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miR-326 Inhibits Gastric Cancer Cell Growth Through Downregulating NOB1

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MicroRNAs (miRNAs) play a crucial role in the development and progression of human cancers, including gastric cancer (GC). The discovery of miRNAs may provide a new and powerful tool for studying the mechanism, diagnosis, and treatment of GC. In this study, we aimed to investigate the role of miR-326 in the development and progression of GC. Quantitative PCR (qPCR) was used to measure the expression level of miR-326 in GC tissues and cell lines. We found that miR-326 was significantly downregulated during GC. In addition, overexpression of miR-326 inhibited GC cell proliferation. Fluorescence-activated cell sorting (FACS) further showed that miR-326 significantly induced GC cell G_2/M arrest. Subsequent dual-luciferase reporter assay identified one of the proto-oncogene NOB1 as a direct target of miR-326, and NOB1 can save growth inhibition caused by miR-326. We also confirmed that the growth inhibition caused by miR-326 is associated with AKT pathway activation. Taken together, our results indicate that miR-326 could serve as a potential diagnostic biomarker and therapeutic option for GC in the near future.

Key words: Gastric cancer (GC); miR-326; NOB1; Proliferation; Cell cycle; AKT

INTRODUCTION

Accounting for more than 700,000 deaths annually, gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer deaths in the world[1,2]. Current studies suggest that GC has a complicated multifactorial and multistage process, involving various genetic and epigenetic changes[3]. Although much progress has been reported in GC, more information regarding the pathogenesis and molecular mechanisms responsible for the progression of metastasis of GC is urgently needed.

It is worth mentioning that mRNA degradation and translational suppression are under the regulation of microRNAs (miRNAs)[4,5]. miRNAs play a crucial role in the development and progression of human cancers, including GC, and they contribute to GC cell metastasis and invasion in various ways[6–8]. By regulating the expression of various oncogenes and tumor suppressor genes at different levels, miRNAs are thought to play a critical role in GC initiation and progression[9–12]. The discovery of new miRNAs may provide a novel and powerful tool to research the mechanism, diagnosis, and treatment of GC. Thus, miRNAs are currently one of the hot topics in the study of GC.

A growing number of studies have shown that these differentially expressed miRNAs produce a similar effect on oncogenes or tumor suppressor genes in tumor initiation and progression by negatively regulating them[13]. For example, Bao et al. found miR-139 to have a low expression in GC cells. Furthermore, overexpression of miR-139 proved to inhibit the proliferation of GC by inhibiting CXCR4 expression[14]. In addition, the expression level of miR-21 in GC samples was found to be significantly higher than in healthy controls because the expression of miR-21 inhibits the expression of the tumor suppressor gene RECK[15,16]. Investigation of the function and mechanism of miR-133a in GC suggested that miR-133a is downregulated in GC and functions as a tumor suppressor partly by inhibiting IGF1R[17]. However, the expression pattern and mechanism of miR-326 in human GC tissues and cells are still unclear.

In this study, we explored the expression levels of miR-326 in GC and the effect of miR-326 on GC growth and process. We demonstrated that miR-326 expression was significantly downregulated in GC tissues and cell lines. Furthermore, overexpression of miR-326 was found to potently inhibit GC cell proliferation. More importantly, fluorescence-activated cell sorting (FACS) showed that miR-326 significantly induced GC cell G_2/M arrest. Subsequent dual-luciferase reporter assay identified the proto-oncogene NOB1 as a direct target of miR-326 and that

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NOB1 can save growth inhibition caused by miR-326. We also confirmed that the cell signaling pathway AKT was involved in growth inhibition caused by miR-326. The present study provides a new understanding of the mechanism regulating miR-326 in AKT and identifies potential avenues for therapeutic intervention for GC.

MATERIALS AND METHODS

Tissue Samples

A total of 15 pairs of primary GC tissues and morphologically normal tissues (located >3 cm away from the tumor) were obtained from the Department of Gastroenterology, General Hospital of People's Liberation Army (PLA), from March 2013 to April 2015. All subjects were diagnosed and confirmed by a pathologist. None of the subjects received any biotherapy or chemotherapy before recruitment to this study. All of the human material was obtained with informed consent, and the present study was approved by the ethics committee of the hospital.

