European Review for Medical and Pharmacological Sciences 2019; 23: 9890-9899

Long non-coding RNA TOB1-AS1 modulates cell proliferation, apoptosis, migration and invasion through miR-23a/NEU1 axis *via* Wnt/β-catenin pathway in gastric cancer

K. JIANG, X.-H. ZHI, Y.-Y. MA, L.-Q. ZHOU

Department of Radiation Oncology, The Affiliated Huai'an Hospital of Xuzhou Medical University, Huai'an No. 2 Hospital, Jiangsu, China

Abstract. – OBJECTIVE: Gastric cancer (GC) is the fourth common cancer worldwide. Long non-coding RNA TOB1 antisense RNA 1 (TOB1-AS1) has been found to participate in the process of GC, while the precise role of TOB1-AS1 is still not understood in GC progression.

MATERIALS AND METHODS: We collected 21-paired GC and para-carcinoma tissue specimens, and the levels of TOB1-AS1 and lysosomal sialidase (NEU1) were detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The protein expression levels of NEU1, β-catenin, c-Myc, Cyclin D1, N-cadherin were determined *via* Western blot. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry was performed to evaluate cell proliferation. Besides, GC cell migration and invasion capacities were identified by transwell assay. Dual-Luciferase reporter assay was employed to examine the interrelation between miR-23a and TOB1-AS1 or NEU1. Finally, the role of TOB1-AS1 was verified *in vivo*.

RESULTS: The levels of TOB1-AS1 were decreased in GC tissues and cell lines. Either TOB1-AS1 or NEU1 upregulation accelerated GC cell apoptosis, hampered proliferation, migration, and invasion. Further, the role of TOB1-AS1 silencing on cell behaviors was abrogated by NEU1 upregulation. TOB1-AS1 and NEU1 exerted their roles via Wnt/β-catenin signaling pathway. Overexpression of TOB1-AS1 blocked GC development *in vivo*. Mechanically, miR-23a was targeted by TOB1-AS1, but directly targeted NEU1.

CONCLUSIONS: TOB1-AS1/miR-23a/NEU1 axis regulated proliferation, apoptosis, migration, and invasion of GC cells via Wnt/β-catenin pathway, providing the evidence for serving TOB1-AS1 as an underlying therapeutic target in human GC treatment.

Key Words:
TOB1-AS1, NEU1, MiR-23a, Wnt/β-catenin pathway, GC.

Introduction

Gastric cancer (GC) is the fourth frequently occurred cancer worldwide, causing about 700,000 mortalities each year[1-4]. Even worse, the type of GC, such as metastatic or non-resectable, is closely related to poor prognosis, and the effect of systemic chemotherapeutic approaches is not as expected. Thereby, a novel biomarker for improving the early diagnosis and prognostic assessment of GC is urgently needed.

Over the past decades, high throughput sequencing was rapidly developed. Researchers were conscious that the partially maximal human genome is transcribed to non-coding RNAs (ncRNAs), which are not functions for protein transcriptions. This evidence indicated that a broad set of RNA modulators are dedicated to targeting a relatively small number of effectors[5-7]. According to the newly uncovered RNA theories, long non-coding RNAs (lncRNAs) have been explored to act as the crucial factors for various cellular behaviors, such as cell-fate advancing of disease[8,9]. Notably, multiple lncRNAs act as competing for endogenous RNA (ceRNA) in regulating gene expression *via* sponging miRNAs. Currently, many functions of lncRNAs have been discovered to be involved in the development of the diseases. Especially, plasmacytoma variant translocation 1 gene (PVT1) silencing caused the repression of cell viability and migration[10]. Urothelial carcinoma associated 1 (UCA1) was induced in GC tissues, and it was associated with cancer cell behaviors, such as cell migration and drug resistance[11,12]. While the target RNA in this study, TOB1 antisense RNA 1 (TOB1-AS1) was little reported, earlier works only found that TOB1-AS1 functioned as a cervical cancer suppressor *via* targeting microRNA-27b[13]. We aimed at investigating the impact of TOB1-AS1 in GC pathogenesis.

