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Original Article

MiR-203a inhibits gastric stem cancer cells by targeting mTOR

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Abstract: To evaluate the role and underlying mechanism of microRNA (miR)-203a in gastric stem cancer cells. Reverse transcription quantitative polymerase chain reaction was used to determine the expression of miR-203a in the adjacent cancerous and non-cancerous tissues. Cell proliferation and cellular drug resistance was detected using Cell Counting Kit-8 and EDU assays, and cell migration and invasion were detected using transwell assays. RNA sequencing and database website predictions confirmed the downstream target genes, which was further confirmed by double-luciferase reporter gene experiments. Attenuated miR-203a expression was observed in gastric cancer, and its expression level was associated with tumor size, tumor node metastasis stage, tissue differentiation, and patient survival. Overexpression of miR-203a in gastric stem cancer cells significantly inhibited cell proliferation, migration, invasion and cellular drug resistance, whereas silencing its expression resulted in the opposite effects. Double-luciferase reporter gene experiments confirmed that miR-203a acts directly on the downstream target gene, mTOR. Cellular mTOR upregulation restores the inhibitory effect of miR-203a overexpression on gastric stem cancer cell proliferation, migration, invasion and drug resistance. MiR-203a inhibits the malignant behavior of gastric stem cancer cells by targeting mTOR.

Keywords: MiR-203a, gastric stem cancer, mTOR, proliferation, migration, invasion

Introduction

Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide [1, 2]. Most GC cases are adenocarcinomas. The mortality rate of GC is second only to lung cancer [3, 4]. Every year, approximately 1 million GC cases are diagnosed and more than 700,000 people die from it [5, 6]. The current 5-year survival rate for patients with GC is less than 20% owing to recurrence and metastasis [7]. Owing to the low rate of early GC diagnosis, most patients with GC are diagnosed at stage II or III [8]. Surgical resection is the only possible cure for GC; however, it is limited to patients with stage I GC [9-12]. Therefore, effectively improving the early diagnosis rate of patients with GC is an important issue in clinical diagnosis, treatment, and scientific research, and we must identify new and effective molecular biological markers for GC.

Therefore, discovering potential targets for the treatment of GC is valuable. MicroRNAs (miRNAs) are short-stranded non-coding RNA that encode RNA by targeting the inhibition of translation or the stability of mRNA [13]. This mature small RNA molecule is an approximately 22-bp-long single-stranded RNA that usually inhibits the expression of protein-coding genes [14]. MiR-203, which is located on human chromosome 14q32.33, is often used as a tumor suppressor and plays a crucial role in the development of many tumor types [14, 15]. One study showed that the inhibitory effect of miR-203a on tumors might be due to its ability to inhibit the selection and expansion of gastric cancer cells in the early stages of development [16]. MiR-203a reportedly plays a tumor-inhibitory role in many types of cancers, such as melanoma [17], B-cell lymphoma [18], laryngeal cancer [19], pancreatic cancer [20], and colon cancer [21]. However, its role in gastric stem cancer cell development and progression remains unclear.

In this study, the expression level of miR-203a in selected GC tissue samples was detected by

Table 1. Primer sequence

	Forward primer	Reverse primer
MTOR	5'-AGAGGAGGAAGCAAGTGTAAGG-3'	5'-CGCGGTTCTCAGACTTCTTCT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-CCTGCTTCACCACCTTCTTGA-3'
Mir 203a	5'-ACACTCCAGCTGGGAAACCT-3'	5'-CTCAACTGGTGTCGTGGA-3'

reverse transcription polymerase chain reaction (RT-PCR) and compared with the patients' clinical data. Next, a gastric stem cancer cell line overexpressing miR-203a and a control group were established, and the cellular proliferation, migration, invasion abilities and cellular drug resistance were detected by Cell Counting Kit (CCK)-8 and transwell assays, respectively. MiR-203a expression was silenced, and the cellular proliferation and migration rates, invasion ability and cellular drug resistance were re-detected. Next, the target genes downstream of miR-203a were predicted and analyzed using RNA sequencing (RNA-seq) and database website predictions, and the analysis was verified using a double-luciferase reporter gene experiment. Target gene expression was upregulated in cells stably overexpressing miR-203a, and the recovery of the effect of upregulated target genes on gastric cancer stem cells was detected using an experimental method. This study aimed to clarify the role of miR-203a in the occurrence and development of gastric cancer stem cells through the above experiments.