Cell Culture

The human GC cell lines, including BGC-823, SGC-7901, HGC-27, and MKN-45, and the human immortal gastric mucosa epithelial cell line GES were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The BGC-823 and GES cell lines were propagated in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria), streptomycin (100 μg/ml), and penicillin (100 U/ml). The HGC-27, MKN-45, and SGC-7901 cell lines were maintained in RPMI-1640 medium (PAA) supplemented with 10% FBS (PAA). All cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Cell Transfection and Treatment

For transfection, the miR-326 mimic, antisense oligonucleotides (ASO) of miR-326 and their negative controls were synthesized by RiboBio (Guangzhou, P.R. China). The NOB1 expression plasmid pc-NOB1 was synthesized by GenePharma (Shanghai, P.R. China) by subcloning the full-length wild-type NOB1 coding sequence into pcDNA3.1. Cells were seeded on a 60-mm dish and incubated for 16 h, and then Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection according to the manufacturer's instructions. Cells were harvested after 48 h of transfection and used for the additional analyses.

A specific inhibitor of p-p38, that is, SB203580, was purchased from Calbiochem (La Jolla, CA, USA), and 10 μM SB203580 was used for treating cells for 2 h to inhibit p38 MAPK activation.

RNA Isolation and Quantitative PCR (qPCR)

Total RNA was extracted from cells and frozen tissues using TRIzol reagent (Invitrogen). cDNA was synthesized using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, USA) according to the manufacturer's instructions. For quantitative PCR (qPCR) analysis, FastSTART Universal SYBR Green Master (ROX) (Roche) was used according to the manufacturer's instructions. Each qPCR was conducted on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were normalized to GAPDH or U6 snRNA expression in each sample, and results were calculated according to the classic $2^{-\Delta\Delta}$Ct method. All primers were synthesized by GenePharma.

Cell Proliferation Assay

For evaluation of cell proliferation, 5×10^4 SGC7901 and BGC-823 cells were seeded in 96-well plates and incubated overnight. After transfection, 20 μl of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and plates were incubated for 4 h at 37°C. Then the reaction was stopped by 150 μl of dimethyl sulfoxide (DMSO), and optical density was detected with a microplate spectrophotometer (ELx800; BioTek, Winooski, VT, USA) at a wavelength of 570 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell Cycle Analysis

To analyze cell cycle, DNA content per duplicate was analyzed using the FACSort Cellquect software (BD Biosciences, San Jose, CA, USA). The transfected cells were seeded into six-well plates and cultured for 2 days. At the end of 2 days, cells were fixed in 75% ice-cold ethanol overnight at 4°C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 15 min at room temperature in the dark and analyzed by fluorescence-activated cell sorting (FACSCalibur; BD Biosciences). The cells were excited at 488 nm, and the emission was collected through a 630-nm filter. In total, 2×10^4 cells were collected from each sample. The cell cycle distribution was evaluated by calculating the proportion of cells in the G_0/G_1, S, and G_2/M stages. In each independent experiment, three parallel wells were made, and the procedures were carried out in triplicate.

Dual-Luciferase Reporter Assay

The 3′-UTR of NOB1 was amplified by PCR and placed in the pmiR-Report vector (Ambion, Grand Island, NY, USA). These vectors were cotransfected with the miR-326 mimic or its negative control into cells using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, firefly and *Renilla* luciferase activities were

measured using the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA)[18].

Western Blotting Analysis

Cells were lysed in lysis buffer (Beyotime, Shanghai, P.R. China). Insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4°C. Protein was subjected to electrophoresis using 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH, USA). Membranes were blocked for 2 h in 5% nonfat dry milk in TBST (10 mM Tris-HCl and 0.05% Tween 20). The membrane was incubated with primary antibodies: cyclin B1 (sc-752; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), cyclin D1 (sc-753; Santa Cruz Biotechnologies), CDK1 (ab18; Abcam, Cambridge, MA, USA), NOB1 (ab87151; Abcam), or GAPDH (sc-25778; Santa Cruz Biotechnologies) overnight at 4°C, and then with secondary antibodies (sc-2985 or sc-2986; Santa Cruz Biotechnologies) for 2 h at room temperature. The dilutions for the primary and secondary antibodies were 1:1,000 and 1:2,000, respectively. The immunoreactive protein bands were visualized using enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, UK).

Statistical Analysis

Statistical analysis was performed with SPSS 13.0 software (version 13.0; Chicago, IL, USA). Student's *t*-test and one-way ANOVA test were used according to the data characteristics. A value of $p<0.05$ was considered statistically significant. The quantitative data are presented as mean ± standard derivation (SD).

