

# miR-320a serves as a negative regulator in the progression of gastric cancer by targeting RAB14

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**Abstract.** Gastric cancer (GC) is one of the most common types of malignancy worldwide, with high morbidity and mortality rates. The dysregulation of microRNAs (miRs) has been found to be involved in the carcinogenesis of GC. The present study aimed to investigate the underlying association between GC and miR-320a. Analysis using reverse transcription quantitative polymerase chain reaction indicated that the expression of miR-320a was downregulated and the expression of RAB14 was upregulated in GC tissues and cells, compared with the corresponding controls. MTT, colony formation assays, and flow cytometric analyses were used to evaluate the effect of miR-320a on cell proliferation and the cell cycle. The ectopic expression of miR-320a using miR-320a mimics suppressed cell viability, inhibited G1/S transition, and induced apoptosis in AGS and MKN45 cells. In addition, RAB14 was identified as a direct target gene of miR-320a, according to the results of bioinformatics analysis and a luciferase reporter assay. Downregulation of RAB14 by RAB14-small interfering RNA inhibited the viability of GC cells, which was similar to the phenotype of miR-320a mimics. Furthermore, the reintroduction of RAB14 partially abrogated the miR-320a-mediated downregulation of RAB14 and rescued the miR-320a-induced effects on GC cell growth. These findings suggest a potential novel therapeutic target for the treatment of GC.

## Introduction

Gastric cancer (GC) is one of leading causes of malignant tumor-associated mortality worldwide due to its high morbidity and mortality rates, and delayed diagnosis. Advances in the development of gastroscopy have contributed to the clinical outcome of patients, however, survival rates remain poor due

to the high rate of metastasis and poor prognosis (1). Therefore, investigations of the pathogenesis of GC are urgently required, in order to identifying and examine novel targets or biomarkers for therapy.

MicroRNAs (miRNAs) are reported to be involved in various tumors by regulating the stability or translational efficiency of its target gene (2). Previous studies have provided evidence that the altered expression of miRNAs in cancer tissues may be a potential prognostic marker due to their functions as either oncogenes or tumor suppressors (3-7). Over previous years, the function of miRNA (miR)-320a in several malignancies had received increased attention due to its effect on the formation, progression and metastasis of tumors. miR-320a is a member of the miR-320 family, which has been described in different malignancies (8). As reported previously, miR-320a suppresses cell proliferation, metastasis and invasion in several types of cancer, including human colon cancer, salivary adenoid cystic carcinoma and colorectal carcinoma, by directly targeting their target genes (9-11). The oncogenic function of miR-320a has also been clarified in prostate cancer cells. A study by Xu *et al* (12) indicated that the expression of miR-320a was promoted ~2-14-fold, in prostate cancer cells, compared with adjacent non-tumor tissues. Until now, several potential miRNAs, including miR-320a, have been indicated as biomarkers in the diagnosis of GC (13,14). Xu *et al* (15) analyzed the miRNA expression profile of 291 patients (103 controls, 94 patients with atrophic gastritis and 94 patients with GC), which indicated that the serum level of miR-320a was a potential biomarker in the diagnosis of older women with GC. The present study aimed to further determine the role of miR-320a in GC tumor samples and cell lines in order to assist in understanding the pathogenesis of GC.

Rab proteins (20-25 kDa), including RAB-1, 3, 5, 27 and 14, are conserved regulators of multiple aspects of intracellular membrane trafficking and dynamics (16). Rab proteins are involved in various cellular events, and different Rab proteins have distinct effects, including RAB-1/RAB-2, which are involved in innate immunity, and vesicle trafficking and maturation in neurons, respectively (17). RAB-14 exerts its function as a target of miR-451 and miR-338-3p in the progression of lung cancer (18,19). In the present study, the association between miR-320a and its targeted gene, RAB14, was investigated to examine the mechanisms underlying the carcinogenesis of GC.

