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Original Article

microRNA-340 influences cell proliferation, apoptosis and invasion by targeting NF-κB1 in gastric cancer

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Abstract: Gastric cancer is a serious threat to human health, and its pathogenesis may be regulated by a variety of mRNAs. Abnormal expression of microRNA-340 has been frequently reported in many malignant neoplasms, while the molecular mechanism of miR-340 has not been explored in gastric cancer. In this study, the mRNA level of miR-340 was determined by real-time PCR in GC cell lines. The miR-340 mimic was transiently transfected into GC cells by using Lipofectamine™ 2000 reagent. The BrdU-ELISA results showed that introduction of miR-340 inhibited cell proliferation. It was demonstrated that miR-340 mimic arrested cell cycle progression and promoted apoptosis of MKN-45 and BGC-823 cells. In addition, the overexpression of miR-340 could inhibit invasion and EMT of MKN-45 and BGC-823 cells. The expression of NF-κB1 was evidently reduced by up-regulation of miR-340. Luciferase reporter assay further confirmed that miR-340 could directly target the 3'UTR of NF-κB1. Moreover, overexpression of NF-κB1 transfected with miR-340 mimic partially reversed the inhibitory of miR-340 mimic in MKN-45 and BGC-823 cells. In conclusion, miR-340 induced cell apoptosis and inhibited invasion by down-regulation of NF-κB1, which might be a potential target in treatment and prevention of gastric cancer.

Keywords: microRNA-340, gastric cancer, proliferation, invasion, NF-κB1

Introduction

Gastric cancer (GC) is still a major cause of morbidity and mortality, with little change in survival rates in the recent 30 years. Recently, previous reports have demonstrated that several genes are closely related to human gastric cancer [1, 2]. However, the precise molecular mechanisms of gastric cancer are not defined yet. As far as we know, the mechanisms of GC are revealed by focusing on known genes, but focusing on unknown microRNAs may also lend insight into the biology of GC.

Many studies have reported that miRNAs are small by the length of 21-23 nucleotides [3, 4]. Moreover, miRNAs can regulate cellular proliferation in a new layer of gene-regulation mechanism, differentiation and apoptosis, as well as cancer initiation and progression [5-7]. Actually, many studies have showed that miRNAs were differentially regulated in diverse cancer types such as colorectal cancer, breast cancer and lung cancer [8-10]. The miR-17-5p was over-expressed in GC cells, and up-regulation of miR-17-5p significantly promoted proliferation, migration, invasion and EMT of GC cells by targeting PTEN signaling [11]. Wang et al. found that miR-20a expression was associated with motility of GC by targeting ZEB1 [12]. MiR-448 promoted proliferation of GC cells through directly regulating ADAM10 [13]. These three miRNAs act as oncogene, whereas some tumor suppressor miRNAs were also studied in GC. For example, Overexpression of miR-199a-5p reversed TGF-β1-induced EMT of OC cells by decreasing klotho expression [14]. The level of miR-129 was markedly down-regulated in GC tissues, and miR-874 functions as a tumor suppressor by inhibiting angiogenesis through STAT3/VEGF-A pathway in gastric cancer [15, 16].

MiR-340 has been involved in regulation of multiple biological processes such as cell proliferation, apoptosis, migration and invasion [17]. In recent years, miR-340 was considered as a tumor suppressor and down-regulated in pros-

tate, esophageal and non-small cell lung cancers [18-26]. Wei et al. found that miR-340 overexpression inhibited proliferation and invasive properties of prostate cancer cells by targeting high-mobility group nucleosome-binding domain 5 [23]. Furthermore, miR-340 was reduced in esophageal cancer cells (ESCCs), and functioned as a tumor suppressor to inhibit growth, colony formation and invasion of ESCCs by down-regulation of phosphoserine aminotransferase 1 [20]. Fernandez found that miR-340 functioned as a tumor suppressor through inhibiting proliferation and inducing apoptosis in colon cancer cells by regulation of p27 [26].

In this paper, down-regulation of miR-340 was frequently observed in MKN-45 and BGC-823 cells. Up-regulation of miR-340 inhibited cell proliferation arrested cell cycle and induced apoptosis of GC cells. In addition, miR-340 overexpression could also inhibit invasion and EMT of GC cells, and NF-κB1 was the direct target of miR-340. Therefore, our results showed critical roles for miR-340 in the pathogenesis and suggested its potential application in treatment of gastric cancer.

Materials and methods

Cell culture and miRNA transfection

Human GC cell lines such as MKN-45, BGC-823 and an immortalized normal human fallopian tube epithelial cell line FTE187 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Ann they were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Co., USA) including 10% fetal bovine serum (FBS) (Gibco Co., USA), 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 on 0.1% gelatin-coated culture flasks.

MiRNA transient transfection

To enhance the expression of miR-340 in MKN-45 and BGC-823 cells, both cells were transfected with miR-340 mimic, which served as the miR-340 group. MKN-45 and BGC-823 cells transfected with miR-negative control (miR-NC) were used as miR-NC group. One day before transfection, cells at about 40 to 60% confluency were changed to the antibiotic-free media. After 24 h, cells were transfected with 50 nM miR-340 mimic using Lipofectamin™ 2000 reagent (Invitrogen, USA) following manufacturer's protocol.