RESULTS

Low miR-326 Expression in GC

To determine the role of miR-326 in GC, we detected the miR-326 level in GC and adjacent nontumor gastric tissues and in cells by qPCR. The expression of miR-326 was significantly downregulated in GC compared with normal gastric tissues and cell lines ($p<0.05$ or $p<0.01$) (Fig. 1A and B).

miR-326 Inhibited GC Cell Proliferation and Induced G_2/M Arrest

We chose BGC-823 and SGC7901 for the next step in our research because they showed relatively low expression of miR-326. The MTT assay showed that overexpression of miR-326 inhibited cell proliferation ($p<0.05$ or $p<0.01$), while downregulation of miR-326 accelerated cell proliferation when compared with the negative control groups ($p<0.05$) (Fig. 2A and B).

Next, we used a fluorescence-activated cell sorter to detect the cell cycle process. Overexpression of miR-326 caused blocking of G_2/M (Fig. 2C and D). We then examined the expression of cell cycle-related protein at the level of mRNA and protein with qPCR and Western blotting. Results showed that the overexpression of miR-326 inhibited the expression of cell cycle proteins ($p<0.01$). Conversely, downregulation of miR-326

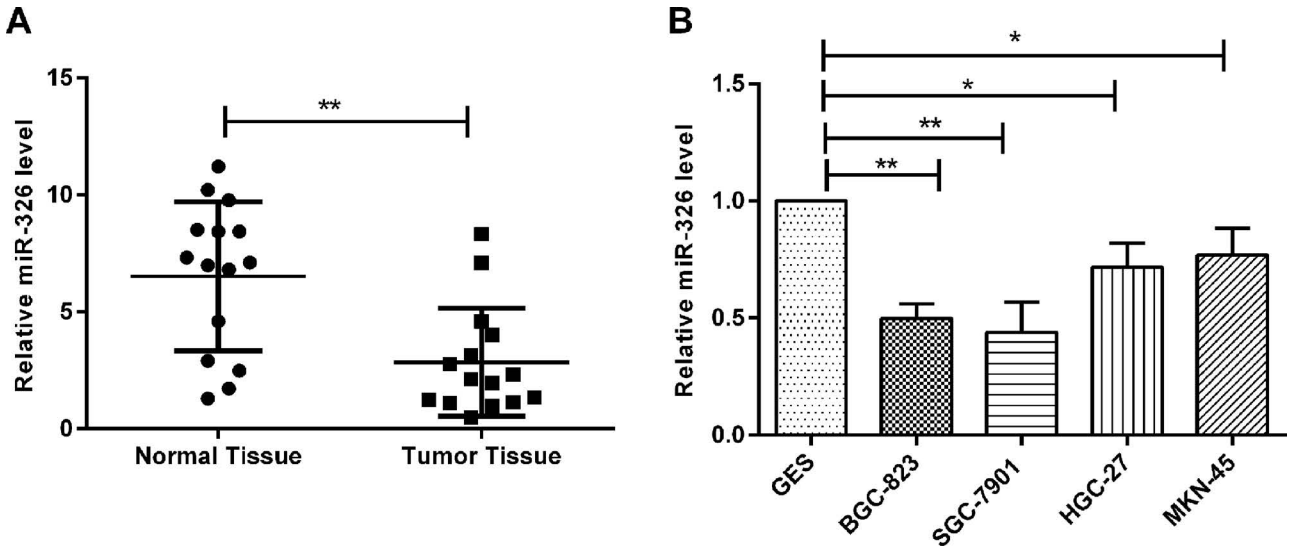

Figure 1. Low expression of miR-326 in gastric cancer (GC). (A) The expression level of miR-326 in GC tissues and the matched normal tissues was measured by qPCR. (B) The expression levels of miR-326 in the HGC-27, BGC-823, SGC-7901, MKN-45, and GES cell lines were measured by qPCR. *$p<0.05$; **$p<0.01$.