Material and methods

Cell lines and experimental materials

The MGC-803 cell lines were obtained from the Cell Bank of Xiangya Medical College of Central South University of China. The miR-203a mimics were synthesized by Jin Weizhi Company. The CCK-8 kit was purchased from Beyotime Company, the transwell chamber and Matrigel were purchased from Millipore, and mTOR primary antibody was purchased from Santa Cruz Biochemical. Beijing Zhongshan Jinqiao Company provided the sheep anti-mouse secondary antibodies labeled with horseradish peroxidase. Israeli BI Company provided the fetal bovine serum (FBS). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco.

Patient data and samples

All clinical data for this project were obtained from pathological GC samples collected from the First Affiliated Hospital of Guilin Medical University in 2012-2015; a total of 95 samples were selected. All patients were diagnosed with GC based on the postoperative histopathological examination. The Ethics Committee of Guilin Medical University approved the study, and informed consent was obtained from all participants.

RT-PCR technology

Total RNA was extracted using the TRIzol method as instructed by the reverse transcription kit and reverse transcribed into cDNA. Quantitative RT-PCR was then performed using TaqMan Universal PCR Master Mix (434437) was then used in an ABI PRISM 7700 Thermocycler (Applied Biosystems) to conduct quantitative RT-PCR (**Table 1**). The reaction conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 59°C for 30 s, and 70°C for 30 s. The relative expression of the target gene was calculated using $^{\Delta\Delta}$Ct method using U6 as the internal reference. We repeated the experiment three times and obtained average values.

Cell culture and flow sorting

High-glucose DMEM containing 10% FBS and double antibodies was cultured in the cell incubator at 5% CO_2, 37°C, and 95% humidity. After staining with CD44, Fluorescent antibody-stained cells were flow-sorted using a FACS Aria II (Becton Dickinson) flow cytometer.

Cell transfection

After being prepared as a single-cell suspension, the cells were inoculated into a six-well plate and cultured until approximately half of the fusion degree of the cells, and the medium was changed to Opti-MEM serum-free medium.

Plasmid DNA was diluted, mixed with Opti-MEM, and incubated at room temperature. Simultaneously, Opti-MEM was used to dilute Lipofectamine 2000 transfection reagent, mixed, and incubated at room temperature. After mixing, the transfection solution was added slowly to the cells. After 12 h, the culture medium was replaced with complete medium and routine culturing was continued in a cell incubator at 5% CO_2, 37°C, and 95% humidity.

Cellular proliferative ability and cell drug resistance detected by CCK-8 and EDU assay

Cells in the logarithmic phase after transfection were resuspended in complete culture medium after trypsin digestion. For different experimental groups, 96-well plates were inoculated with the prepared single-cell suspensions. A 100-mL single-cell suspension was added to each well, the equivalent of 2000-3000 cells/mL/well. In the experiment, there were five replicate holes in each group. After 24 h of culture in a cell incubator, 10 μl of the CCK-8 chromogenic agent was added to each well, and the cells were incubated for an hour.

Absorbance was measured at 450 nm using an enzyme-labeling instrument. One trial was carried out per day and the test was conducted for four consecutive days. We defined Day 1 as the first day when we began the CCK8 assay. EDU assay was conducted by using EDU assay Kit according to the manufacturer's instructions.

Transwell assay to measure cell migration and invasion ability

Cells in the logarithmic phase after transfection were resuspended in DMEM containing 1% FBS after trypsin digestion and the cell density was adjusted to 5×10^5 cells/mL. A transwell chamber was placed in a 24-well plate. Next, 100 mL of the single-cell suspension was added to the chamber, while 500 mL of complete medium was added to the lower 24-well plate. After 48 h of culture, the compartment was removed and the culture medium discarded. After formaldehyde fixation, crystal violet staining, and rinsing with phosphate-buffered saline, the cells on the outer filter membrane of each chamber were observed under an inverted microscope. Five visual fields were randomly selected from each chamber and the cells counted.

For the transwell invasion test, the diluted Matrigel gel was added to the transwell chamber, which was placed in a 24-well plate and incubated in a cell incubator for 4-6 h. The remaining experimental procedures were consistent with those described above. Finally, an inverted microscope was used to observe and count the cells on the filter membrane of the chamber.

RNA-seq sequencing and database analysis

The TargetScan (http://www.targetscan.org/) and miRDB (http://www.mirdb.org/miRDB/) databases and RNA-seq were used to predict the downstream target genes of miR-203a. These results suggested that miR-203a targets mTOR.