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## Materials and methods

**Patients and clinical specimens.** A total of 21 pairs of GC tissue clinical samples and matched non-tumor adjacent tissue samples were obtained from patients (15 males, 6 females; 57.3±17.25 and 60.5±9.17 years old, respectively; stage I, n=3; stage II, n=4; stage III, n=6; stage IV, n=8) at The Fifth Central Hospital of Tianjin between February 2014 and November 2015. None of the patients had received radiotherapy or chemotherapy prior to undergoing macroscopic curative resection. The present study was approved by the ethics committee of the Fifth Central Hospital of Tianjin (Tianjin, China) and all patients provided written informed consent.

**Cell culture.** The human GC cell lines (MKN28, MGC803, SGC7901, BGC823, AGS and MKN45) and normal gastric mucosa cell line (GES) were obtained from the cell bank of the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, China). The cells were maintained in DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 mg/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Bioinformatics analysis.** To investigate the putative protein of miR-320a, PicTar ([http://pictar.mdc-berlin.de/cgi-bin/new\\_PicTar\\_vertebrate.cgi](http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi)), TargetScan (<http://www.targetscan.org>) and miR Base (<http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl>) were used to predict the potential target gene of miR-320a.

**Cell transfection.** In order to investigate the function of RAB14, synthesized RAB14-small interfering (si)RNA (cat. no. sc-76312; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and corresponding control siRNA (siRNA-NC; cat. no. sc-36869; Santa Cruz Biotechnology, Inc.) were synthesized. miR-320a mimics, anti-miR-320a and their corresponding controls (miR-NC and anti-NC) were also synthesized by GenePharma (Shanghai, China). The AGS and MKN45 cells were transfected with 50 nM RAB14-siRNA, 100 nM miR-320a mimics, 100 nM anti-miR-320a, or corresponding controls, with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**Luciferase reporter assay.** The full-length 3'untranslated region (UTR) of RAB14 was obtained from GenPharma and subsequently ligated to the psi CHECK-2 dual-luciferase reporter (Promega Corporation, Madison, Wisconsin, USA) to generate RAB14-3'UTR. The QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to generate the mutated miR-320a binding site (RAB14-3'UTRm). The cells were seeded in 24-well plates (1.5-2.0×10<sup>3</sup>/well) 1 day prior to transfection and were then co-transfected with the miR-320a mimics, anti-miR-320a and the corresponding controls, in addition to RAB14-3'UTR or RAB14-3'UTRm. At 48 h post-transfection, the cells were harvested, lysed and measured using a dual luciferase reporter assay (Promega Corporation). The luciferase activity was normalized to Renilla luciferase activity.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from the tissue samples and cell lines using an miRNA kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miRNA (5 ng) was reverse transcribed to obtain cDNA, using the SuperScript III reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The qPCR analysis was performed in triplicate using SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China) on an Applied Biosystems 7500 Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total RNA was extracted from the cells and reverse-transcribed using reverse transcription (RT)-PCR kits (Applied Biosystems) with an oligo d(T)16 primer to obtain the cDNA. For the qPCR analysis, specific primers were designed as follows: RAB14, forward, 5'-CGCTCGAGATGGCAACTG CACCATACAAC-3' and reverse, 5'-CGGAATTCCTAGCAG CCACAGCCTTCTC-3'; miR-320a, forward, 5'-GTTGGA TCCGGCGTTTCCTTCCGACATG-3' and reverse, 5'-GCT GAATTCGTCCACTGCGGCTGTTCC-3'; U6 small nuclear RNA, forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. U6 small nuclear RNA or β-actin forward, 5'-CTCCATCCTGGCCTCGCTGT-3' and reverse, 5'-GCTGTCACCTTCACCGTTCC-3' served as the internal controls. The PCR thermocycling conditions were as follows: 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. The relative expression of target genes was determined using the 2<sup>-ΔΔC<sub>q</sub></sup> method (20).

**Cell proliferation analysis.** MTT and colony formation assays were used to evaluate the effect of miR-320a on cell proliferation. The MTT assay was performed using the Cell Proliferation kit I (cat. no. 11465007001; Roche Diagnostics, Basel, Switzerland). The procedures were performed according to the manufacturer's protocol. Briefly, 2×10<sup>3</sup> cells (AGS and MKN45 cells) were seeded into 96-well plates in quadruplicate and transfected with miR-320a mimics/anti-miR-320a or the corresponding controls. Subsequently, 0.5 mg/ml MTT reagent was added 48 h following transfection and incubated for 2 h at 37°C. Following incubation, the mixture was subjected to lysis with 100% DMSO and measured at 590 nm using the Tecan SpectraFluor microplate reader (Tecan Group Ltd., Männedorf, Switzerland). For the colony formation assay, a total of 1,500 transfected cells were placed in six-well plates in quadruplicate and maintained in DMEM containing 10% FBS for 2 weeks. The medium was replaced every 3 days. After 14 days, the colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma; Merck Millipore, Darmstadt, Germany) in PBS for 15 min. Five fields were randomly chosen and counted under an inverted microscope.