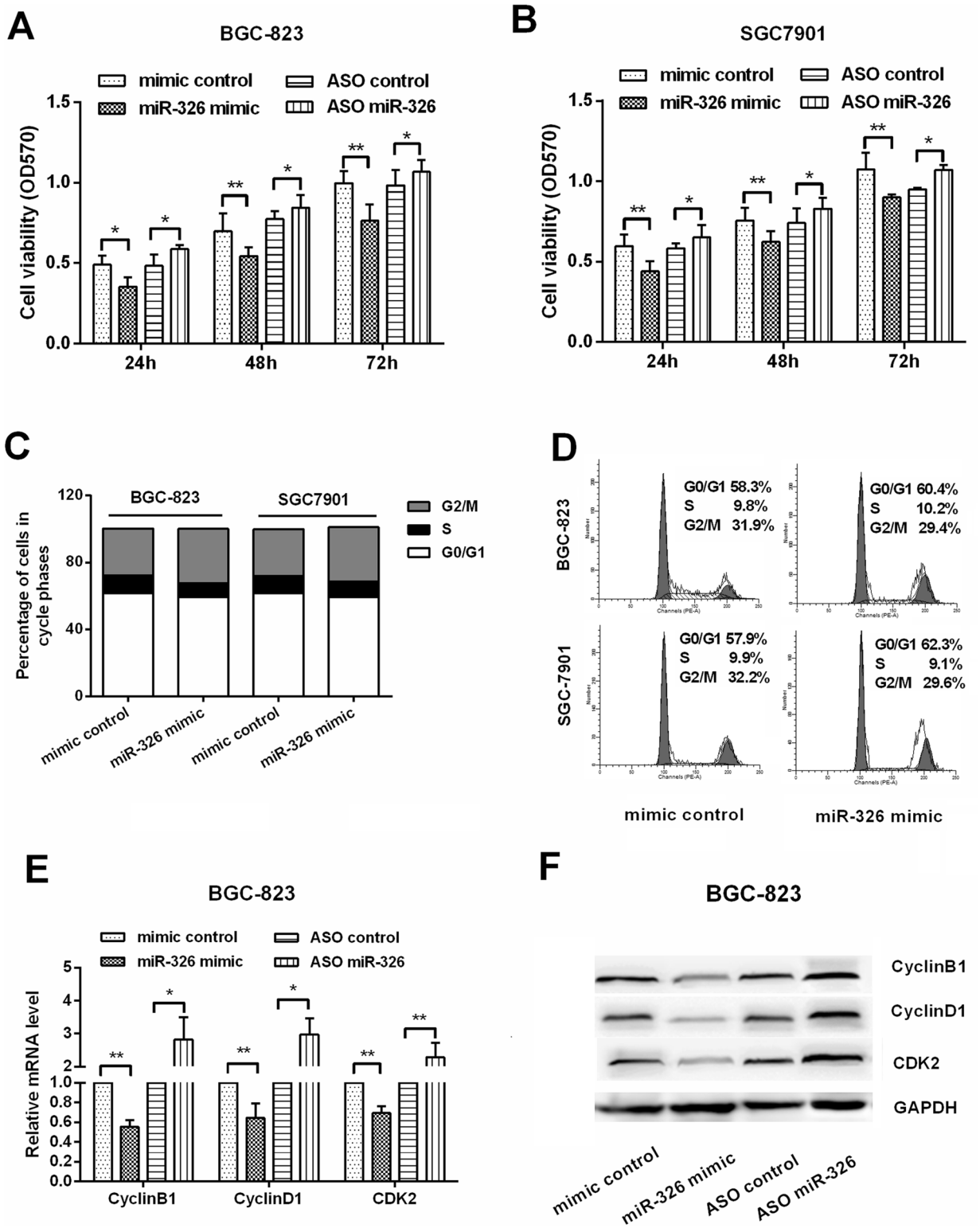

Figure 2. miR-326 inhibited gastric cancer (GC) cell proliferation and induced G_2/M arrest. (A, B) BGC-823 and SGC-7901 cells were transfected with miR-326 mimic, ASO of miR-326, or their negative control, and then the transfected cells were incubated for 24–72 h. After the incubation, cell viability was determined by MTT. (C, D) After transfection, cell cycle distribution was evaluated by calculating the proportion of cells in the G_0/G_1, S, and G_2/M stages. FACSort Cellquect software was used to analyze DNA content per duplicate. (E, F) After transfection, the mRNA and protein level expression of cell cycle-related factors, including cyclin B1, cyclin D1, and CDK2, was measured by qPCR and Western blotting, respectively. *$p<0.05$; **$p<0.01$.

promoted the expression of cell cycle protein when compared with the negative control groups ($p<0.05$ or $p<0.01$) (Fig. 2E and F).

NOB1 Was the Direct Target of miR-326

In order to determine the detailed mechanism of miR-326 suppression in GC, NOB1 was predicted as the target gene of miR-326 by using the microRNA.org (www.microrna.org) database (Fig. 3A). Results of the luciferase reporter assay demonstrated that miR-326 could indeed target NOB1 3′-UTR (Fig. 3B and C). In the meantime, qPCR and Western blot results demonstrated that miR-326 could negatively control the expression level of NOB1 (Fig. 3D and E). In combination, these data indicate that NOB1 might be a direct target gene of miR-326.