Reverse transcription polymerase chain reaction

Total RNA of MKN-45 and BGC-823 cells were extracted by using Trizol reagent (Life Technologies, CA). Two microgram RNA was used for gene-specific reverse transcription polymerase chain reaction (RT-PCR) using one-step RT-PCR kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protocols. The following primers were used: miR-340, forward: 5'-CCGGGATCCGCAAACTCAGCTTTAC-3' and reverse: 5'-CGGAATTCGTGGCGACCGTGATACC-3'; E-cadherin, forward 5'-TACACTGCCCAGGAGCCAGA-3' and reverse: 5'-TGGCACCAGTGTCCGGATTA-3'; N-cadherin, forward: 5'-CGAATGGATGAAAGACCCATCC-3' and reverse: 5'-GGAGCCACTGCCTTCATAGTCAA-3'; Vimentin, forward: 5'-GCTGAATGACCGCTTCGCCAACT-3' and revere: 5'-GCTCCCGCATCTCCTCCTCGTA-3'; U6, forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH, forward: 5'-GAGTCAACGGATTTGGTCGTATTG-3' and reverse: 5'-CCTGGAAGATGGTGATGGGATT-3'. U6 snRNA and GAPDH mRNA were used to normalize. Each sample was assessed in triplicate.

Cell proliferation assay

To study the role of miR-340 mimic in proliferation of MKN-45 and BGC-823 cells, 5×10^3 cells were seeded in 96-well plate and allowed to grow for 24 h in complete medium. The medium was then removed and the cells were transfected with miR-340 mimic or miR-NC for 24 h at 37°C. Cell Proliferation ELISA-BrdU (colorimetric) Kit (Roche Diagnostics, USA) was used to detect the cells proliferation according to the manufacturer's protocols.

Cell cycle analysis

To detect cell cycle distribution, the MKN-45 and BGC-823 cells were transfected with miR-340 mimic for 24 h. After transfection, MKN-45 and BGC-823 cells were collected by trypsinization, washed with ice-cold PBS, and fixed in ice-cold 70% methanol overnight. Then, cells were centrifuged, suspended in ice-cold phosphate buffer saline (PBS), and incubated with RNase (Sigma, USA) for 30 min at 37°C, and then were

incubated with propidium iodide (PI; Sigma, USA) at room temperature for 30 min. The analyses of cell cycle distribution were performed by FACScan flow cytometer (BD Biosciences, USA).

Annexin V-FITC/PI analysis

MKN-45 and BGC-823 cells were transfected with miR-340 mimic for 24 h. After transfection, cells were harvested and washed twice in PBS and double-stained with Annexin V-FITC and PI by using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA) following the manufacturer's protocols. Then, each sample was quantitatively analyzed at 488 nm emission and 570 nm excitation by FACSCalibur flow cytometer (BD Biosciences, USA).

Transwell invasion assay

Transwell Matrigel invasion assay using Transwell chambers (8-mm pore size; Corning, USA) precoated with Matrigel (BD Biosciences, USA) that included extracellular matrix proteins was used to evaluate cell invasion. In brief, after serum-starved for 24 h, 2×10^5 cells were suspended in 100 μl serum-free DMEM, and then were seeded in the top chamber, and 600 μl DMEM containing 10% FBS was added to the lower chamber. After 24 h incubation at 37°C in a 5% CO_2 atmosphere, cells that remained in the upper surface of the membrane were removed by cotton swabs and penetrating cells were fixed in methanol, and then stained with 0.1% crystal violet. Cell invasion was quantified by counting cells on the lower surface using phase contrast microscopy.

Wound healing assay

Cultured plates were seeded on the back of the line before the label, cell digestion after access to 12-well plate, perpendicular to the orifice to create cell scratches. Absorb the cell culture medium, rinse the orifice plate three times with PBS, and wash away the scratches generated cell debris. Add serum-free medium, the culture plate into the incubator culture, every 4-6 hours to take pictures. Analyze the experimental results based on the collected image data.

Western blot analysis

To extract the proteins, MKN-45 and BGC-823 cells were washed twice in cold PBS, and then lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, China) with protease inhibitor cocktail (Merk, Germany). The protein concentration of cell lysates was quantified by BCA Kit (Beyotime Institute of Biotechnology, China), and equal quantities (50 μg) of proteins were separated by SDS-PAGE on 10% gels, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in 5% shimmed milk diluted with Tri Buffered Saline Tween-20 (TBST) (in mmol/L: Tris-HCl 20, NaCl 150, PH 7.5, 0.1% Tween 20) at room temperature for 1 h and incubated overnight at 4°C with primary anti-NF-κB1 antibody (1:1000; Cell Signaling Technology Inc, USA). The membranes were then incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (1:1000; Cell Signaling Technology Inc, USA) for 2 h. The proteins were visualized using ECL-plus reagents (Beyotime Institute of Biotechnology, China). The density of the bands was measured using the Image J software (USA), and values were normalized to the densitometric values of GAPDH (1:1000; Cell Signaling Technology Inc, USA) in each sample.

Luciferase reporter assay

MKN-45 and BGC-823 cells (2×10^5/well) were seeded in 24-well plates and incubated overnight before transfection. Cells were cotransfected with pMIR-EGFR-3'UTR wild-type or mutant reporter plasmid, miR-340 mimic or miR-NC, and pRL-SV40 renilla plasmid (Promega, USA) using Lipofectamine 2000. At 48 h after co-transfection, both firefly and renilla luciferase activities were quantified using a dual luciferase reporter system (Promega, USA) following the manufacturer's protocols. Each treatment was performed in triplicate in three independent experiments.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Comparisons between more than two groups determined using Student's t-tests and one-way analysis of variance (ANOVA) followed by Bonferroni t-tests, and $P<0.05$ was considered statistically significant. Student's t-tests and one-way analysis of variance (ANOVA) followed by Bonferroni t-tests.