Double-luciferase reporter gene detection

A wild-type and mutant mammalian target of rapamycin (mTOR) 3' untranslated region (3'UTR) sequence was constructed, and the wild-type and mutant sequences were inserted into the pmir-Glo reporter vector. The miR-203a mimic (or miR-203a-NC) and reporter gene plasmid vectors were co-transfected into the gastric cancer stem cell. After 48 h of transfection, luciferase activity was calculated using a double-luciferase reporter system (Promega, Madison, WI, USA), and the relative luciferase activity was compared.

Statistical analysis

In this experiment, SPSS (version 24.0) was used to analyze the data. The data are expressed by mean ± standard deviation. The Student's t-test was used for comparisons between two independent groups, and one-way analysis of variance (ANOVA) was used for comparisons between multiple groups. This was repeated thrice, and the average value was calculated. This difference was statistically significant ($P<0.05$).

Results

MiR-203a expression is attenuated in GC tissues and related with clinicopathological characteristics and prognosis

Using quantitative RT-PCR, we examined the miR-203a expression levels in GC and non-can-

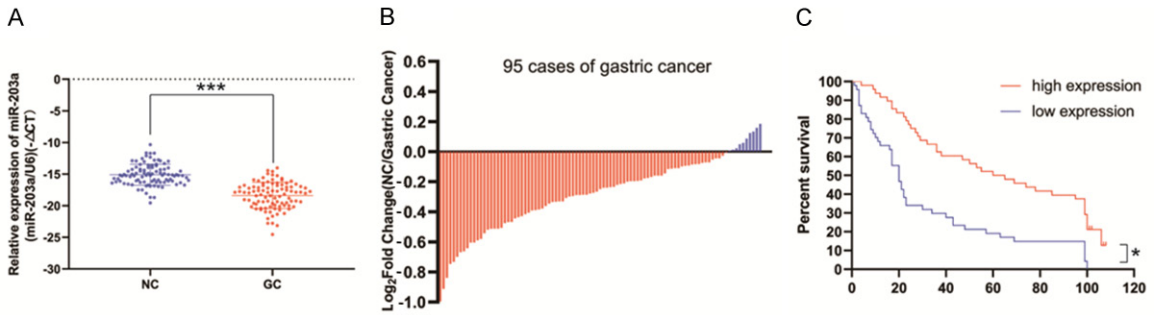

Figure 1. MiR-203a was downregulated in gastric cancer and associated with patient prognosis. A. Expression of miR-203a in gastric cancer (GC) tissues (n = 95) versus non-cancer (NC) adjacent tissues (n = 95). B. The fold change of the ratio of miR-203a expression in GC tissues and adjacent tissues. C. Survival curve of patients with low versus high miR-203a expression levels. The comparisons were performed using *t*-test and pairwise *t*-tests (* means P<0.05, * means P<0.01, * means P<0.001).

Table 2. Clinical characteristics of patients

Clinicopathological feature	miR-203a		*P* value
	High expression (n = 48)	Low experssion (n = 47)	
Gender			
Female	25	19	
Male	24	28	0.301
Age (years)			
≤60	27	25	
>60	21	22	0.225
Pathological grading			
Poor	17	32	
Well	31	15	0.005
AJCC seventh clinical stage (TNM stage)			
I+II	20	29	
III+IV	28	18	0.018
Tumor size (cm)			
≥5	22	31	
<5	26	16	0.010

cerous adjacent tissues. Its expression in GC tissues was significantly lower than that in paracancerous tissues (**Figure 1A**, **1B**). When we combined the PCR results with the patients' clinical data, we found that miR-203a expression was correlated with tumor size, tumor node metastasis (TMN) stage, and tissue differentiation degree (P<0.05) but not with age or sex (P>0.05) (**Table 2**). In addition, a survival analysis demonstrated that low miR-203a expression was associated with poor overall survival (**Figure 1C**). This suggests that miR-203a may be related to malignancy and the malignant biological behavior of gastric cancer stem cells.

MiR-203a inhibited biological behaviors of gastric cancer stem cells

First, $CD44^+$ MGC-803 cells were isolated by flow cytometric sorting and expanded (**Figure 2A**). To explore the role of miR-203a in gastric cancer stem cell, we used the liposome transfection technique to introduce miR-203a mimics into human gastric cancer stem cell line $CD44^+$ MGC-803, respectively, to establish an overexpressed miR-203a group and control cell lines. After successful transfection, we first detected the proliferative ability of cells in each group using the CCK-8 and EdU assays. Cell proliferation was significantly lower in the overexpression versus control group (**Figure 2B**,