NOB1 Saved the Growth Inhibition Caused by miR-326

Next, we explored the function of NOB1 in BGC-823 cells. MTT analysis determined that NOB1 promoted cell growth ($p<0.05$ or $p<0.01$) (Fig. 4A). Moreover, NOB1 was proven to be able to accelerate the process

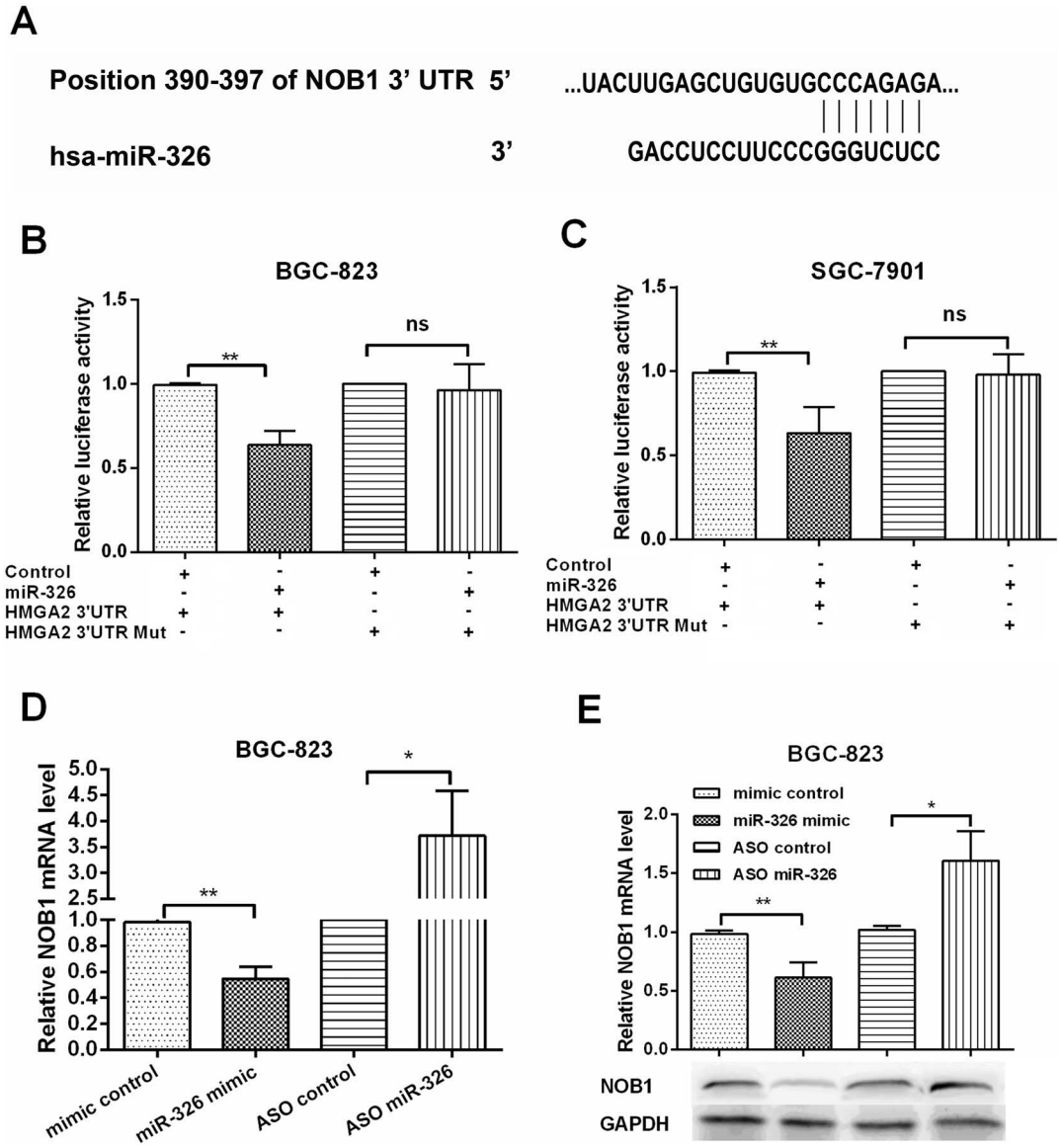

Figure 3. NOB1 was the direct target of miR-326. (A) The microRNA.org (www.microrna.org) database was used to predict the target of miR-326, and NOB1 was found to be a target of miR-326. (B, C) miR-326 significantly reduced the luciferase reporter activity by targeting NOB1 3′-UTR. (D, E) BGC-823 cells were transfected with miR-326 mimic, ASO of miR-326, or their corresponding controls, and then both mRNA and protein level expression of NOB1 in cells were analyzed by qPCR and Western blotting. *$p<0.05$; **$p<0.01$; ns, no significance.