Corresponding Author: Liqing Zhou, MD; e-mail: zlq_hill@126.com

Besides, microRNAs (miRNAs), which unusually interact with lncRNAs[14], have been reported to be related to many types of cancers[15,16]. For example, microRNA-34a is associated with p-53-mediated inhibition of GC[17]. MicroRNA-223 induces GC cell motility by regulating Erythrocyte Membrane Protein Band 4.1 Like 3 (EPB41L3)[18]. MicroRNA-96 functions as a tumor suppressor *via* curbing Kirsten rat sarcoma viral oncogene (KRAS) in pancreatic cancer[19]. In addition, microRNA-23a (miR-23a) modulates epithelial-mesenchymal transition (EMT) of lung cancer cells induced by transforming growth factor-β (TGF-β) *via* modifying E-cadherin[20]. MiR-23a accelerates gastric adenocarcinoma cell growth *in vitro*[21]. The potential role of miR-23a in the tumorigenesis may interact with TOB1-AS1. Over the past years, Neuraminidase 1 (NEU1) was found to belong to the NEU family. Of which NEU consists of four members, NEU1, NEU2, NEU3, and NEU4. NEU1 is located in the region of chromosome 6p21.3, and its upregulation could cause cytotoxicity and cell necrosis[22,23]. Further, reports[24] showed that high malignant was closely related to accumulated NEU expression in ovarian cancer. NEU1 was also involved in the inflammatory response in monocytes[25]. Hence, we presumed that NEU1 acted as the target gene of miR-23a, and it was thus researched in this study.

Herein, we investigated the roles of TOB1-AS1, miR-23a, and NEU1 in the progression and initiation of human GC. Further, their partial molecular mechanism in the development of GC was also explored *in vitro*.

Patients and Methods

GC Patient Specimens and Cell Culture

A set of samples, including GC tissue samples (n=21) and paired noncancerous tissues (n=21), were received from the Affiliated Huai'an Hospital of the Xuzhou Medical University, Huai'an No. 2 Hospital and used for subsequent quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot assays. None of the participators previously underwent pre-surgery chemotherapy or radiotherapy. Written informed consent was signed by each patient, and this research was approved by the Ethics Committee of the Affiliated Huai'an Hospital of the Xuzhou Medical University, Huai'an No. 2 Hospital.

In the assay, human GC cell lines (AGS and MKN74) were obtained from COBIOER (Shanghai, China), and the normal control cells (NCM460) were purchased from Be Na Culture Collection (Beijing, China). AGS cells were grown in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Sigma-Aldrich, St. Louis, MO, USA), MKN74 cells were cultured with Ham's F-12K (Kaighn's) Medium (F-12K; HyClone, South-Logan, UT, USA), and NCM460 cells were grown in McCoy's 5A (HyClone, South-Logan, UT, USA). In addition, all medium was supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% antibiotics (Gibco, 100 U/mL of penicillin and 10 mg/mL of streptomycin) before use. All cell lines were incubated in a 5 % CO_2 incubator at 37°C.

QRT-PCR

The GC tissues were maintained at -80°C and then homogenized with liquid nitrogen. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate and extract the total RNA from GC tissues and cell lines based on the manufacturer's descriptions. Then, the first-strand cDNA was reversely transcribed by using the total RNA and PrimeScript RT Reagent kit (TaKaRa, Dalian, China), followed by performing real time-PCR utilizing SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR cycle was carried out with a real-time quantitative machine (CFX96; Bio-Rad Laboratories, Hercules, CA, USA). The primers of TOB1-AS1, miR-23a, NEU1, and the reference primers of U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were listed: TOB1-AS1 (forward: 5'-GCCAGGCCTAGAAGCTTTTG-3', reverse 5'-TCTTCCCACCCCTTCTCCTA-3'); miR-23a (forward: 5'-ATCACATTGCCAGGGATTTCC-3', reverse 5'-CCAGTGCAGGGTCCGAGGT-3'); NEU1 (forward: 5'-AGCCTACTGTTTATCTTGCTTCTGC-3', reverse 5'-GATGTGCATTGACAGAACCATAGAGA-3'); GAPDH (forward: 5'-CGACTTATACATGGCCTTA-3', reverse 5'-TTCCGATCACTGTTGGAAT-3'); U6 (forward: 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'). The relative levels of TOB1-AS1 and NEU1 were standardized using GAPDH as internal control, and the relative level of miR-23a was normalized with endogenous reference of U6. Their levels were calculated *via* $2^{-\Delta\Delta Ct}$ method.

