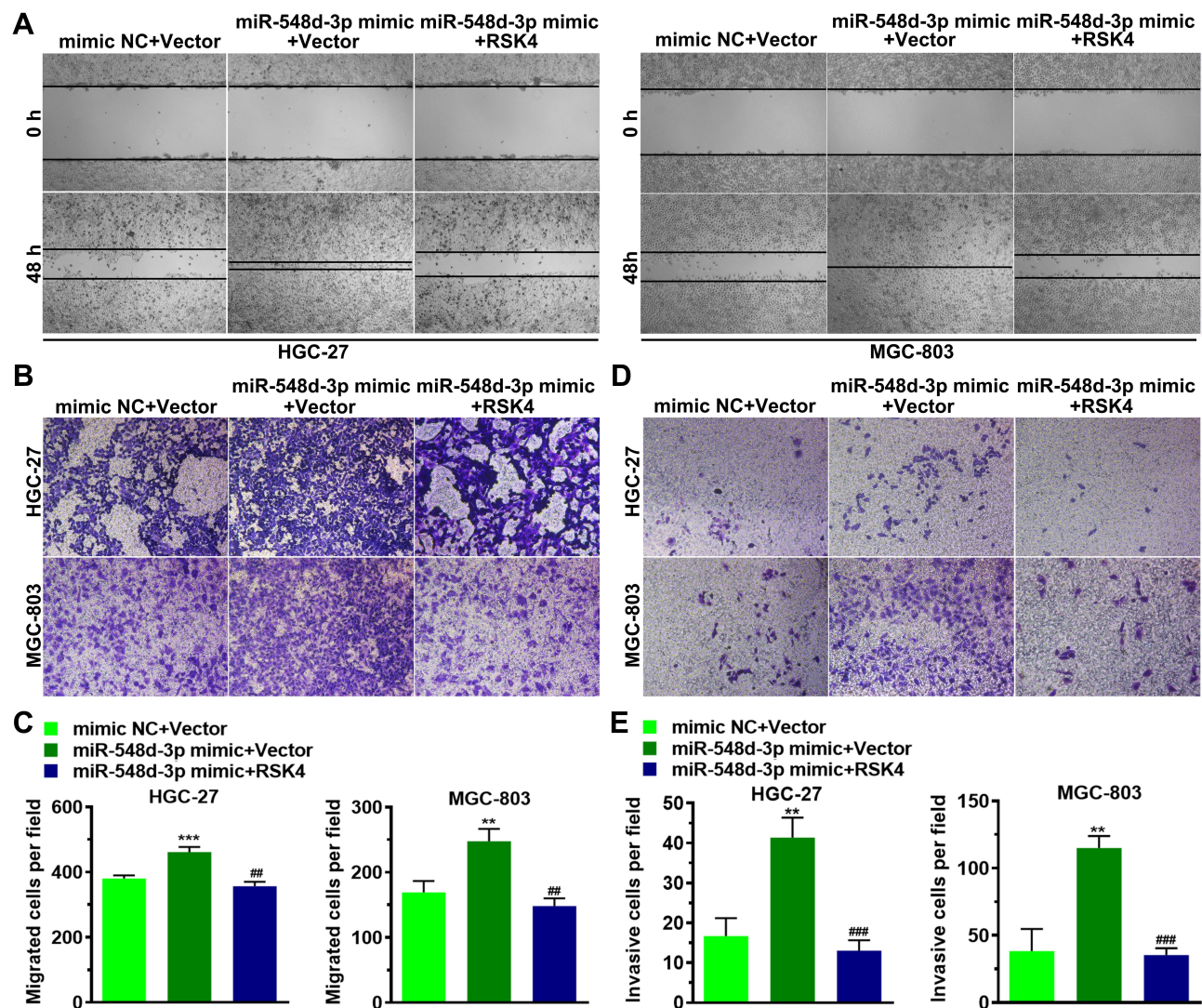
## Discussion

Gastric cancer is a common malignant tumor and one of the main causes of cancer-related deaths.<sup>3</sup> Non-coding RNA is dysregulated in the process of carcinogenesis and is considered useful in the diagnosis and treatment of cancer.<sup>3,34</sup> miRNAs, a type of non-coding small RNA with 18 to 25



**Figure 3** RSK4-targeting miR-548d-3p weakens apoptosis. **(A)** After HGC-27 and MGC-803 cells were treated as described above, apoptosis was detected using annexin V-FITC/PI apoptosis detection kit. **(B)** Histogram of apoptosis percentage. \*\* $p < 0.01$  vs mimic NC + Vector; # $p < 0.05$ , ## $p < 0.01$  vs miR-548d-3p mimic + Vector. **Abbreviation:** PI, propidium iodide.