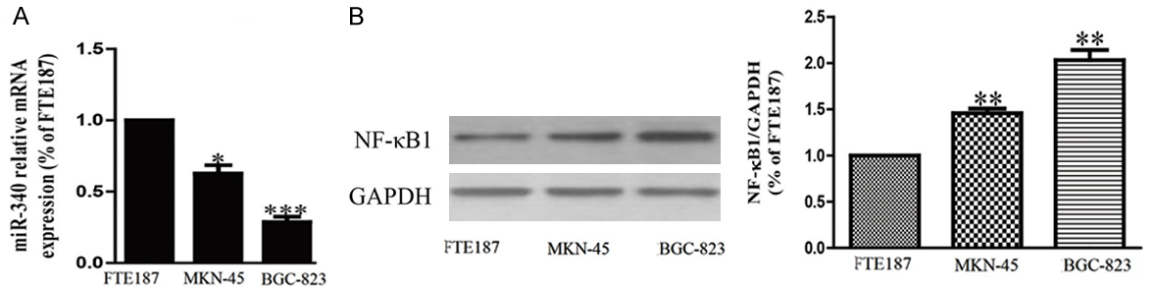


Figure 1. The altered expression of miR-340 and NF-κB1 were shown in GC cell lines. A. The relative mRNA level of miR-340 in MKN-45, BGC-823 and FTE187 cells by RT-PCR. B. NF-κB1 protein level in MKN-45 and BGC-823 cells compared with FTE187 cells were determined by western blotting. GAPDH was detected as a loading control. All data are presented as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.0001 vs. FTE187.

Results

The level of miR-340 was decreased in GC cell lines

Many reports have showed that miR-340 was reduced in prostate, esophageal and non-small cell lung cancers. However, the level of miR-340 in GC is still unclear. Therefore, the level of miR-340 was determined by RT-PCR in five GC cell lines such as MKN-45 and BGC-823 and an immortalized normal human fallopian tube epithelial cell line FTE187. Our findings demonstrated that expression of miR-340 was evidently reduced in all GC cell lines compared to that in normal human fallopian tube epithelial cell line FTE187 (**Figure 1A**). Moreover, we found that NF-κB1 was predicted by using the online database (TargetScan 6.3) to be a direct target of miR-340. Then, among these GC cell lines, MKN-45 and BGC-823 cells were used to study further. Our results showed that the protein level of NF-κB1 in MKN-45 and BGC-823 cells was significantly increased compared with FTE187 cell (**Figure 1B**).

miR-340 inhibited proliferation, arrested cell cycle and induced cell apoptosis of MKN-45 and BGC-823 cells

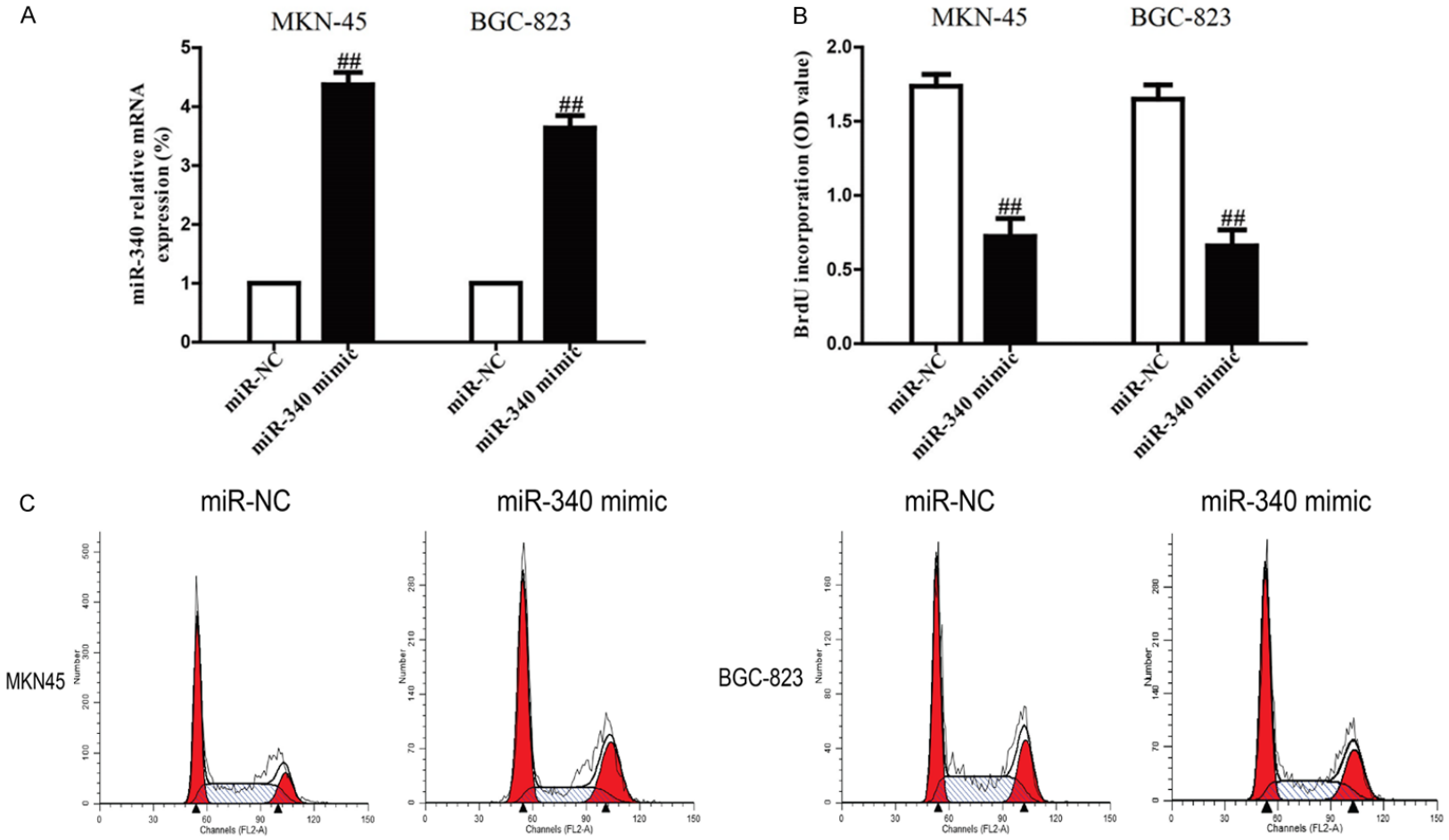
However, the decreased expression of miR-340 in GC cell lines indicated that miR-340 might act as a tumor suppressor in GC. We found that level of miR-340 was evidently up-regulated after transfection with miR-340 mimic compared to miR-NC group (**Figure 2A**). Our data indicated that we could availably enhance miR-340 level in MKN-45 and BGC-823 cells. To investigate the role of miR-340 in proliferation of GC cells, MKN-45 and BGC-823 cells were transfected with miR-340 mimic or miR-NC. Results from BrdU-ELISA assay showed that overexpression of miR-340 clearly suppressed the viabilities of MKN-45 and BGC-823 cells (**Figure 2B**). These findings indicated that up-regulation of miR-340 had available anti-proliferative effect in both MKN-45 and BGC-823 cells.