Western Blot

The proteins harvested from GC patient specimens and cell lines (AGS and MKN74) were iso-

lated utilizing 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and these separated proteins were transfected onto the surface of the polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). The blots were subsequently blocked with 5% skim milk (Sangon, Shanghai, China) for 2 h at room temperature followed by special coverage antibodies at 4°C overnight. On the next day, the membranes were incubated with secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 40 min at room temperature. The protein bands were appeared and visualized using enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA), and the protein gray value was quantified *via* the software of Image-Pro Plus 6.0. In addition, primary antibodies were listed: NEU1 (1:4000, 67032-1-Ig) was obtained from ProteinTech (Chicago, IL, USA), primary antibodies except NEU1 were purchased from Abcam (Cambridge, UK), including β-catenin (1:7500, ab32572), c-Myc (1:1000, ab32072), Cyclin D1 (1:150, ab16663), N-cadherin (1:500, ab18203), and β-actin (1:6000, ab8226).

Transient Transfection

The miR-23a mimic (miR-23a) and its blank control (miR-NC) were gained from GenePharma (Shanghai, China), and vectors were also designed and synthesized in GenePharma, including pcDNA3.1-mediated TOB1-AS1 (pcDNA-TOB1-AS1) and NEU1 (pcDNA-NEU1), their negative control (pcDNA-control), small interfering RNA (siRNA) against TOB1-AS1 (si-TOB1-AS1), and its negative control (si-control). These described vectors and oligonucleotides were transfected into AGS and MKN74 cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the producer's instructions.

Cell Proliferation Assay

The cell growth and viability of GC cells (AGS and MKN74) were determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In short, GC cells with 1×10^5 cells/well were seeded on a 96-well plate. After incubation for 0 h, 24 h, 48 h, and 72 h, MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was introduced into each well containing transfected cells, and then, incubated for 4 h. Subsequently, the medium was abandoned, and dimethyl sulfoxide (DMSO, 200 μL/well; Sigma-Aldrich, St. Louis, MO, USA) was used to dissolve the formazan crystals. After gently shook for 15 min, the OD value was assessed by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Flow Cytometry Assay

Apoptotic cells were distinguished from the total cells using reagent of Annexin V-fluorescein isothiocyanate/Propidium Iodide (Annexin V-FITC/PI) Apoptosis Detection Kit (BestBio, Shanghai, China) and flow cytometry assay. After AGS and MKN74 cells were treated accordingly, they were washed with ice-cold phosphate-buffer solution (PBS; HyClone, South-Logan, UT, USA) twice. Then, the reagents of Annexin V-FITC and PI were added into cell suspension according to the producer's manuals. After gently blended, the mixture was darkly incubated for 10 min at room temperature. The stained cells were discerned by a FACScalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell Assay

Transwell chamber assay was employed to evaluate the capacities of cell migration and invasion. For cell invasion assay, AGS and MKN74 cells (2×10^4) were plated into the upper chamber (8 μm pore size, BD Biosciences, Franklin Lakes, NJ, USA) of a transwell with Matrigel-coated (BD Biosciences, Frank) previously and cultured for 2 h at 37°C. Then, the cells on the upper surface of the membranes of the upper chamber were discarded by using a cotton swab, and the penetrating cells were stained with 0.5% crystal violet, and these stained cells were counted. Besides, the detection of cell migration capacity was according to the protocol of cell invasion assay except for the Matrigel coating.

Dual-Luciferase Reporter Assay

The common sequences of TOB1-AS1 (wildtype and mutant) and miR-23a, and the binding sites between miR-23a and wildtype or mutant of NEU1 were totally amplified and inserted into pmirGLO (Promega, Madison, WI, USA), thereby fabricated the Luciferase reporter vectors of TOB-AS1 (wildtype named as WT-TOB1-AS1 and mutant named as MUT-TOB1-AS1) and NEU1 (wildtype named as WT-NEU1 and mutant named as MUT-NEU1). For the Dual-Luciferase reporter assay, AGS and MKN74 cells were co-transfected with Luciferase reporter vectors (WT-TOB1-AS1, MUT-TOB1-AS1, WT-NEU1 or MUT-NEU1), and the *Renilla* vector (pRL-CMV; Promega, Madison, WI, USA), as well as miR-23a mimic or control. After 48 h transfection, the firefly activities and *Renilla* fluorescence intensities were analyzed utilizing the Dual-Luciferase report assay system (Promega, Madison, WI, USA). The action of *Renilla* acted as the internal reference for firefly activity.