**Analysis of cell cycle and apoptosis.** Flow cytometric analysis was performed to investigate the effect of the overexpression of miR-320a on cell cycle and apoptosis. Firstly, the AGS and MKN45 cells were transfected with miR-320a mimics and associated control at indicated concentrations. The transfected cells were collected, fixed in 75% ethanol at -20°C for 16 h, and incubated with 1 mg/ml RNase A at 37°C for 30 min. Following incubation, the cells were stained with propidium

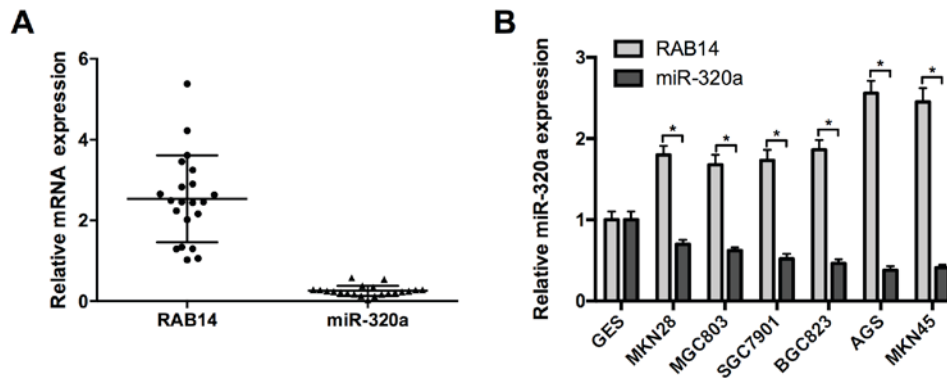


Figure 1. miR-320a is downregulated and RAB14 is upregulated in GC tissues and cell lines. (A) Expression levels of miR-320a and RAB14 were detected in 21 GC tissues and adjacent non-cancerous gastric tissues using reverse transcription-quantitative polymerase chain reaction analysis. The difference in expression level between miR-320a and RAB14 in adjacent non-cancerous gastric tissues was significant ( $P=0.002$ ). (B) Expression of miR-320a and RAB14 mRNA in GC cell lines and normal gastric mucosa (GES) cells. \* $P<0.05$ . GC, gastric cancer; miR, microRNA.

iodide (50 mg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) and examined using a FACScan system (BD Biosciences, San Jose, CA, USA) and equipped with ModFit LT version 2.0 (Verity Software House, Inc., Topsham, ME, USA), as previously reported (21). An Annexin V-Fluorescein isothiocyanate apoptosis detection kit (Oncogene Research Products, Boston, MA, USA) was used to determine cell apoptosis, as previously described (22). At least five visual fields were selected for the observation of each sample under a fluorescence microscope.

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation, and statistical analysis was performed using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA). Differences were compared using one-way analysis of variance and post hoc Scheffé tests.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

### *Expression of miR-320a and RAB14 in GC tissues and cell lines.*

In the present study, 21 tissues from cases of GC and adjacent non-cancerous gastric tissues were analyzed to determine the correlation between expression levels of miR-320a and RAB14 in human GC. The results revealed that the expression level of miR-320a was significantly decreased and that of RAB14 was significantly increased in GC tissues (Fig. 1A). Furthermore, it was found that the levels of miR-320a in the GC cell lines were lower and the levels of RAB14 were higher, compared with those in the normal gastric mucosa cell line (Fig. 1B). Poorly differentiated GC cell lines (AGS and MKN45) with a low expression of miR-320a exhibited higher expression levels of RAB14, whereas the well-differentiated GC cell lines (MKN28 and MGC803) exhibited high expression levels of miR-320a and lower expression levels of RAB14 (Fig. 1B).