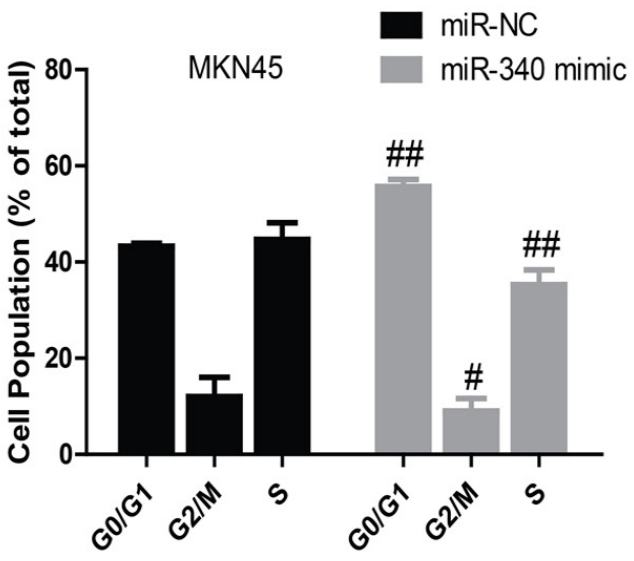
Because miR-340 mimic obviously inhibited proliferation of MKN-45 and BGC-823 cells, we speculated that introduction of miR-340 could arrest cell cycle of GC cells. Our data confirmed this tentative by flow cytometry. We demonstrated that up-regulation of miR-340 dramatically enhanced the percentage of cells in the G1/G0 peak and reduced the percentage of cells in the S peak in both MKN-45 and BGC-823 cells compared with cells transfected with miR-NC (**Figure 2C**). Thus, overexpression of miR-340 might inhibit GC cell proliferation by impeding the G1/S cell cycle transition.

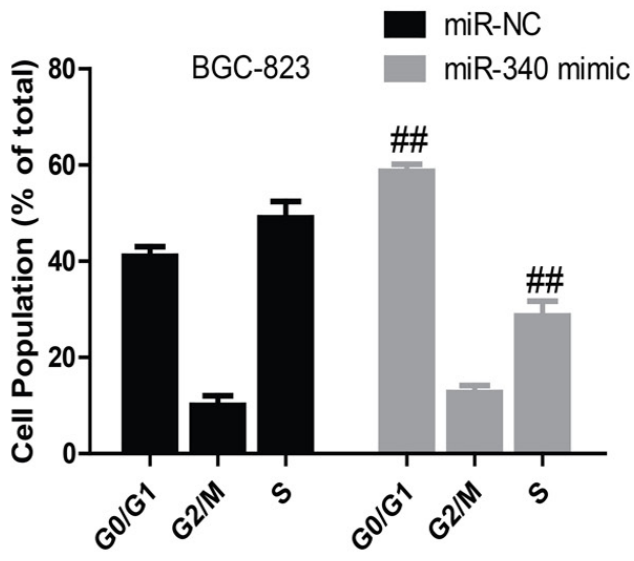
For studying whether pro-apoptotic effect was involved in miR-340-induced anti-proliferative effect, we use flow cytometry analysis to detect the total apoptosis rates of MKN-45 and BGC-823 cells. We found that the number of apoptotic MKN-45 and BGC-823 cells was significantly higher in miR-340 group than that in miR-NC group (**Figure 2D**).

MiR-340 overexpression restrained the invasive capacities of GC cells

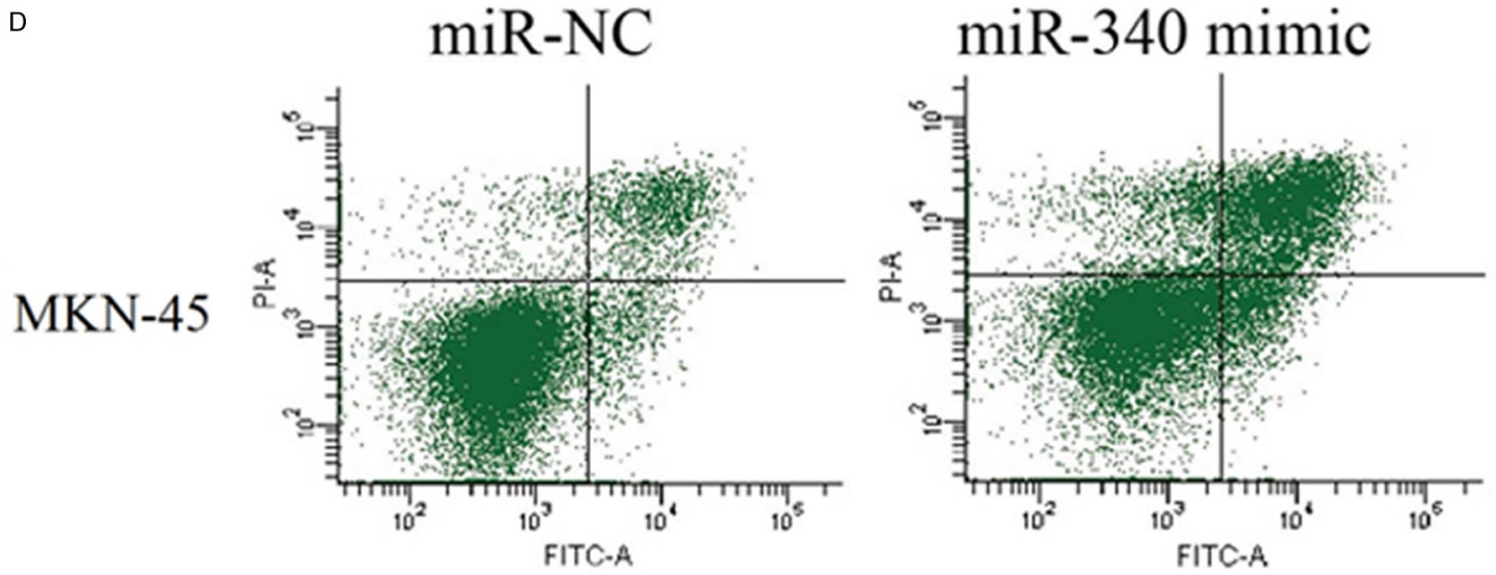
To understand whether introduction of miR-340 had a negative role in invasion of GC cells, we transfected miR-340 mimic into BGC-823 cells, and the invasive abilities were evaluated by Transwell invasion assay. We found that the