Xenograft Experiments

Eight-week-old male mice (C57BL/6, n=10/group) weighing about 20-25 g were purchased from the Chinese Academy of Medical Sciences Laboratory Animal Center (Beijing, China). All the mice were raised under Specific Pathogen Free (SPF) conditions with free access to fodder and water. The experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the Affiliated Huai'an Hospital of the Xuzhou Medical University, Huai'an No. 2 Hospital. In this assay, the mice were subcutaneously injected with AGS cells, which transfected with pcDNA-TOB1-AS1 or pcDNA-control. The tumor volumes of mice were measured at 7 days, 14 days, 21 days, and 28 days post-injection; tumor volumes were calculated *via* volume=length×width^2×0.05 formula. After mice were sacrificed at day 28, tumor weights were also measured and analyzed.

Statistical Analysis

Experimental data from at least three independent assays were processed utilizing SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and exhibited as the means ± standard deviation (means ± SD). Then, Student's *t*-test was performed to compare the difference between two sets of data, which came from two groups, and One-way analysis of variance followed by Tukey's test was employed to compare the differences among multiple groups. *p*-value less than 0.05 was considered to be statistically significant.

Results

Expression Levels of TOB1-AS1 and NEU1 Were Decreased in GC Tissues

Expression levels of TOB1-AS1 and NEU1 in 21 paired GC tissues and matched noncancerous tissues were measured by qRT-PCR. Their data exhibited that both of TOB1-AS1 and NEU1 expression levels were clearly reduced in GC tissues (Figure 1A and 1B). Furthermore, the protein expression of NEU1 was also distinctly restrained in GC cells compared with matched noncancerous tissues (Figure 1C). Subsequently, the correlation of the TOB1-AS1 level with NEU1 expression displayed an evidently positive association (Figure 1D). This evidence indicated that the aberrant expression of TOB1-AS1 possibly involved in GC progression.

Upregulation of TOB1-AS1 Improved Cell Apoptosis, Suppressed Proliferation, Migration and Invasion in GC Cells

To further investigate the biological function of TOB1-AS1 in GC progression, the expression of TOB1-AS1 was measured in GC cells, and the result showed low expression of it in AGS and MKN74 cells (Figure 2A). What's more, the overexpression vector of TOB1-AS1 was introduced into AGS and MKN74 cells, and its efficiency was verified by qRT-PCR. The results exhibited that TOB1-AS1 expression was markedly improved in both AGS and MKN74 cells (Figure 2B). At the same time, cell proliferation was clearly reduced by upregulating TOB1-AS1 *in vitro* (Figure 2C). Subsequently, flow cytometric analysis explored that the overexpression of TOB1-AS1 could significantly enhance cell apoptotic rate in AGS and MKN74 cells (Figure 2D). In addition, the transwell assay was aimed to assess the role of TOB1-AS1 in the migration and invasiveness. The results proved that elevated expression of TOB1-AS1 efficiently impeded cell migration and invasion *in vitro* (Figure 2E and 2F). From the above, we revealed that the upregulation of TOB1 could significantly accelerate cell apoptosis, hamper proliferation, migration, and invasion in GC cells.

NEU1 Overexpression Enhanced Cell Apoptosis, Curbed Proliferation, Migration and Invasion in GC Cells

Firstly, the mRNA level and protein expression of NEU1 in GC cells were evaluated, and the results showed that both mRNA and protein levels were evidently reduced in AGS and MKN74 cells (Figure 3A and 3B). Then, the AGS and MKN74 cells were transfected with the overexpression vector of NEU1 or its control; the results indicated that the expression of NEU1 was remarkably increased in the pcDNA-NEU1 group compared with pcDNA-control in the aspects of mRNA level and protein expression (Figure 3C and 3D). Moreover, the overexpression of NEU1 could notably hinder cell proliferation *in vitro* (Figure 3E). However, NEU1 upregulation strikingly augmented the apoptotic cell rate *via* flow cytometric analysis (Figure 3F). In the meanwhile, the transwell assay results displayed that NEU1 upregulation significantly decreased the abilities of migration and invasiveness in AGS and MKN74 cells (Figure 3G and 3H). These descriptions indicated that NEU1 may act as a tumor suppressor and that its overexpression specially accelerated cell apoptosis, restrained proliferation, migration, and invasion in GC cells.