**Figure 4** RSK4-targeting miR-548d-3p strengthens cell migration and invasion. HGC-27 and MGC-803 cells were treated as described above. (A and B) Cell migration was measured by wound healing assay (A) and transwell assay (B). (C) Histogram of migrated cells per field tested by transwell assay. (D) Invasiveness of cell was tested by using transwell assay. (E) Histogram of invasive cells per field tested by transwell assay. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs mimic NC + Vector; ## $p < 0.01$ ; ### $p < 0.001$  vs miR-548d-3p mimic + Vector.

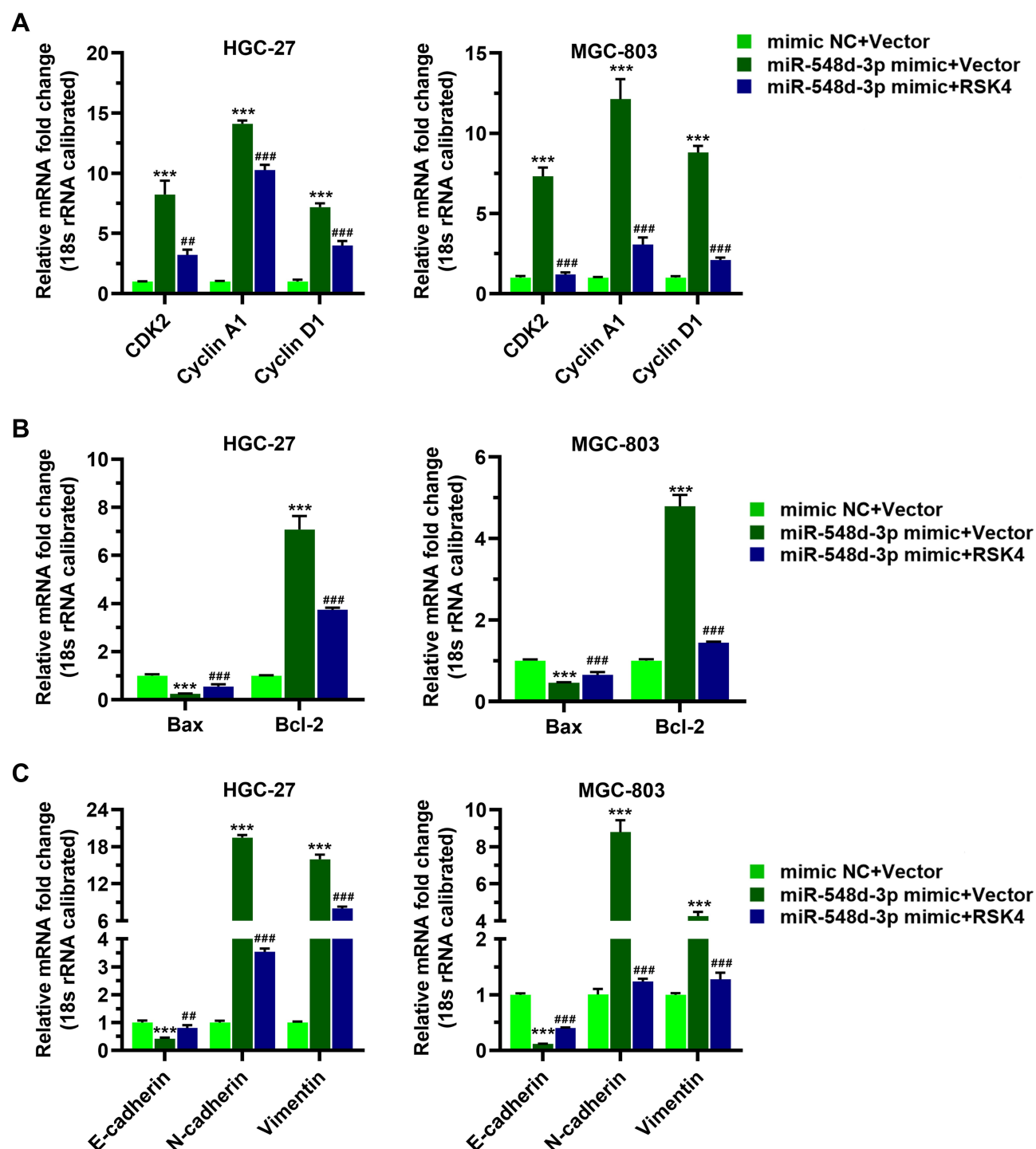
nucleotides, promote mRNA degradation or inhibit translation by binding to 3'UTR of the target mRNA, which causes changes in miRNA levels to seriously affect physiological and pathological processes.<sup>35–37</sup> Furthermore, miRNA is involved in a variety of diseases, including cancer and cardiovascular diseases, and is considered an important therapeutic target for these diseases.<sup>35</sup> miR-548 is involved in certain cancers and is considered a potential target for the treatment of cancers.<sup>38–40</sup> The present study confirmed that miR-548d-3p targets RSK4 to increase the proliferation of gastric cancer cells and the occurrence of tumors in vivo, and may be a potential target for the treatment of gastric cancer.