A
MKN-45
BGC-823
miR-340 relative mRNA expression (%)
##
miR-NC
miR-340 mimic
B
MKN-45
BGC-823
BrdU incorporation (OD value)
##
miR-NC
miR-340 mimic
C
miR-NC
miR-340 mimic
MKN45
BGC-823
Number
Channels (FL2-A)


miR-NC
miR-340 mimic
MKN45
Cell Population (% of total)
80
60
40
20
0
##
#
##
G0/G1
G2/M
S
G0/G1
G2/M
S


miR-NC
miR-340 mimic
BGC-823
Cell Population (% of total)
80
60
40
20
0
##
##
G0/G1
G2/M
S
G0/G1
G2/M
S

D


miR-NC
miR-340 mimic
MKN-45
PI-A
FITC-A
PI-A
FITC-A

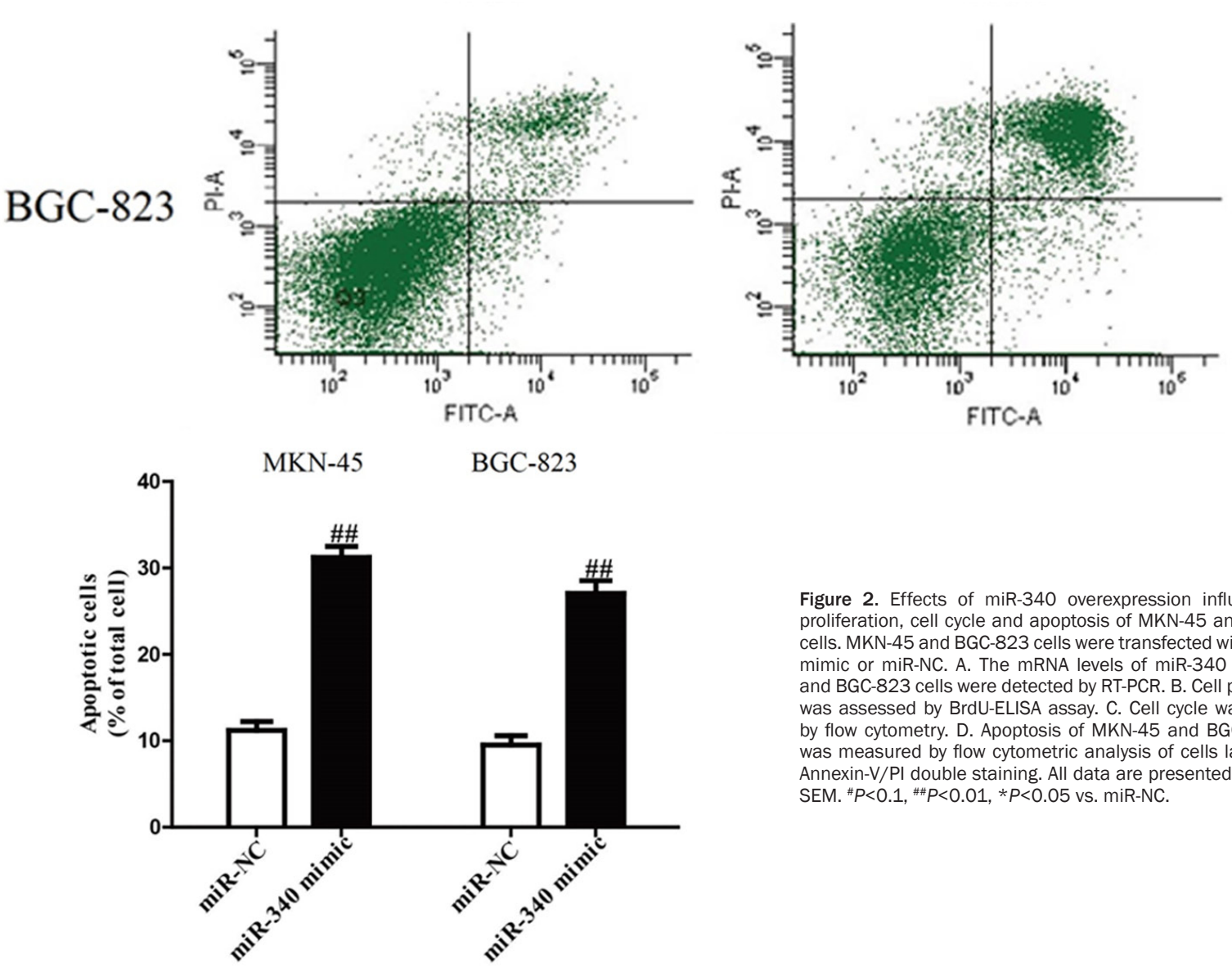


Figure 2. Effects of miR-340 overexpression influenced the proliferation, cell cycle and apoptosis of MKN-45 and BGC-823 cells. MKN-45 and BGC-823 cells were transfected with miR-340 mimic or miR-NC. A. The mRNA levels of miR-340 in MKN-45 and BGC-823 cells were detected by RT-PCR. B. Cell proliferation was assessed by BrdU-ELISA assay. C. Cell cycle was detected by flow cytometry. D. Apoptosis of MKN-45 and BGC-823 cells was measured by flow cytometric analysis of cells labeled with Annexin-V/PI double staining. All data are presented as mean ± SEM. $^{\#}P<0.1$, $^{\#\#}P<0.01$, $^{*}P<0.05$ vs. miR-NC.

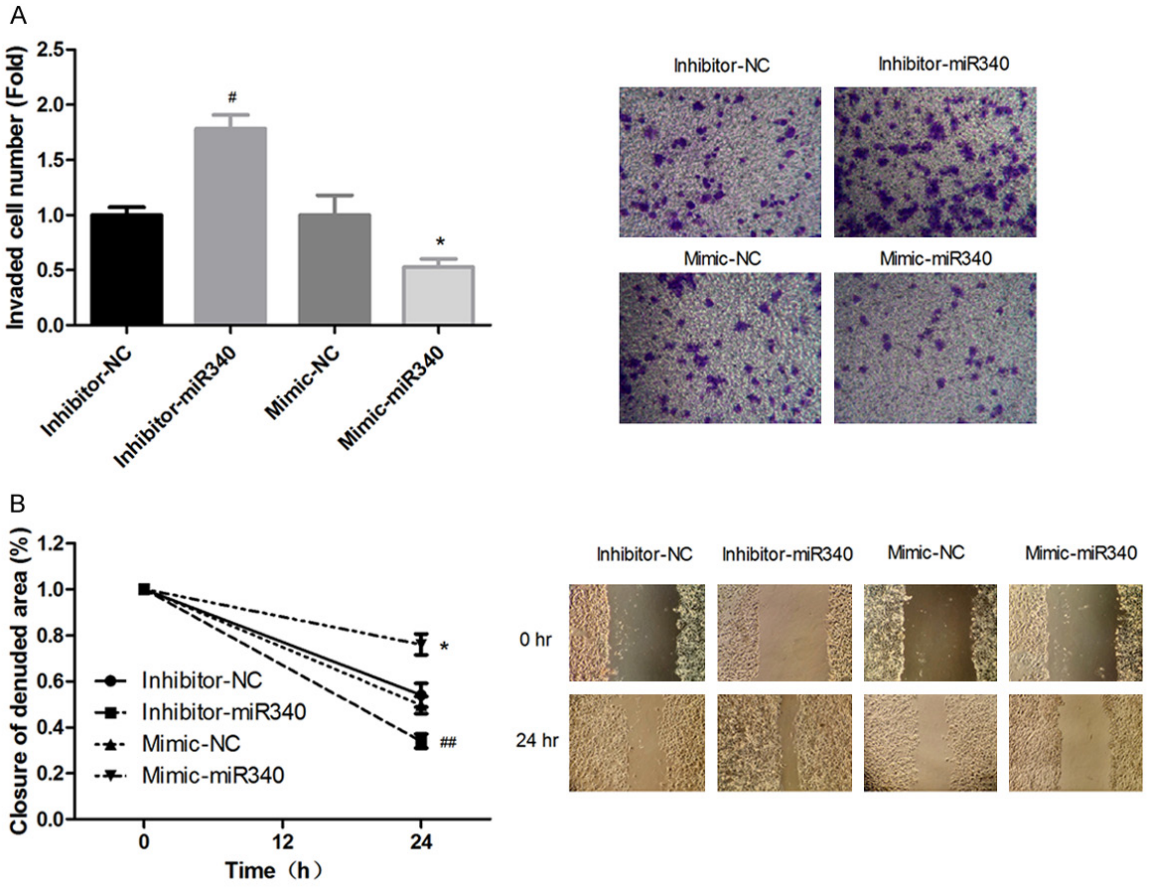