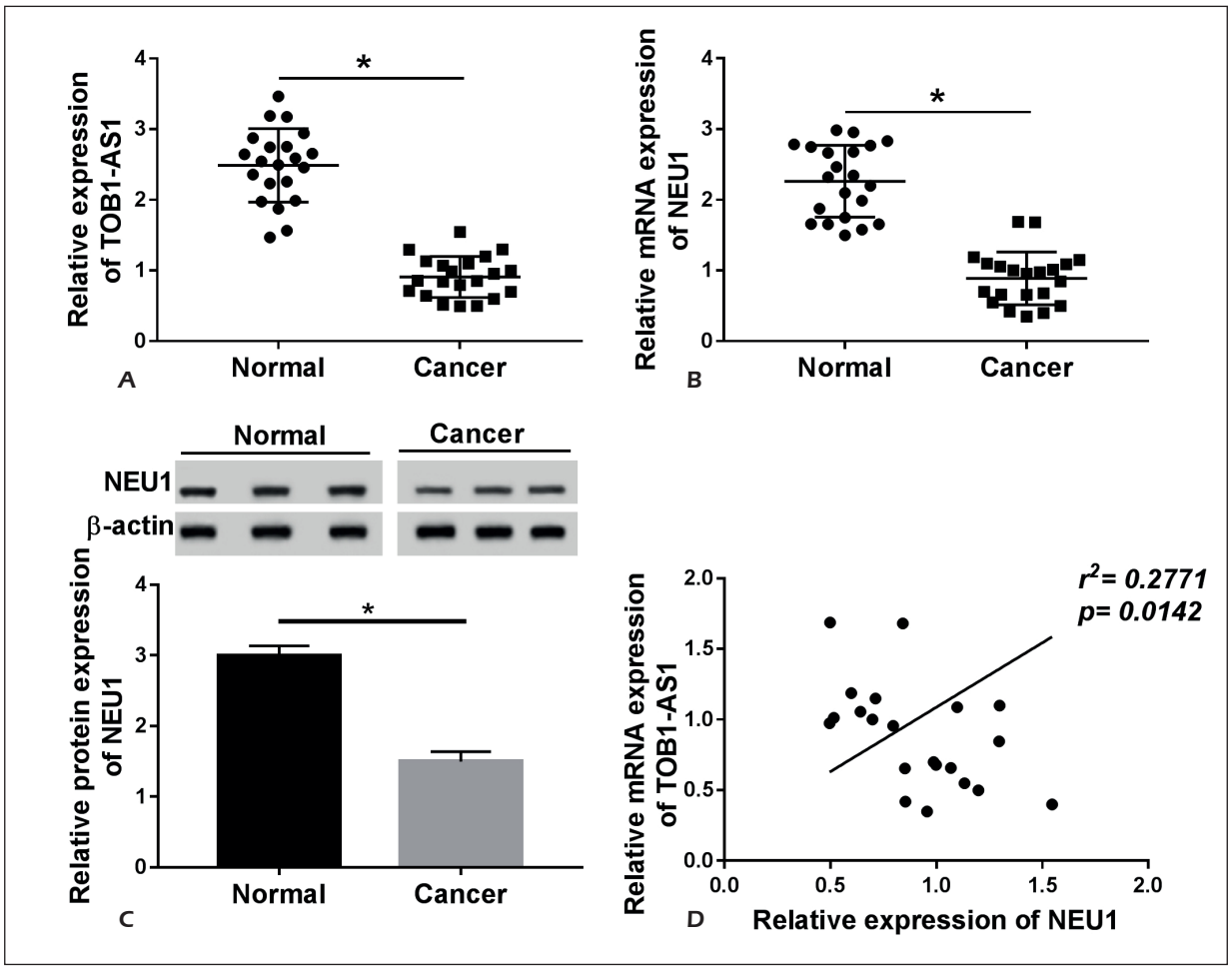

Figure 1. Expression of TOB1-AS1 and NEU1 were decreased in GC tissues. **A**, and **B**, MRNA levels of TOB1-AS1 and NEU1 were determined by qRT-PCR. **C**, The protein expression of NEU1 was estimated using Western blot. **D**, The correlation between TOB1-AS1 and NEU1 was analyzed by qRT-PCR. *$p<0.05$.