Previous studies have revealed that miR-548d is an inhibitor of pancreatic cancer.<sup>17</sup> miR-548d-3p inhibits

osteosarcoma by down-regulating KRAS, and miR-548 inhibited the growth of breast cancer cells by down-regulating ECHS1.<sup>38,41</sup> Interestingly, in breast cancer cells, miR-548d-3p down-regulates the expression of TP53BP2, thereby promoting the proliferation of breast cancer cells.<sup>42</sup> These findings indicate that miR-548d-3p plays different roles in different situations. Nonetheless, our study found that miR-548d-3p promotes gastric cancer by inhibiting the expression of RSK4.

Overexpression of RSK4 in breast cancer cells reduces the proliferation, migration, and angiogenesis, and increases apoptosis.<sup>43</sup> Compared with patients with hypermethylation of RSK4, patients with hypomethylation of RSK4 showed longer disease-free survival, indicating that

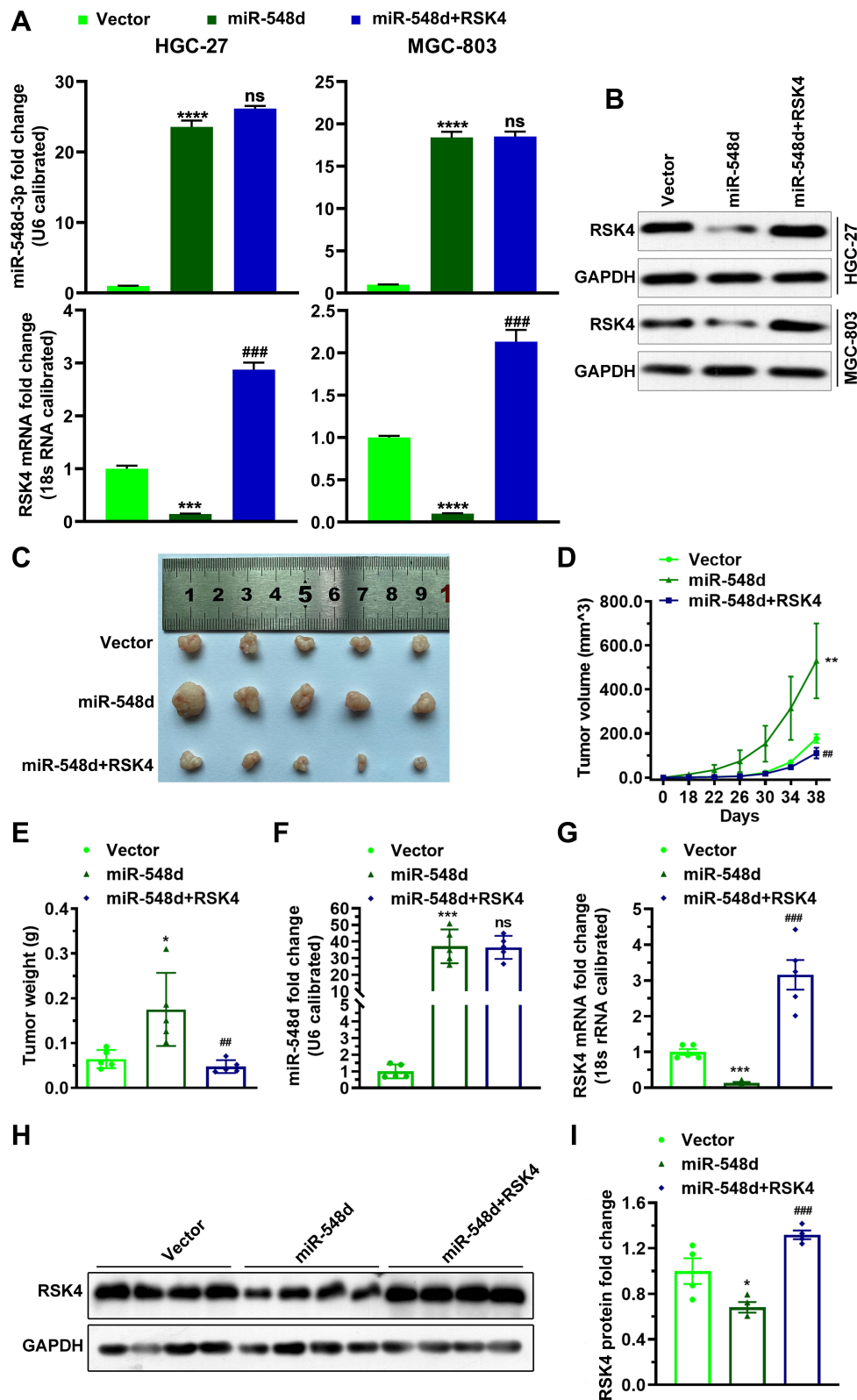




**Figure 5** RSK4-targeting miR-548d-3p regulates the mRNA levels of molecules related to cell cycle, apoptosis, and epithelial-mesenchymal transition. HGC-27 and MGC-803 cells were treated as described above. (A) The mRNA levels of CDK2, cyclin A1, and cyclin D1 were tested by RT-PCR. (B) RT-PCR was used to detect the mRNA levels of Bax and Bcl-2. (C) The mRNA levels of N-cadherin, Vimentin and E-cadherin were determined by RT-PCR. \*\*\* $p < 0.001$  vs mimic NC + Vector; ## $p < 0.01$ ; ### $p < 0.001$  vs miR-548d-3p mimic + Vector.

hypomethylation of the RSK4 promoter may potentially contribute novel prognostic markers of breast cancer patients.<sup>43</sup> In addition, overexpression of RSK4 reverses the resistance of human breast cancer cells to adriamycin

through the PI3K/Akt signaling pathway.<sup>44</sup> The expression of RSK4 is low in non-small cell lung cancer tissues and is related to the clinical and pathological staging of non-small cell lung cancer.<sup>45</sup> RSK4, which is overexpressed



**Figure 6** miR-548d promotes tumor growth by targeting RSK4. (**A** and **B**) The cell lines with stable overexpression of miR-548d or of both miR-548d and RSK4 were detected by RT-PCR and Western blot. (**C**) Picture of the formed tumor. (**D**) The graph of tumor volume. (**E**) Histogram of tumor weight. (**F**) RT-PCR was used to detect the level of miR-548d in the formed tumor. (**G**) RT-PCR was used to detect the mRNA level of RSK4 in the tumor. (**H** and **I**) Western blot analysis was used to detect the protein level of RSK4 in the tumor. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs Vector. ### $p < 0.01$ , #### $p < 0.001$  vs miR-548d.

**Abbreviation:** ns, not significant.

in colorectal cancer cell lines, destroys the growth, metastasis, and invasion of tumor cells by inhibiting epithelial-mesenchymal transition. Similarly, in gastric cancer, reducing the expression of RSK4 inhibits cell apoptosis, leading to enhanced cell proliferation, migration, and invasion, and promotes tumor occurrence in vivo.<sup>1</sup> These results indicate that RSK4 has a tumor suppressor effect in a variety of cancers. However, in lung adenocarcinoma, RSK4 is highly expressed, and is closely related to TNM stage, tumor diameter, lymph node metastasis, and distant metastasis, and is considered useful for the diagnosis of lung adenocarcinoma, as well as predicting the progression of this disease.<sup>46</sup> Molecular studies showed that overexpression of RSK4 can enhance the invasion and migration ability of clear cell renal cell carcinoma cell lines by regulating the expression of CD44 and MMP-9.<sup>47</sup> Thus, RSK4 plays different roles in different tumors, reflecting the diversity and complexity of RSK4 functions.

In a previous study, lowering RSK4 expression in breast cancer cells increased cyclin D1 mRNA levels and decreased E-cadherin mRNA levels.<sup>27</sup> This is consistent with our results that overexpression of miR-548d-3p reduces RSK4 expression, thereby increasing cyclin D1 mRNA level and decreasing E-cadherin mRNA level. RSK4 overexpression has been reported to increase E-cadherin mRNA levels in SW480 and HCT116 cells.<sup>26</sup> Similarly, we found that E-cadherin mRNA level was elevated after RSK4 supplementation.

## Conclusion

We identified the RSK4-targeting miRNA, miR-548d-3p, and investigated its function in gastric cancer. Our findings reveal that miR-548d-3p accelerates the proliferation of gastric cancer cells and tumorigenesis by reducing the expression of RSK4.

## Abbreviations

miRNAs, microRNAs; RT-PCR, real-time PCR; UTR, untranslated region; RSKs, ribosomal S6 kinases; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; ANOVA, analysis of variance.

## Ethics Committee Approval

This study was approved by the Institutional Ethics Committee of Zhuhai People's Hospital (Zhuhai Hospital Affiliated with Jinan University). All of the experiments using animals were performed in accordance with a

protocol approved by the Animal Care and Use Committee of Jinan University.

## Disclosure

The authors have declared that no competing interest exists.

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