Figure 3. Overexpression of miR-340 influenced the invasion and migration of BGC-823 cells by transfection with mimic-miR340, miR-NC, inhibitor-NC and inhibitor-miR340. A. The invasion of BGC-823 cells through the membrane was stained and quantified. B. The migration of BGC-823 cells was detected by the Wound healing assay. Data represent means ± SEM. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. miR-NC.

number of BGC-823 cells invading through the Transwell membrane was evidently less in miR-340 group compared to miR-NC group (**Figure 3A**). The migration of BGC-823 cells was further weakened in the interference expression of miR-340 (**Figure 3B**). Our data indicated that up-regulation of miR-340 influenced the invasion of GC cells.

Effects of miR-340 overexpression on EMT-related molecules of GC cells

Next, to know whether EMT contributed to inhibition of OC cell invasion by up-regulation of miR-340, we studied the effects of miR-340 overexpression on the mRNA levels of EMT markers in MKN-45 and BGC-823 cells by qRT-PCR. Introduction of miR-340 in MKN-45 and BGC-823 cells led to up-regulation of E-cadherin that was an epithelial marker, and down-regulation of N-cadherin and Vimentin that were the mesenchymal markers at mRNA levels (**Figure 4**). Taken together, our results indicated that up-regulation of miR-340 could markedly restrain the invasive ability of GC cells partly by regulation of EMT.