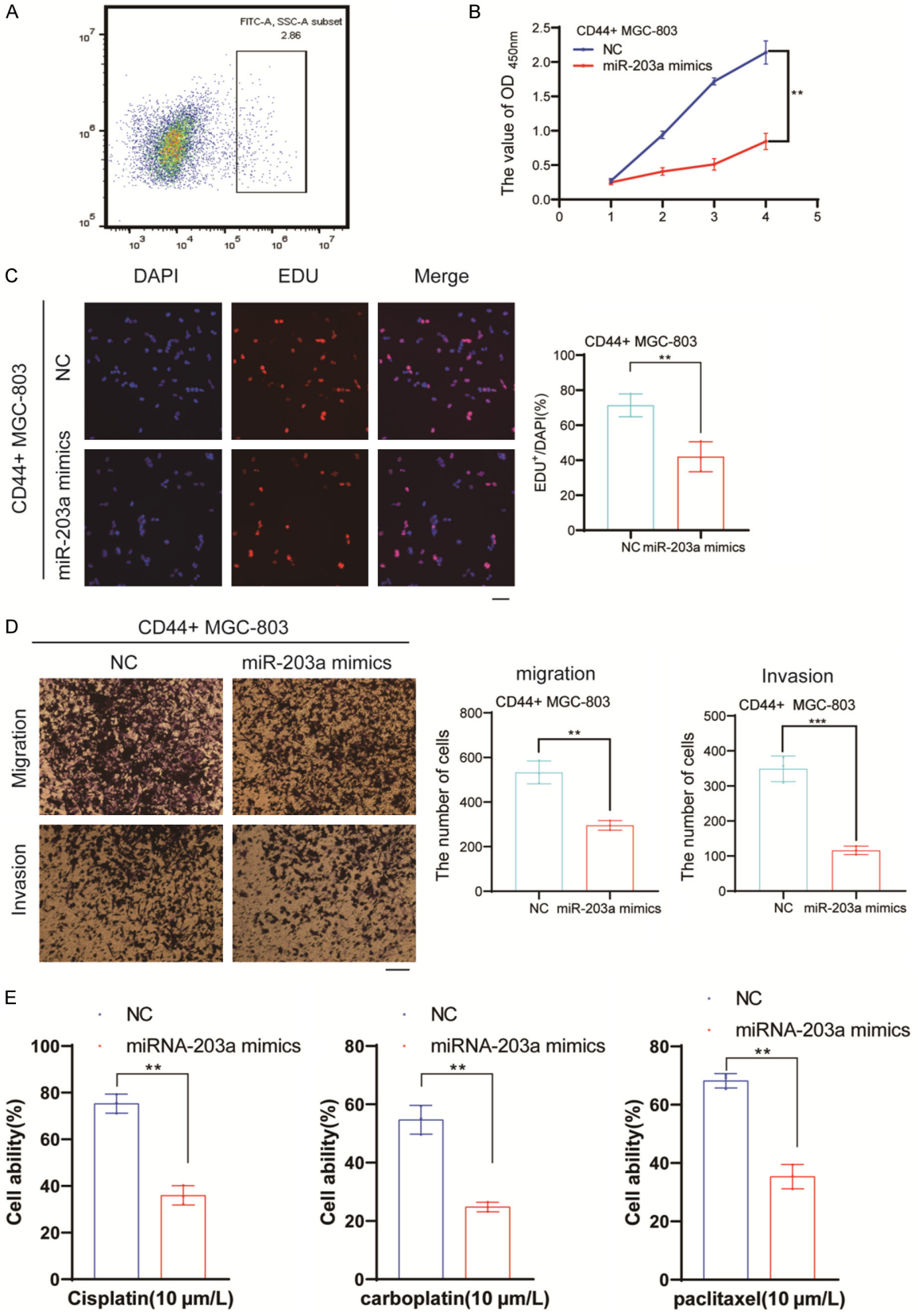

Figure 2. MiR-203a overexpression inhibited gastric stem cancer cell proliferation, migration, invasion and cell drug resistance. A. $CD44^+$ MGC-803 cells were isolated by flow cytometric sorting. B. Growth curves of gastric cancer

stem cells measured by Cell Counting Kit-8 assays transfected with miRNA-203a mimics. C. The ability of cell proliferation was tested by EDU transfected with miRNA-203a mimics. D. The ability of cell migration and invasion after transfection with miRNA-203a mimics examined by transwell. E. The ability of drug resistance of cells transfected with miRNA-203a mimics examined by CCK8. The comparisons were performed using *t*-test and pairwise *t*-tests (* means P<0.05, * means P<0.01, * means P<0.001; the scar bar: 50 μm).