Impacts of TOB1-AS1 Silencing on Cell Proliferation, Apoptosis, Migration and Invasion Were Totally Reversed by NEU1 Upregulation

Based on the biological roles of TOB1-AS1 and NEU1 in GC development, the regulatory mechanism between them was subsequently investigated. Firstly, TOB1-AS1 siRNA or relative control was transfected into AGS and MKN74 cells; the knockdown efficiency lived up to our expectation (Figure 4A). Next, si-control, si-TOB1-AS1, si-TOB1-AS1+pcDNA-control or si-TOB1-AS1+pcDNA-NEU1 into GC cells, the mRNA level and protein expression of NEU1 were retarded by si-TOB1-AS1, whereas this inhibitory impact was recovered by introducing the overexpression vector of NEU1 (Figure 4B and 4C). Moreover, the promotor effect of TOB1-AS1 silencing on cell proliferation was restored by transfecting pcDNA-NEU1 (Figure 4D). Apoptotic rate, which was suppressed by si-TOB1-AS1, was regained *via* pcDNA-NEU1 mediation (Figure 4E). After that, the transwell assay was conducted to estimate cell migration and invasion in AGS and MKN74 cells. The results demonstrated that TOB1-AS1 detection facilitated cell migration and invasion, but the acceleration effects of si-TOB1-AS1 on the ability of migration and invasiveness in GC cells were rescued *via* NEU1 upregulation (Figure 4F and 4G). In short, the role of TOB1-AS1 knockdown in cell proliferation, apoptosis, migration, and invasion was abrogated by NEU1 overexpression in GC cells.

MiR-23a Was Targeted by TOB1-AS1, But Directly Targeted NEU1

According to the above introductions, there was an interaction between TOB1-AS1 and NEU1,