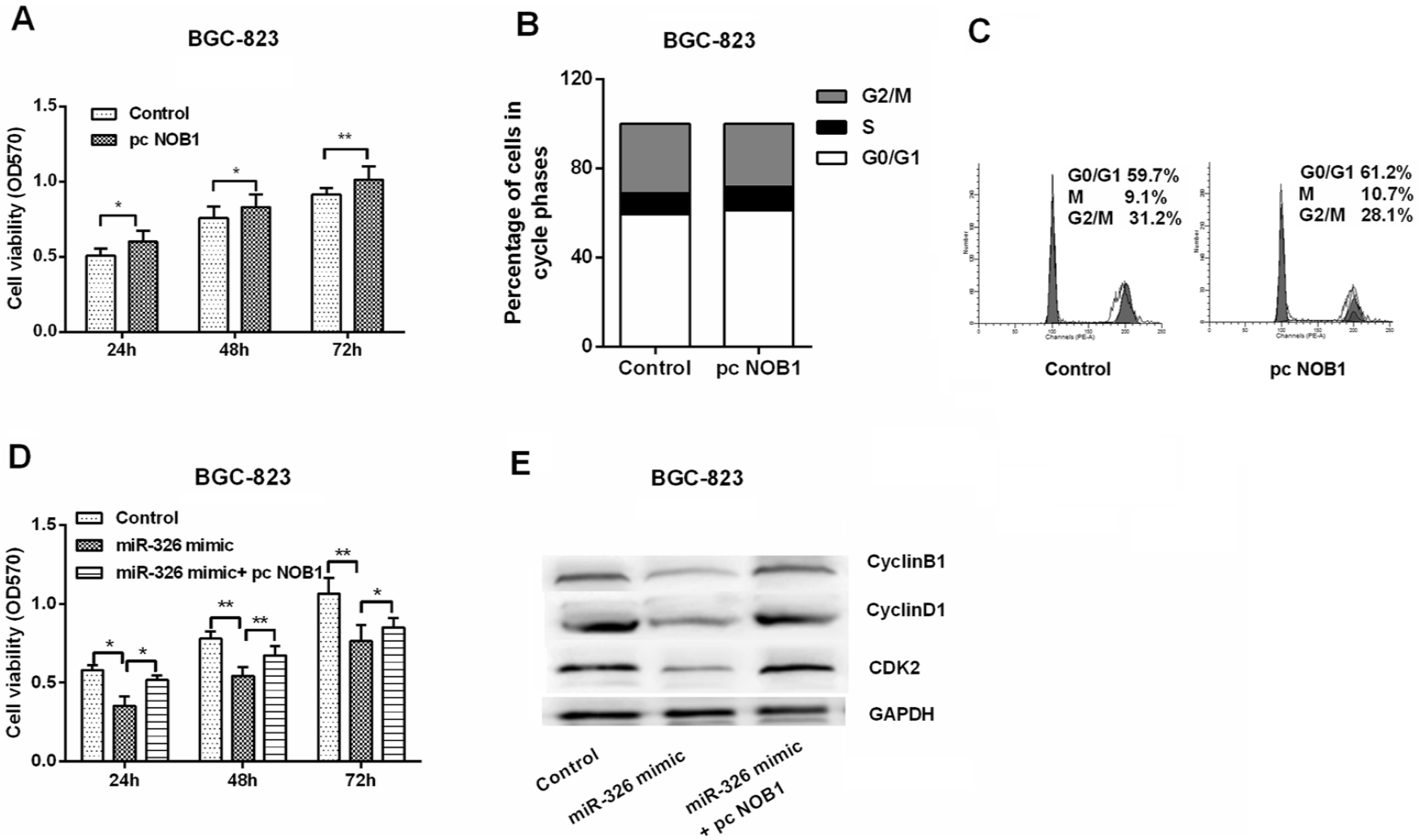

Figure 4. NOB1 rescued the growth inhibition caused by miR-326. (A) BGC-823 cells were transfected with pc-NOB1, and then cell viability was determined by MTT. (B, C) After transfection, the cell cycle distribution was evaluated by calculating the proportion of cells in the G_0/G_1, S, and G_2/M stages. FACSort Cellquect software was used to analyze DNA content per duplicate. (D, E) Furthermore, BGC-823 cells were transfected with miR-326 mimic and/or pc-NOB1. Subsequently, cell viability was measured by MTT, and the protein expression of cell cycle-related proteins, including cyclin B1, cyclin D1, and CDK2, was detected by Western blotting. $^*p<0.05$; $^{**}p<0.01$.

of cell cycle (Fig. 4B and C). Overexpression of NOB1 in BGC-823 could save the growth inhibition caused by miR-326 (Fig. 4D and E).

Growth Inhibition Caused by miR-326 Was Associated With AKT Pathway

On the basis of the research results achieved above, we carried out the exploration of AKT, the growth-related pathway. We found that miR-326 overexpression upregulated the expression of AKT and p38, indicating that miR-326 may be associated with the AKT pathway (Fig. 5A). Next, we used SB203580 (p38 inhibitor) for further research. By using MTT, flow cycle detection, and Western blot for the test of cell cycle proteins, the ability of SB203580 to save growth inhibition caused by miR-326 was confirmed. Therefore, we can conclude that the growth inhibition caused by miR-326 is associated with the AKT pathway.

DISCUSSION

A large number of studies have shown that miRNAs are involved in almost all cell biological processes including individual development, cell proliferation, apoptosis, cell differentiation, cell migration, and invasion[11,19]. Takacs and Giraldez found that miR-430 participated in zebrafish brain development by coordinating the stereotypical cell divisions[20]. miR-181 was widely acknowledged as a tumor suppressor in the pathogenesis of acute myeloid leukemia. Accumulating evidence indicates that miR-181 exhibits a significant role in the survival