2C). We then tested the cellular migration and invasion ability using transwell cell migration and invasion experiments to count the number of transmembrane cells under a microscope. We found that the cellular migration and invasion abilities in the overexpression group were significantly lower than those in the control group (**Figure 2D**). Next, we tested the resistance of cells to different chemotherapy drugs. The result showed that the cellular drug resistance in the overexpression group were significantly lower than those in the control group (**Figure 2E**). These results suggested that miR-203a inhibits the biological behavior of gastric cancer stem cells.

Silencing miR-203a promoted biological behaviors of gastric cancer stem cells

To further investigate the role of miR-203a in gastric cancer stem cells, we used liposome transfection to transfer siRNAs constructed in vitro to silence miR-203a expression in $CD44^+$ MGC-803 cells, thereby establishing cell lines with low miR-203a expression. The results showed that the low expression group had a significantly higher proliferation ability than the control group (**Figure 3A**, **3B**). We also found that cells in the low expression group were significantly more able to migrate, invade and had better cellular drug resistance than cells in the control group (**Figure 3C-E**). These results suggested that silencing miR-203a promoted the biological behaviors of gastric cancer stem cells.

MiR-203a binds directly to mTOR in cells to exert biological effects

To explore the potential molecular mechanism underlying the role of miR-203a in cells and identify its possible downstream target genes, we first performed RNA-seq of gastric cancer stem cells overexpressing miR-203a and control cells. We then analyzed the mRNA with differential expression levels between the two types of cells (**Figure 4C**), which revealed that mTOR was the target gene. Therefore, to further verify our findings, we used a double-luciferase reporter gene experiment to construct wild-type and mutant mTOR 3'UTR sequences interacting with miR-203a, then clone formed them into a luciferase reporter vector and co-transfected them with miR-203a mimics and unrelated control sequences into gastric cancer stem cells. The double-luciferase reporter system was used to detect and analyze the binding of miR-203a and mTOR 3'UTR. We found that the luciferase activity decreased significantly after the co-transfection of the miR-203 mimic and reporter gene vector with wild-type mTOR 3'UTR sequence, while miR-203 had no effect on luciferase activity of the reporter gene vector with mutant mTOR 3'UTR sequence (**Figure 4A**, **4B**). These results suggest a direct interaction between miR-203 and mTOR in gastric cancer stem cells.

Upregulation of mTOR can restore tumor inhibition caused by high miR-203a expression

We further explored whether miR-203a could regulate the malignant behavior of gastric cancer stem cells by targeting mTOR. We examined the proliferation, migration, invasion and cell drug resistance of gastric cancer stem cells stably overexpressing miR-203a after mTOR upregulation. We found that mTOR upregulation restored the inhibitory effects of miR-203a on cell proliferation (**Figure 5A**, **5B**), migration, and invasion (**Figure 5C**, **5D**) and cell drug resistance (**Figure 5E**). These results suggest that miR-203a inhibits the physiological activity of gastric cancer stem cells by acting on mTOR.

Discussion

The number of new cases of GC in China accounts for more than 40% of the global total annually [22-24]. Surgical resection is the primary treatment for GC; however, the survival rate is less than 30% [25]. Because the early diagnosis rate of GC is low, most patients are diagnosed with stage II or III disease [8]. Therefore, it is necessary to identify new molecular targets to inhibit GC occurrence and progression.