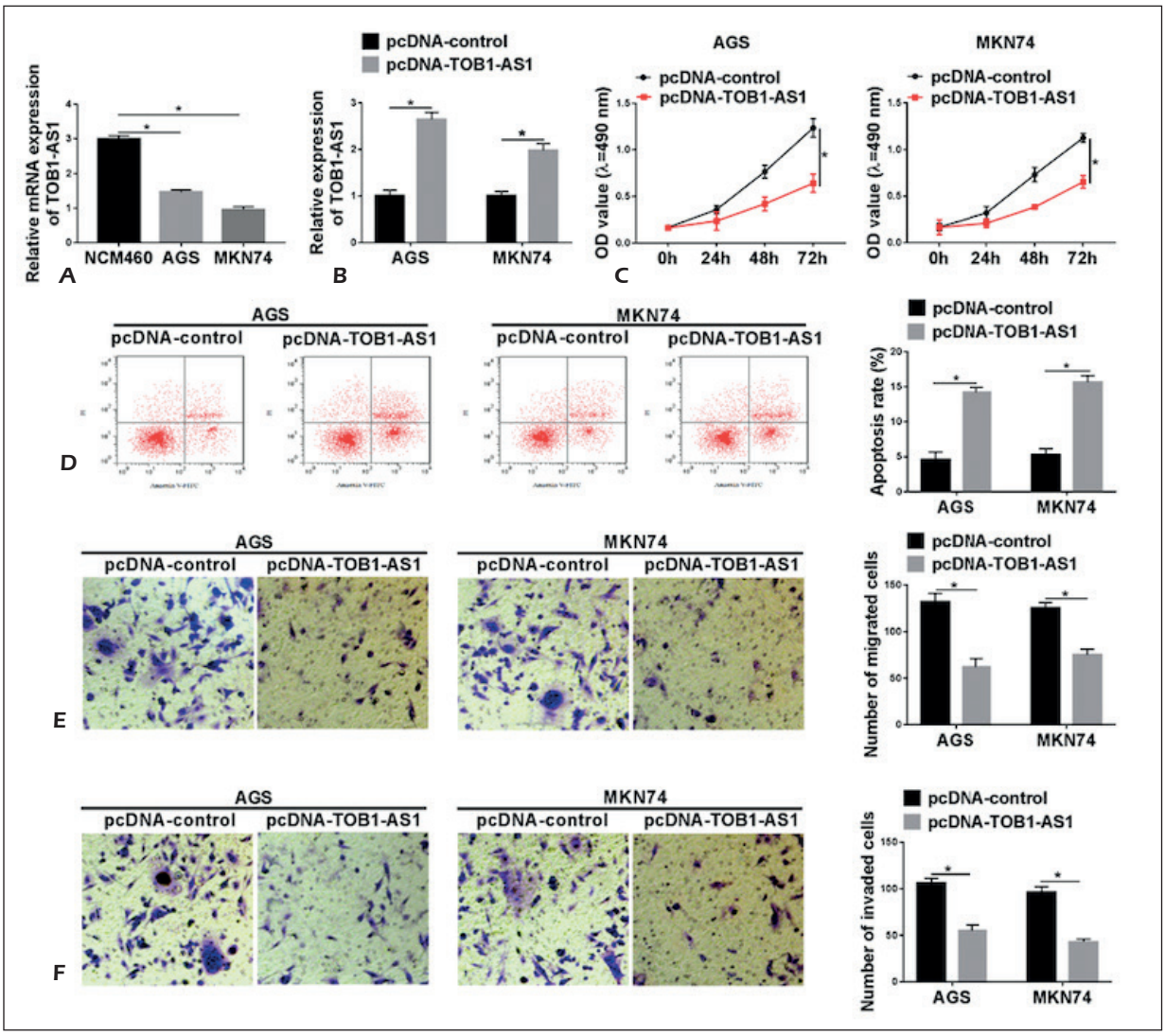

Figure 2. Upregulation of TOB1-AS1 improved cell apoptosis, suppressed proliferation, migration, and invasion in GC cells. **A**, The mRNA level of TOB1-AS1 was measured by qRT-PCR in GC cells. AGS and MKN74 cells were transfected into pcDNA-TOB1-AS1 and pcDNA-control, respectively, (**B**) and the efficiency of pcDNA-TOB1-AS1 transfection was identified using qRT-PCR. **C**, MTT assay was performed to estimate the proliferation of AGS and MKN74 cells at the absorbance of 490 nm. **D**, Impact of TOB1-AS1 overexpression on cell apoptosis of AGS and MKN74 cells was detected by flow cytometry. **E**, and **F**, Transwell assay was aimed to evaluate GC cells migration and invasion in vitro (x200). *$p<0.05$.

while the direct target miRNAs of TOB1-AS1 were still needed to be found. StarBase software was used to predict the binding sites between TOB1-AS1 and its targeted miRNAs; the results showed that miR-23a might be targeted by TOB1-AS1, and miR-23a was also predicted to target NEU1 (Figure 5A and 5C). Subsequently, Dual-Luciferase reporter assay was employed to confirm the relationship between miR-23a and TOB1-AS1 or NEU1. The decreased Luciferase activity in wildtype and no significant difference of the fluorescence intensity in the mutant proved that miR-23a was sponged by TOB1-AS1, while it could target NEU1 (Figure 5B and 5D). Then, the molecular mechanisms of miR-23a and TOB1-AS1 in modifying NEU1 were researched *in vitro*. After transfection with miR-NC, miR-23a, miR-23a+pcDNA-NC, or miR-23a+pcDNA-TOB1-AS1 in AGS, and MKN74 cells, the mRNA level and protein expression of NEU1 were detected. The results showed that miR-23a mimic was visibly constrained in both aspects of NEU1 mRNA level and protein expression, while these inhibiting effects were abolished by overexpression of TOB1-AS1 in AGS and MKN74 cells (Fig-