**miR-320a suppresses cell viability and growth, inhibits G1/S transition and induces apoptosis in GC cells.** To investigate the effect of miR-320a on cell viability and growth, the present study performed overexpression and knockdown experiments in two GC cell lines, AGS and MKN45. Successful overexpression

and knockdown of miR-320a in the cells was confirmed using RT-qPCR analysis (Fig. 2A). A cell colony formation assay confirmed that miR-320a mimics significantly decreased the numbers of colonies, compared with the cells transfected with miR-NC, whereas anti-miR-320a significantly increased the numbers of colonies, compared with the cells transfected with anti-NC (Fig. 2B). The results of the MTT assay showed that the miR-320a mimics decreased cell viability, whereas anti-miR-320a increased cell viability in a time-dependent manner (Fig. 2C and D).

The present study subsequently aimed to determine whether the overexpression of miR-320a affected the cell cycle progression and apoptosis of GC cell lines. Cell cycle distribution was analyzed using flow cytometry and the findings revealed that the increased expression of miR-320a inhibited the G1/S transition, resulting in an increase in the population of cells in the G0/G1 phase, and a decrease in cells in the S phase (Fig. 2E). In addition, the results of the analysis of apoptosis indicated that the overexpression of miR-320a induced cell apoptosis (Fig. 2F).

**miR-320a negatively regulates the expression of RAB14 by directly targeting its 3'UTR.** The results of the bioinformatics analysis using TargetScan and miRanda suggested that RAB14 was a potential target of miR-320a. A luciferase reporter assay was also used to determine whether RAB14 was a direct target of miR-320a in GC cells. The target region sequence of RAB14 3'UTR (3'UTRw) or the mutant sequence (3'UTRm) was cloned into a luciferase reporter vector (Fig. 3A). The findings revealed that the luciferase activity of the RAB14 3'UTRw construct was downregulated in the cells transfected with miR-320a; luciferase activity was upregulated in the anti-miR-320a group, but not in the RAB14 3'UTRm construct (Fig. 3B), confirming that RAB14 was a direct target of miR-320a. To further support this finding, the mRNA levels of RAB14 were assessed in the AGS cells, which showed that miR-320a mimics suppressed the mRNA expression of RAB14, whereas anti-miR-320a upregulated the mRNA expression of RAB14, as determined using RT-qPCR analysis (Fig. 3C). Western blot analysis was also used to detect the protein expression of RAB14 in the presence of miR-320a mimics or anti-miR-320a