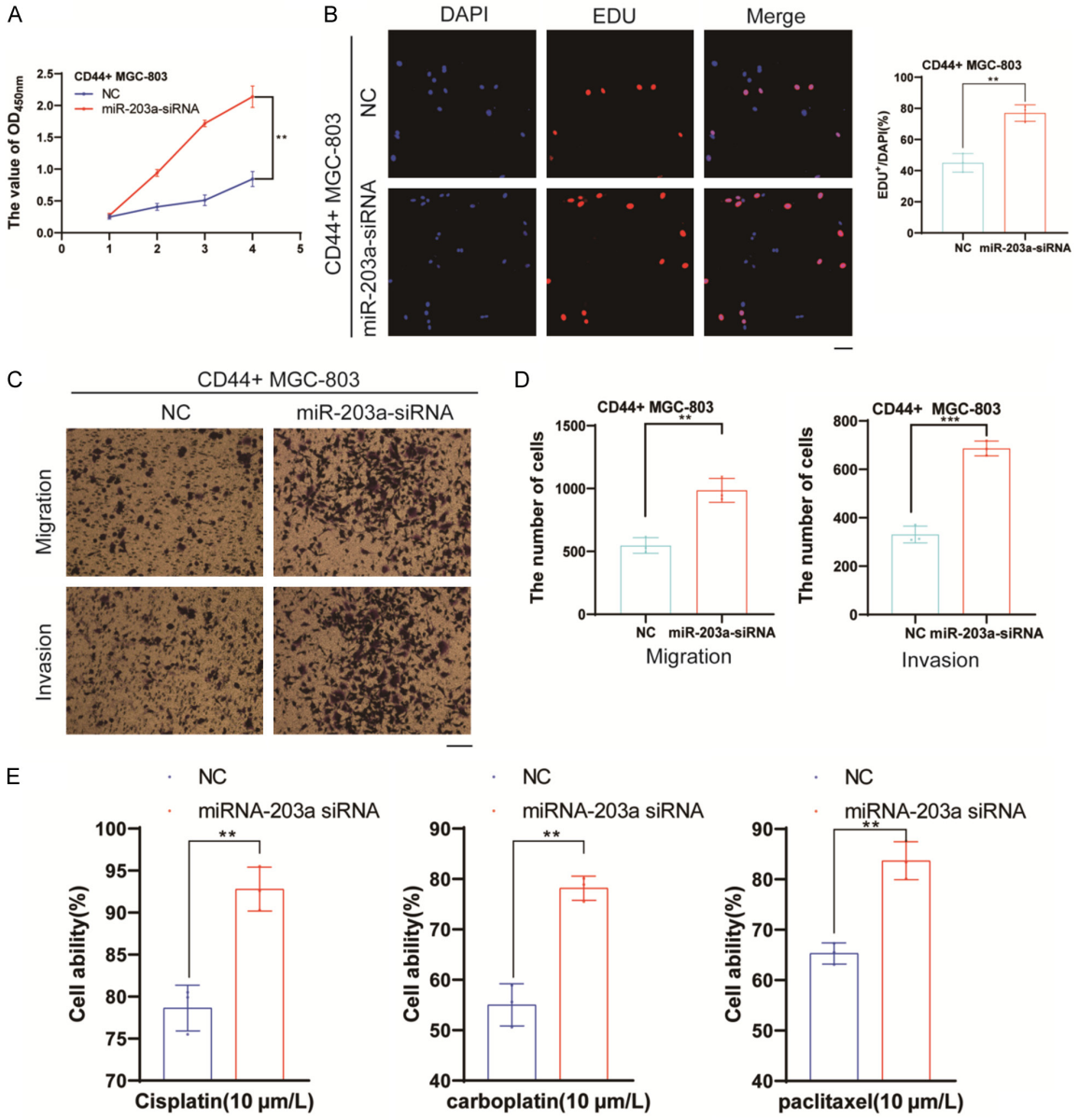

Figure 3. MiR-203a knockdown promoted proliferation, migration, and invasion of gastric stem cancer cells transfected with miRNA-203a siRNA. A. Cell proliferation measured by CCK8 transfected with miRNA-203a siRNA. B. The ability of cell proliferation was tested by EDU transfected with miRNA-203a siRNA. C, D. The ability of cell migration and invasion of cells transfected with miRNA-203a siRNA examined by transwell. E. The ability of drug resistance of cells transfected with miRNA-203a siRNA examined by CCK8. The comparisons were performed using *t*-test and pairwise *t*-tests (* means P<0.05, * means P<0.01, * means P<0.001; the scar bar: 50 μm).

MiR-203a is a short-stranded non-coding RNA located on human chromosome 14q32.33 [13, 14]. A preliminary understanding of its function was provided by a study reporting that the absence of miR-203a can influence epidermal formation during skin development [25-27]. Most studies of miR-203a to date focused on its biological significance as a tumor suppressor. Moreover, miR-203a plays a critical role in the development and occurrence of many tumor types. Miao et al. [20] reported that miR-203a inhibits the migration and invasion of pancreatic cancer cells by targeting caveolin-1. Pal et al. [28] reported that miR-203a inhibits human glioma by negatively regulating GAS41 expression. Zhao et al. [19] showed that miR-203a plays an antitumor role in and is expected to become an early diagnostic marker of laryngeal carcinoma. However, there are few reports on the relationship between miR-203a and GC.