NF-κB1 was a direct target of miR-340 in GC cells

Because NF-κB1 was a binding target of miR-340 predicted by the TargetScan 6.3, the protein expression of NF-κB1 was determined by Western blotting in MKN-45 and BGC-823 cells transfected with miR-340 mimic. Our results demonstrated that NF-κB1 expression was apparently decreased after up-regulation of miR-340 (**Figure 5A**). To further confirm whether a direct target of miR-340 was NF-κB1, 3'-UTR of NF-κB1 was cloned into a luciferase

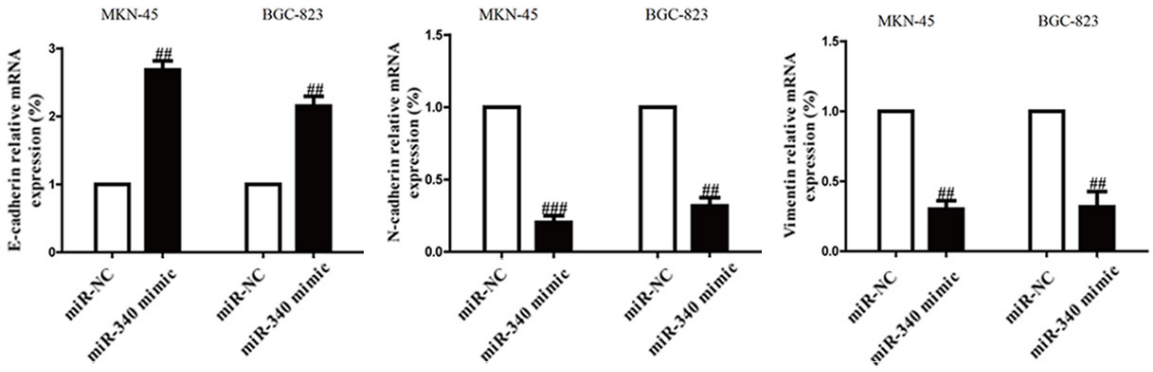


Figure 4. Overexpression of miR-340 regulated the mRNA levels of EMT-related molecules in GC cells. MKN-45 and BGC-823 cells were transfected with miR-340 mimic and miR-NC. The mRNA levels of E-cadherin, N-cadherin and Vimentin were determined by qRT-PCR in MKN-45 and BGC-823 cells. GAPDH was detected as a loading control. All data are presented as mean ± SEM. $^{##}P<0.01$, $^{###}P<0.001$ vs. miR-NC.

reporter vector and the putative miR-340 binding site in the NF-κB1 3'-UTR was mutated (**Figure 5B**). Luciferase reporter assay was used to determine the effect of miR-340 mimic. Our data displayed that introduction of miR-340 dramatically suppressed the luciferase activity of pMir-NF-κB1 3'-UTR WT (**Figure 5C**). The effect of miR-340 was abolished by mutation of the miR-340-binding site in the NF-κB1 3'-UTR, which suggested that miR-340 directly and negatively regulated NF-κB1.

Discussion

In recent years, many reports had shown the miRNAs are dysregulated in multiple types of cancers [27, 28]. It was critical for identification of cancer-specific miRNAs and their targeted to understand their roles in oncogenesis and defined novel therapeutic targets [29-36]. In this paper, we focused on the function of miR-340 in the pathogenesis of human gastric cancer. Firstly, we detected miR-340 level in GC cell lines by real-time RT-PCR assay. We found that the level of miR-340 was significantly reduced in cancer cells compared to the normal human fallopian tube epithelial FTE187 cells.

Many reports on the function of miR-340 deregulation in human tumorigenesis had been showed. Interestingly, making use of miRNA expression analysis and real-time TaqMan PCR, we also found that level of miR-340 was significantly reduced in gastric cancer cells compared to relevant normal cells. In addition, several reports had demonstrated that miR-340 was also evidently down-regulated in colorectal cancer, breast cancer, glioblastoma and oral squamous cell carcinoma [17, 26, 37, 38]. Therefore, aberrant regulation of miR-340 was altofrequent of in multiple types of cancer cells and tissues, indicating that decreased miR-340 might play a critical role in tumorigenesis.

It had been presumed that many miRNAs were decreased in cancers, suggesting that they might normally function as tumor suppressor genes. Hence, we hypothesized that miR-340 was an inhibitory factor of growth in human gastric cancer cells. Because level of miR-340 was down-regulated in cancer cells and tissues, we expected that up-regulation of miR-340 would lead to arresting cell growth. Using the BrdU-ELISA assay, we found that MKN-45 and BGC-823 cells transfected with the miR-340 mimic exhibited decreased growth compared to cells transfected with miR-NC. Cell cycle analyses also showed that the percentage of cells in the G1-phase was increased and the percentage of cells in the S-phase was decreased in cells transfected with miR-340 mimic compared to cells transfected with miR-NC. Moreover, flow cytometry analysis demonstrated that miR-340 mimic could evidently induced apoptosis of MKN-45 and BGC-823 cells compared with miR-NC group. It has been well known that cell cycle progression and apoptosis are regulated by numerous proteins. In addition, Transwell assay showed that miR-340 mimic dramatically inhibited the invasion of MKN-45 and BGC-823 cells compared with miR-NC group. Furthermore, we determined the change of EMT markers in MKN-45 and BGC-