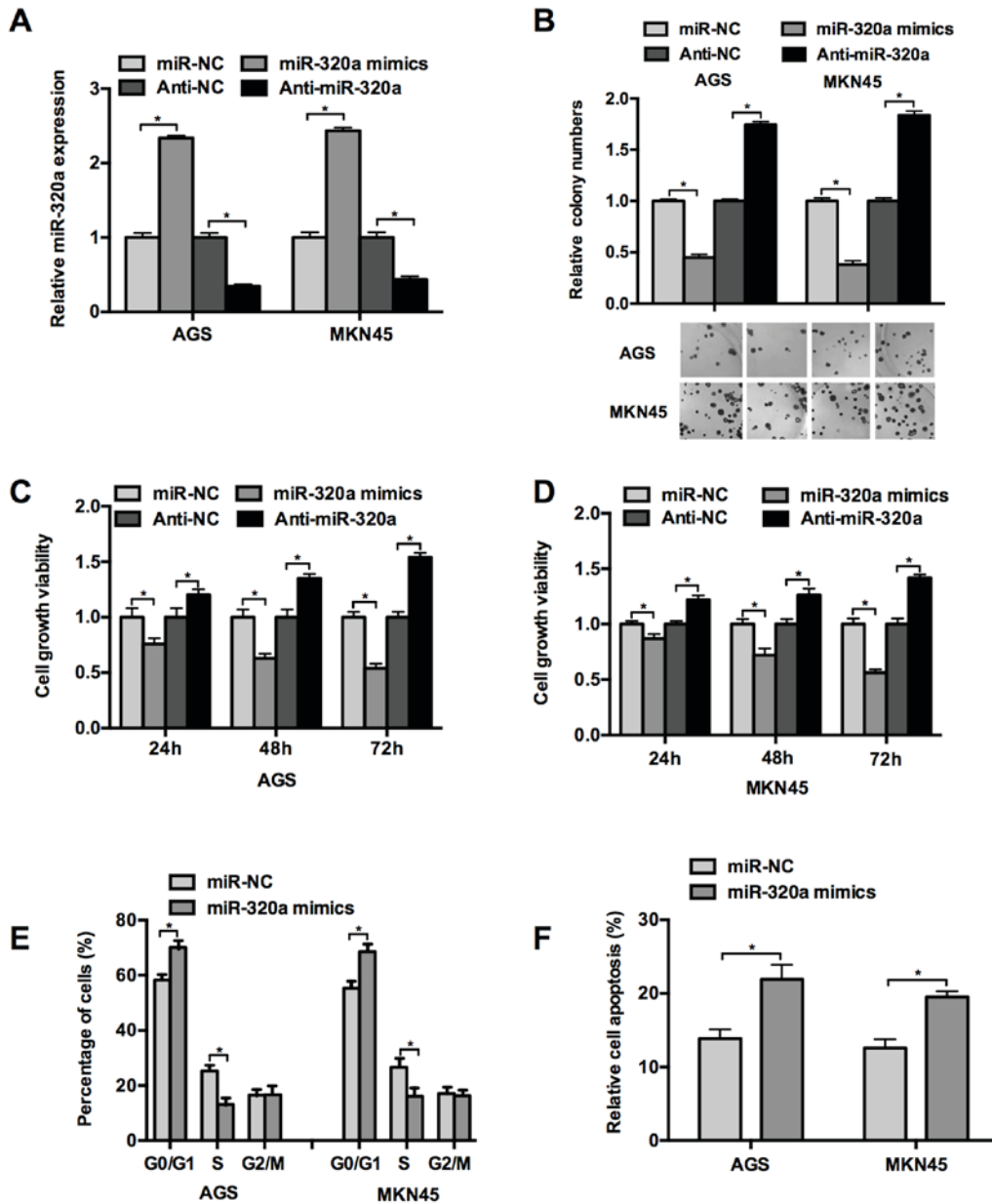


Figure 2. miR-320a regulates cell viability, growth, cell cycle and apoptosis in AGS and MKN45 cell lines. (A) miR-320a was upregulated in cells transfected with miR-320a mimics and was downregulated in the cells transfected with anti-miR-320a. (B) Growth of cells was suppressed in cells transfected with miR-320a mimics, and was accelerated in cells transfected with anti-miR-320a. (C and D) MTT assays revealed the viability of cells transfected with miR-320a mimics was inhibited, whereas cell viability was promoted by transfection with anti-miR-320a. (E) Flow cytometric analysis, revealed that transfection with miR-320a mimics resulted in a significant increase in the population of cells in the G0/G1 phase, and a decrease in the population of cells in the S phase. (F) Transfection with miR-320a mimics resulted in a significant increase of apoptosis in cells. \*P<0.05. miR, microRNA; NC, negative control.

in AGS cells. The protein expression of RAB14 was significantly downregulated following transfection with miR-320a mimics, whereas the protein expression of RAB14 was significantly upregulated following transfection with anti-miR-320a (Fig. 3D and E). Taken together, these results suggested that RAB14 is a potential target of miR-320a, and that miR-320a may negatively regulate the gene expression of RAB14 in GC cells.

*RAB14 silencing suppresses the viability and growth of AGS and MKN45 cells.* To examine the function of RAB14 in GC, the AGS and MKN45 cells were transfected with RAB14-siRNA.

The results of the MTT assay revealed that the knockdown of RAB14 inhibited the viability of the AGS and MKN45 cells (Fig. 4A) in a time-dependent manner. Furthermore, the cell colony formation assay suggested that the downregulated expression of RAB14 inhibited the growth capability of the GC cells (Fig. 4B). Therefore, RAB14 silencing appeared to induce a similar phenotype to that of the miR-320a mimic-transfected GC cells. These results suggested that miR-320a downregulated RAB14, thus suppressing GC cell viability and growth.