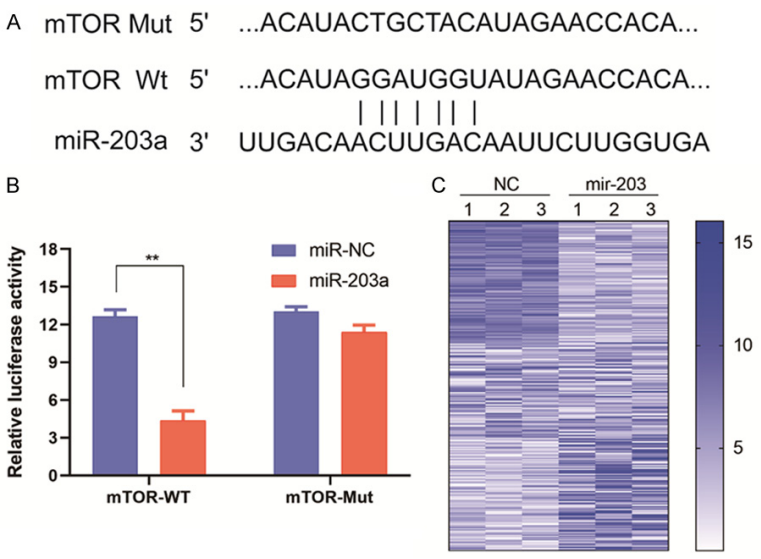

Figure 4. MTOR was the target gene of miR-203. A. TargetScan and miRDB databases indicated that mammalian target of rapamycin (mTOR) might be the target of miR203a. B, C. The luciferase activity decreased significantly after the co-transfection of miR-203a mimic with wild-type mTOR 3' untranslated region (3'UTR) sequence, whereas no difference was seen in luciferase activity after the co-transfection of the miR-203a mimic with the mutant mTOR 3'UTR sequence. The comparisons were performed using *t*-test and pairwise *t*-tests (* means P<0.05, * means P<0.01; the scar bar: 50 μm).

Given the low diagnostic and high mortality rates of GC, it is necessary to explore the role of miR-203a. Here we detected the expression level of miR-203a in selected GC tissues and their paracancerous tissues using RT-PCR and analyzed the correlation between the results and the patients' clinical data. In GC, miR-203a expression is low and related to tumor size, TNM stage, and tissue differentiation. Therefore, we determined that miR-203a expression may be related to the high malignancy of GC and the malignant biological behavior of gastric cancer stem cells. Therefore, we established a miR-203a overexpressing cell line and a control cell line using a liposome transfection technique. CCK-8 and transwell assays were used to analyze cell proliferation, migration, invasion and cell drug resistance. These results demonstrated that miR-203a overexpression inhibited gastric cancer stem cell proliferation, migration, invasion and cellular drug resistance. To further investigate its effect, we silenced miR-203a expression in cells using siRNA; the results were similar to those in the overexpression group. After miR-203a silencing, cell proliferation, migration, invasion and cell drug resistance improved. Accordingly, we conclude that miR-203a is expressed at low levels in GC and can regulate gastric cancer stem cell proliferation, migration, invasion and cellular drug resistance.

MTOR exists as two kinds of complexes: mTORC1 and mTORC2. The former contains the regulation-related protein mTOR, while the latter contains rapamycin-insensitive mTOR chaperone qZ [29]. To identify the downstream target genes of miR-203a, we identified the possible target gene mTOR using RNA-seq and database website prediction analyses. To verify the direct interaction between miR-203a and mTOR in the cells, we used a double-luciferase reporter assay. Our results indicate a direct interaction between miR-203a and mTOR in gastric cancer stem cells. To explore the effect of mTOR upregulation in gastric cancer stem cells overexpressing miR-203a, we performed a series of rescue experiments and found that the inhibition of proliferation, migration, invasion, and cellular drug resistance of gastric cancer stem cell induced by miR-203a overexpression was eliminated after mTOR upregulation. This indicates that miR-203a directly inhibits gastric cancer stem cell proliferation, migration, and invasion and drug resistance via mTOR.

In summary, here we found that miR-203a inhibits the malignant behavior of gastric cancer stem cells. As a tumor suppressor in GC, miR-203a is often downregulated and associated with tumor size, TNM stage, and tissue differentiation. The overexpression of miR-203a in gastric cancer stem cells negatively regulated cell proliferation, migration, invasion and drug resistance by directly acting on mTOR. Follow-up in vivo experiments are needed to study the mechanism of action of miR-203a in gastric cancer stem cells, which can provide new ideas for its clinical diagnosis, treatment, and prognosis to improve the 5-year survival rate. However, we have not yet developed therapeutic approaches specifically targeting miR-