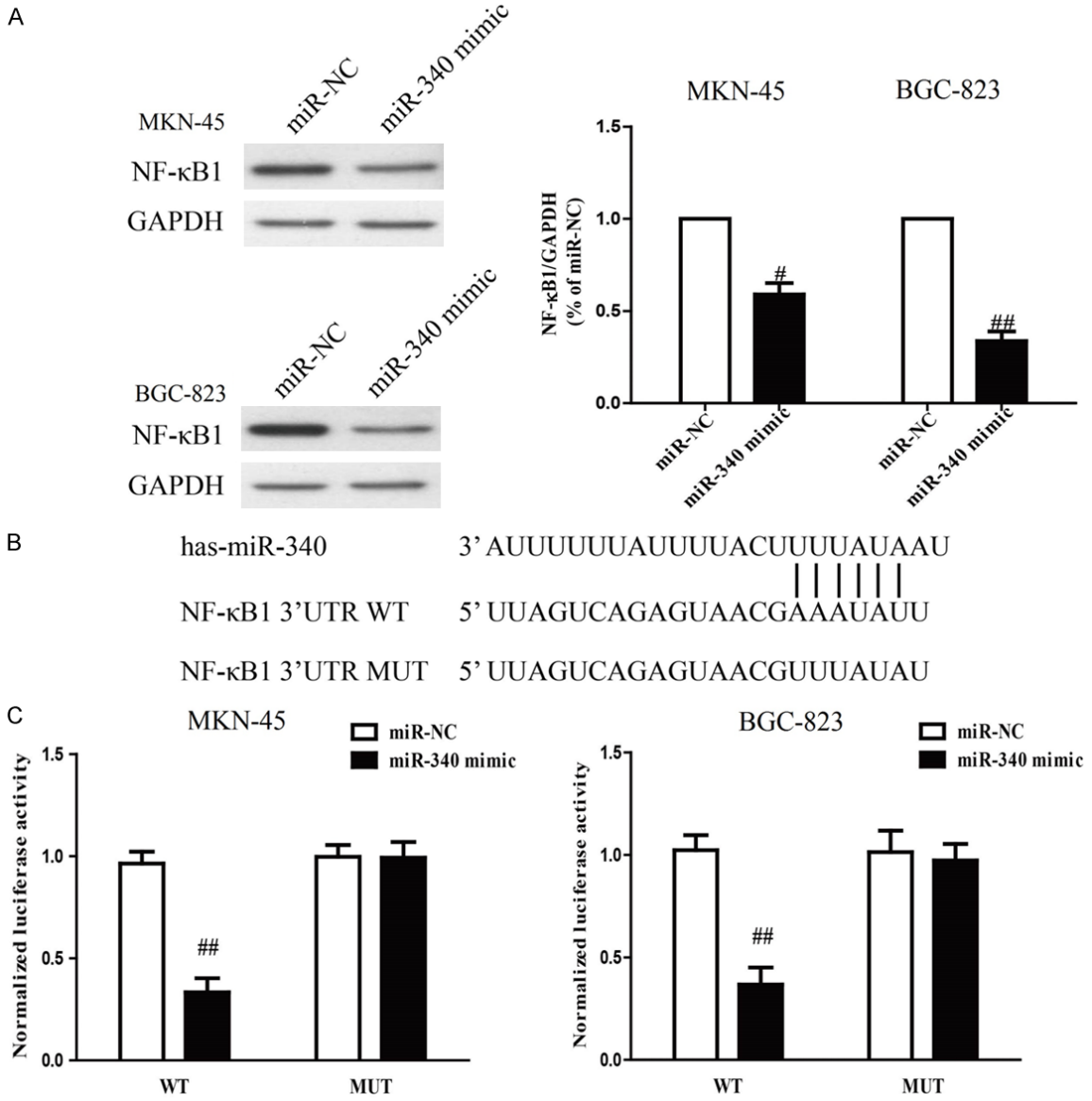


Figure 5. NF-κB1 was a direct target of miR-340. MKN-45 and BGC-823 cells were transfected with miR-340 mimic or miR-NC. A. The protein expression of NF-κB1 was determined by Western blot. GAPDH was detected as a loading control. B. Schematic representation of NF-κB1 3'UTRs showing putative miRNA target site. C. The analysis of the relative luciferase activities of NF-κB1-WT, NF-κB1-MUT in GC cells. All data are presented as mean ± SEM. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. miR-NC.

823 cells transfected with miR-24 mimic. Our results showed that up-regulation of miR-340 could markedly suppress invasive ability of BC cells by dramatically up-regulating the epithelial marker E-cadherin and down-regulating the mesenchymal marker N-cadherin and Vimentin, which supported that miR-340 might suppress EMT process to restrain cell invasion and metastasis.

It had been reported that miR-340 affects cell growth, metastasis and apoptosis of cancer cells possibly by targeting CDK6, ROCK1 and cMet [22-26, 37]. Although bioinformatic tools might help to reveal putative mRNA targets of miRNAs, experimental procedures were required for their validation. In our paper, we showed that miR-340 targeted the NF-κB1 mRNA, thus revealing a possible mechanism associated with ovarian oncogenesis.

Actually, NF-κB1, a member of the Rel/NF-κB transcription factor family, played critical roles in the regulation of immune responses, embryo

and cell lineage development, cell-cycle progression, cell apoptosis, and tumorigenesis [22-24, 35, 36, 39, 40]. Our data showed that NF-κB1 was a target of miR-340. First, using western blotting, we confirmed that introduction of miR-340 could cause the significant decrease in NF-κB1 protein level. In addition, we found that the ability of miR-340 to regulate NF-κB1 expression was direct, because it bound to the 3'UTR of NF-κB1 mRNA with complementarity to the miR-340 seed region. Moreover, the luciferase activity of NF-κB1 3'-UTR was specifically responsive to miR-340 up-regulation. However, mutation of the miR-340 binding site abolished the effect of miR-340 on the regulation of luciferase activity. In this study, overexpression of NF-κB1 could also rescue OC cells from inhibition of cell growth, invasion and EMT caused by miR-340. However, the underlying mechanisms by which NF-κB1 affected growth, invasion and EMT of OC cells remain to be established.

In summary, we showed that miR-340 was evidently decreased in GC cells. Introduction of miR-340 inhibited proliferation, invasion, EMT and induced apoptosis of GC cells through directly targeting NF-κB1. This novel miR-340/NF-κB1 axis might provide new insights into the molecular mechanisms underlying progression and metastasis of GC, and overexpression of miR-340 might be a potential therapeutic strategy for the treatment of GC in the future.

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Disclosure of conflict of interest

None.

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