*Reintroduction of RAB14 rescues miR-320a-induced effects on GC cell growth.* If RAB14 serves as a direct functional target



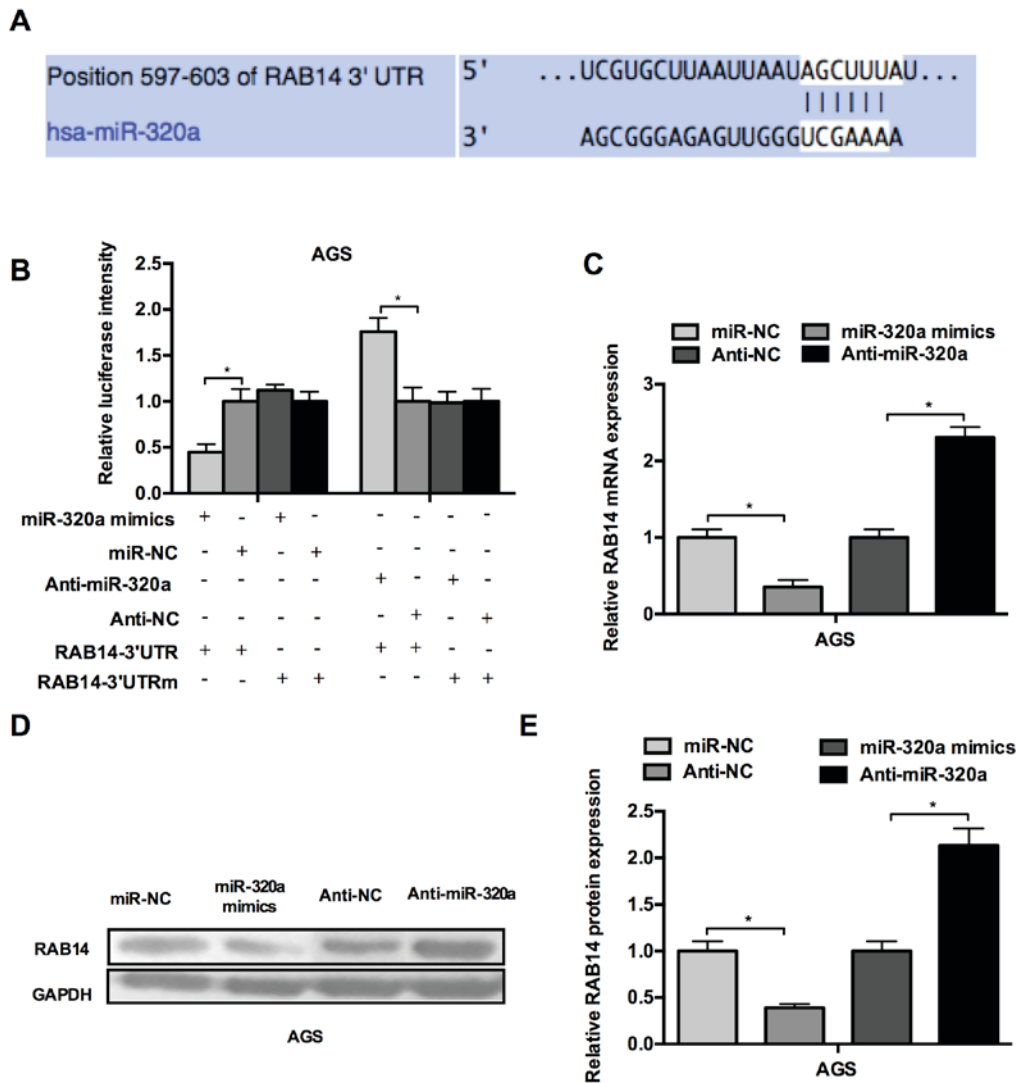


Figure 3. miR-320a negatively regulates the expression of RAB14. (A) Diagram of RAB14 3'UTR targeted by miR-320a. (B) Luciferase assay, in which AGS cells were transiently co-transfected with 3'UTRw/m with miR-320a mimics or anti-miR-320a, as indicated. (C) miR-320a mimics inhibited the expression of RAB14 at the mRNA level in AGS cells, anti-miR-320a increased the expression of RAB14 at the mRNA level. (D and E) miR-320a mimics inhibited the expression of RAB14 at the protein level in AGS cells; anti-miR-320a increased the expression of RAB14 at the protein level. \* $P < 0.05$ . miR, microRNA; UTR, untranslated region; UTRw, UTR wild-type; UTRm, UTR mutant; NC, negative control.