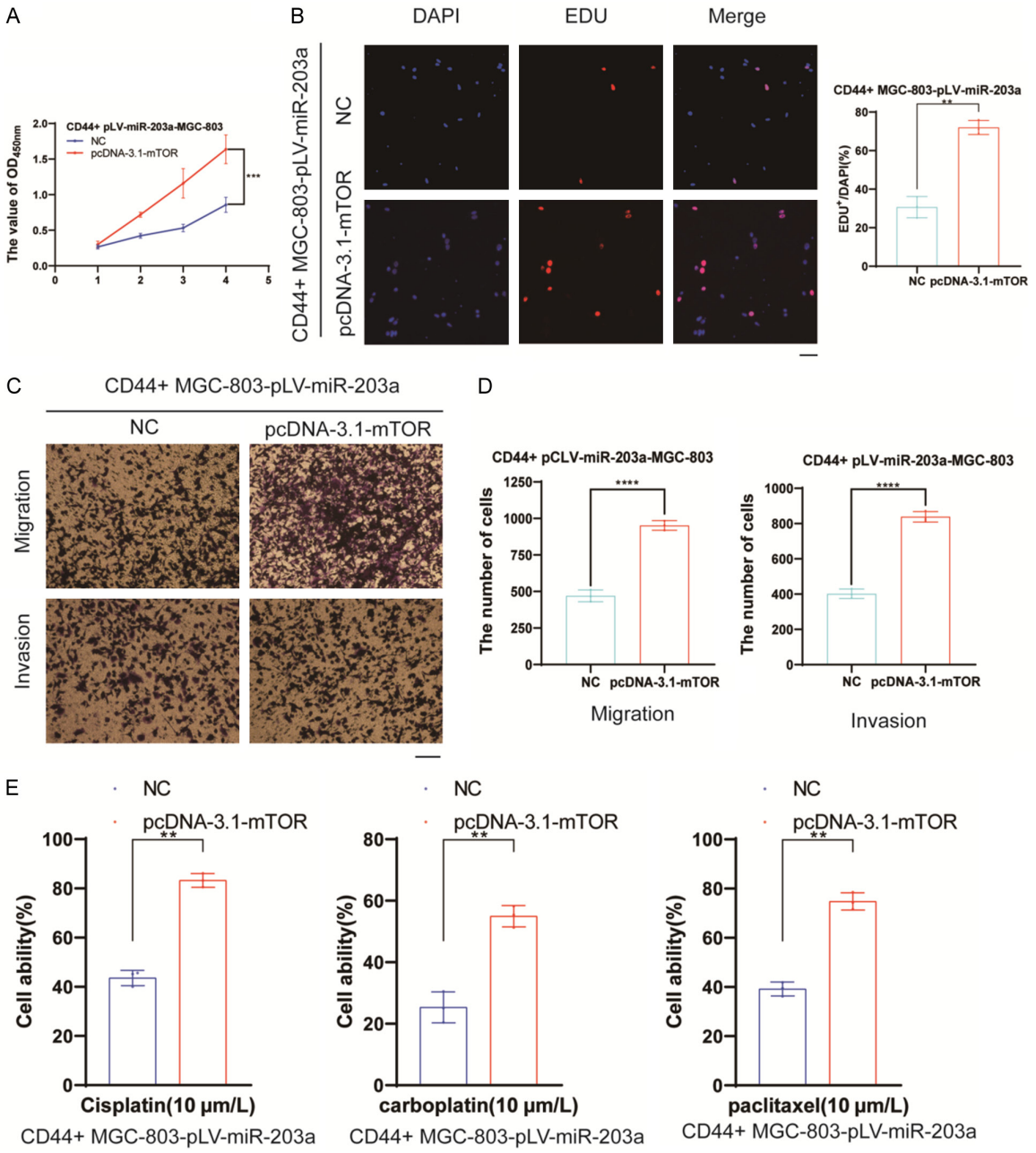

Figure 5. Upregulation of mTOR restored tumor inhibition caused by high miR-203a expression. A. Cell proliferation after transfection with pcDNA-3.1 mTOR measured by CCK8. B. The ability of cell proliferation after transfection with pcDNA-3.1 mTOR tested by EDU. C, D. The ability of cell migration and invasion after transfection with pcDNA-3.1 mTOR examined by transwell. E. The ability of drug resistance of cells transfected with pcDNA-3.1 mTOR examined by CCK8. The comparisons were performed using *t*-test and pairwise *t*-tests (* means P<0.05, * means P<0.01, * means P<0.001; the scar bar: 50 μm).

203a. This will be the focus of our future research efforts.

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Disclosure of conflict of interest

None.

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