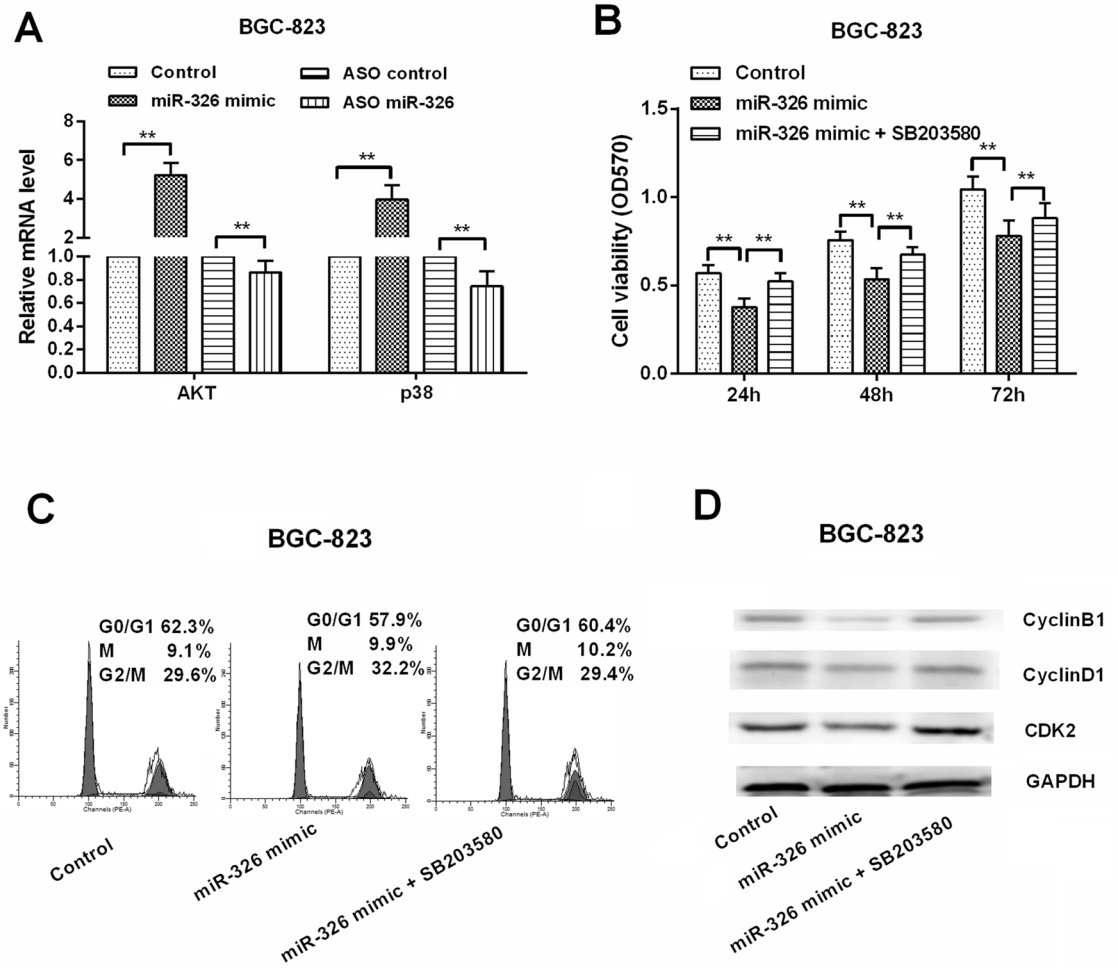

Figure 5. miR-326 inhibited cell growth via the AKT pathway. (A) BGC-823 cells were transfected with miR-326 mimic, ASO of miR-326, or their corresponding controls, and then the mRNA level expression of AKT and p38 was measured by qPCR. (B) BGC-823 cells were transfected with miR-326 mimic and/or treated with SB203580, and then cell viability was detected by MTT. (C, D) BGC-823 cells were first transfected with miR-326 mimic and/or treated with SB203580, and then cell cycle distribution was evaluated by calculating the proportion of cells in the G_0/G_1, S, and G_2/M stages. FACSort Cellquect software was used to analyze DNA content per duplicate. Meanwhile, the expression of cell cycle-related proteins, including cyclin B1, cyclin D1, and CDK2, was detected in cells by Western blotting. $^{**}p<0.01$.

of patients with acute myeloid leukemia[21]. Numerous studies have firmly established that miRNAs play a very important role in the research of GC. In recent years, researchers have found some abnormal expression of miRNA in GC. For instance, downregulation of miR-141, miR-143, and miR-145, as well as upregulation of miR-27, was found in GC cells[22–24]. Zhao et al. proved that miR-638 suppressed cell proliferation in GC by targeting Sp2 with decreasing expression of cyclin D1[25]. In the present study, we researched the role of miR-326 in GC. qPCR was used to measure the expression level of miR-326 in GC tissues and cell lines. We found that miR-326 was significantly downregulated in GC tissues and cell lines, including the HGC-27, BGC-823, SGC-7901, MKN-45, and GES cell lines. This discovery has greatly accelerated our understanding of the relationship between the mechanism and progression of GC and miRNAs.