of miR-320a, it was hypothesized that the reintroduction of RAB14 into miR-320a-expressing cells antagonizes the effects of miR-320a. To confirm this hypothesis, miR-320a mimics were co-transfected with pcDNA3-RAB14 plasmid, which did not contain the RAB14 3'UTR, into AGS cells. Western blot analysis indicated that the overexpression of RAB14 partially abrogated the miR-320a-mediated downregulation of RAB14 (Fig. 4C). Similarly, the colony formation assay confirmed that the overexpression of RAB14 restored the inhibited cell growth induced by the miR-320a mimic (Fig. 4D). These findings demonstrated that the reintroduction of RAB14 abrogated miR-320a-induced cellular behaviors, suggesting that RAB14 is a functional mediator of miR-320a in GC cells.

## Discussion

Cancer is characterized by abnormal and uncontrolled cell proliferation, which is caused not only by the dysregulation

of several important proteins, but also by a systemic change in the miRNA profile (23). GC is reported to be closely associated with the abnormal expression of miRNAs, including miR-375, miR-215 and miR-106a (24-26). Various miRNAs have been identified as important regulators of tumorigenesis and cancer progression through diverse molecular pathways (27). In GC, miRNAs have been described to contribute to several biological processes, including cell proliferation, migration, invasion and metastasis via regulating its target gene (28,29). For example, miR-106a was reported to be significantly upregulated in GC, and enhanced cell proliferation by inhibiting cell apoptosis (30). Similarly, miR-21, which is frequently elevated in GC, accelerated gastric tumor invasion through targeting phosphatase and tensin homolog via the 3'UTR (31). However, the crosstalk between miRNAs and GC is diverse. miR-1182, which is downregulated in GC tissues, has been shown to repress the cell proliferation and metastasis of GC cells (32). In the present study, miR-320a

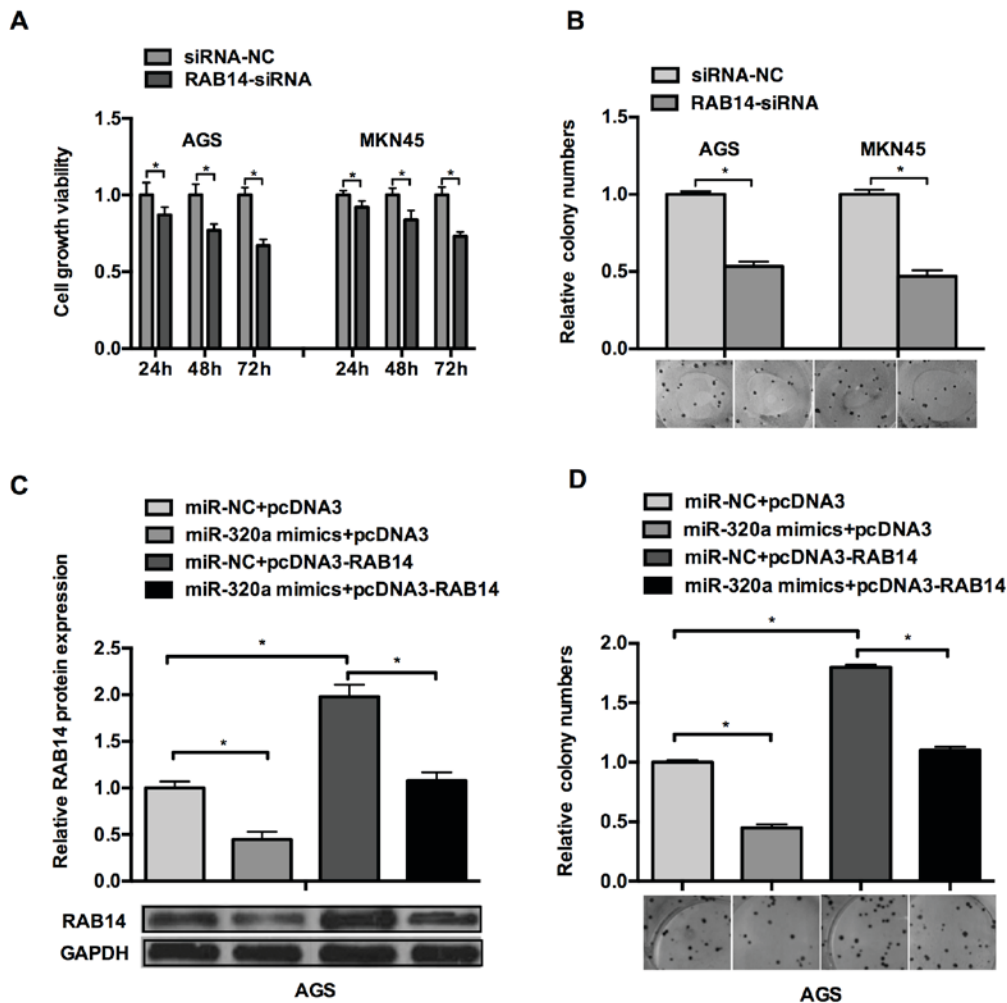


Figure 4. RAB14 is essential for GC cell viability and growth, and is involved in the miR-320a-induced inhibition of cell growth. (A) MTT assays revealed the viability of AGS or MKN45 cells were significantly inhibited by transfection with RAB14-siRNA. (B) Colony formation assays revealed the cell growth ability of AGS and MKN45 cells was significantly decreased by transfection with RAB14-siRNA. (C) Western blot analysis showed that the protein of RAB14 was significantly inhibited by transfection with miR-320a and increased by transfection with pcDNA3-RAB14, whereas co-transfection with miR-320a mimics and pcDNA3-RAB14 reversed this trend. (D) Colony formation assays showed that cell growth was suppressed by transfection with miR-320a mimics and pcDNA3-RAB14 reversed this trend. \* $P < 0.05$ . miR, microRNA; siRNA, small interfering RNA; NC, negative control.

was significantly downregulated in human GC cell lines and clinical samples, compared with that in immortalized normal human proximal tubule epithelial cells and adjacent normal tissues. Furthermore, the promoted expression of miR-320a inhibited cell viability, inhibited G1/S transition and induced cell apoptosis of the GC cells. By contrast, the knockdown of miR-320a promoted cell viability and inhibited apoptosis. Therefore, it was hypothesized that miR-320a is a potential tumor suppressor.

The exact mechanism underlying miR-320a in GC remains to be fully elucidated. The present study hypothesized that miR-320a may exert tumor suppressive or oncogenic effects through target genes, as with other miRNAs. In order to understand the underlying regulatory mechanisms, it is essential to investigate the targets of miR-320a. In the present study, bioinformatics analysis was used for target gene prediction. Considering the overlap of the genes identified by TargetScan, miRBase targets and PicTarget, RAB14 was selected as a potential target for further validation.

RAB14 is a member of the RAS oncogene family of small GTPases, which contains >170 members (33). In addition to the most widely analyzed small G protein, Ras, which is important in human oncogenesis, the RAB14 protein may be involved in human cancer (34). Wang *et al* (18) reported that RAB14 was involved in miR-451-induced tumor suppressor effects in lung cancer. In the present study, a luciferase reporter assay, western blot analysis and RT-qPCR analysis were performed, which confirmed that miR-320a directly targeted RAB14 through interaction with the binding site in the 3'UTR, which may explain the miR-320a-induced inhibition of GC cell viability and growth. In addition, inhibition of the expression of RAB14 was associated with a significant decrease in cell viability and growth. The results of the present study found that the overexpression of RAB14 rescued the miR-320a-mediated downregulation of RAB14 and cell growth inhibition. RAB14 silencing induced a similar phenotype to that of the GC cells transfected with the miR-320a mimic. These results suggested that

miR-320a was involved in GC cell viability and growth via downregulating RAB14. These results indicate a potential target for GC therapy and demonstrate a potential regulatory mechanism in the miR-320a/RAB14 axis in GC.

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