NIN1/RPN12 binding protein 1 homolog (NOB1) is a newly discovered gene, closely related to the regulation of cell cycle and transcription. NOB1 encodes proteins including a PIN (PilTamino terminus) structure domain, a ZNRD1 (zinc) domain structure, and PIN structure domain and transcription[26]. NOB1 was reported to be involved in several kinds of cancer, such as oral squamous cell carcinoma, ovarian cancer, papillary thyroid carcinoma, colorectal cancer, etc.[27–30]. In this study, we found that NOB1 was a direct target gene of miR-326, and miR-326 could negatively control the expression level of NOB1. Furthermore, we demonstrated that NOB1 is implicated in the regulatory effects of miR-326 on cell cycle. Thus, we firmly verified that by targeting NOB1, miR-326 participates in the inhibition of cell growth in GC.

Alterations of the signaling pathway play a very important role in the regulation of multiple cellular functions in GC, including cell growth and proliferation[31,32]. Li et al. found that apoptosis of GC cells has proven to be firmly correlated with processes that are regulated by p38[33]. In this work, we studied the AKT cell signaling pathway in GC cells. It was shown that expression of AKT and p38 was upregulated when miR-326 was overexpressed, while SB203580 (p38 inhibitor) blocked this response. On the other hand, cell cycle-related proteins, including cyclin B1, cyclin D1, and CDK/1, showed negative correlations with miR-326 expression.

In conclusion, the current study provided new evidence that miR-326 may function as a tumor suppressor in GC by repressing the expression of cell cycle protein. Furthermore, NOB1 was directly targeted and could save the growth inhibition caused by miR-326. Our findings that miR-326 serves as a tumor suppressor gene in GC show that miR-326 might be used as a potential biomarker in the diagnosis and treatment of GC.

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