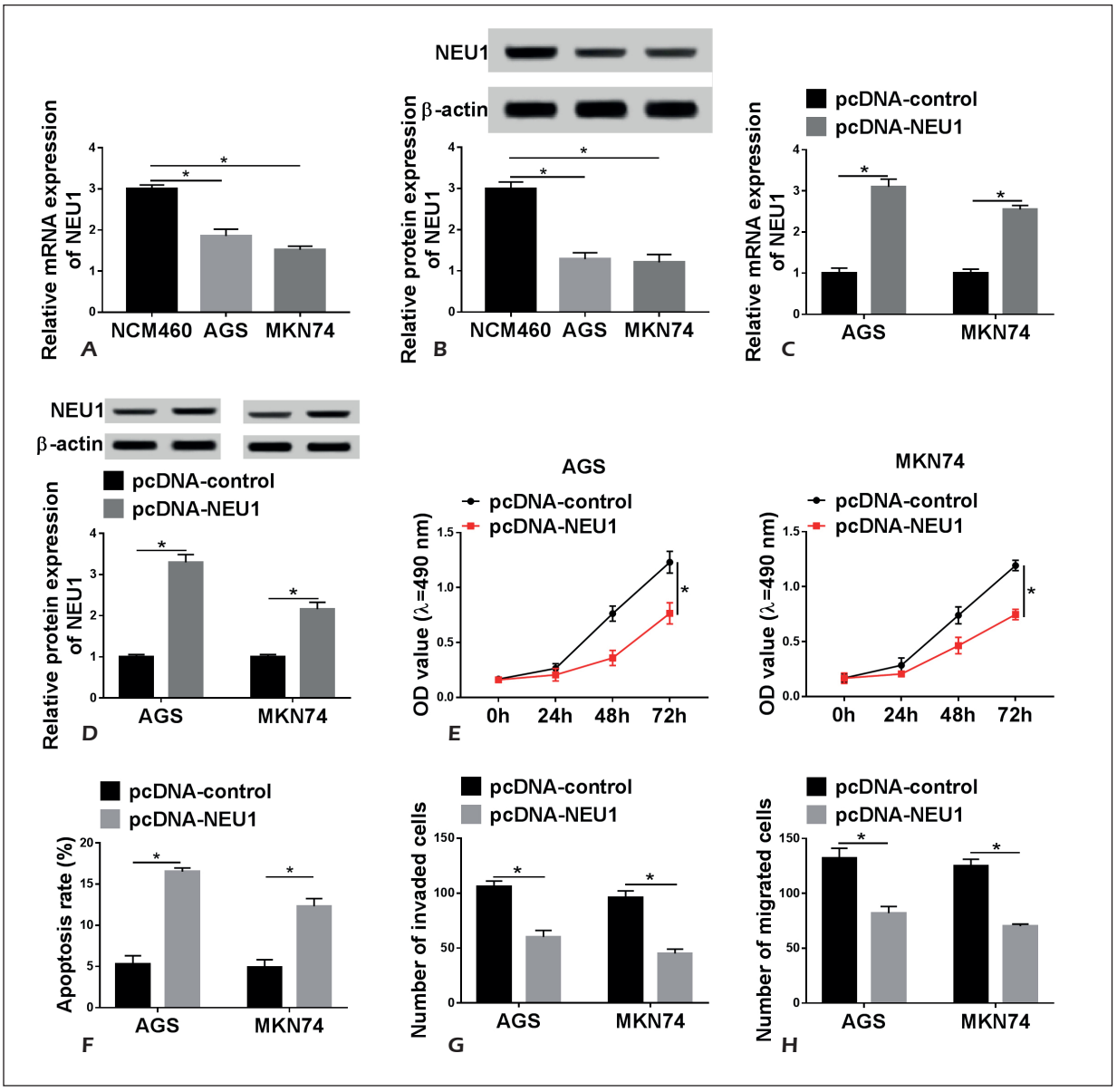

Figure 3. NEU1 overexpression enhanced cell apoptosis, curbed proliferation, migration, and invasion in GC cells. **A**, and **B**, MRNA and protein levels of NEU1 were examined via qRT-PCR and Western blot assays, respectively. The vector of pcDNA-NEU1 or pcDNA-control was introduced into AGS and MKN74 cells, (**C** and **D**) NEU1 mRNA and protein levels analysis using qRT-PCR and Western blot assays, respectively. **E**, MTT assay was carried out to analyze the ability of cell proliferation in vitro. **F**, Apoptotic cell numbers were counted utilizing flow cytometry. **G**, and **H**, transwell assay was used to assess migration and invasion abilities of AGS and MKN74 cells. *p<0.05.

ure 5E and 5F). In brief, TOB1-AS1 served as the "competing endogenous RNA (ceRNA)" of miR-23a to regulate NEU1 in the two GC cells.

TOB1-AS1 and NEU1 Exerted Their Roles Via Wnt/β-Catenin Signaling Pathway

In this study, we researched the regulatory mechanisms of TOB1-AS1, miR-23a, and NEU1 in GC cell behaviors. Subsequently, the research paid attention to find the signaling pathway in these processes. After si-control, si-TOB1-AS1, si-TOB1-AS1+pcDNA-control, or si-TOB1-AS1+pcDNA-NEU1 transfection, the levels of β-catenin, c-Myc, Cyclin D1, and N-cadherin were applied to represent the level of the Wnt/β-catenin signal pathway. QRT-PCR and Western blot assay results found that the Wnt/β-catenin pathway was trig-

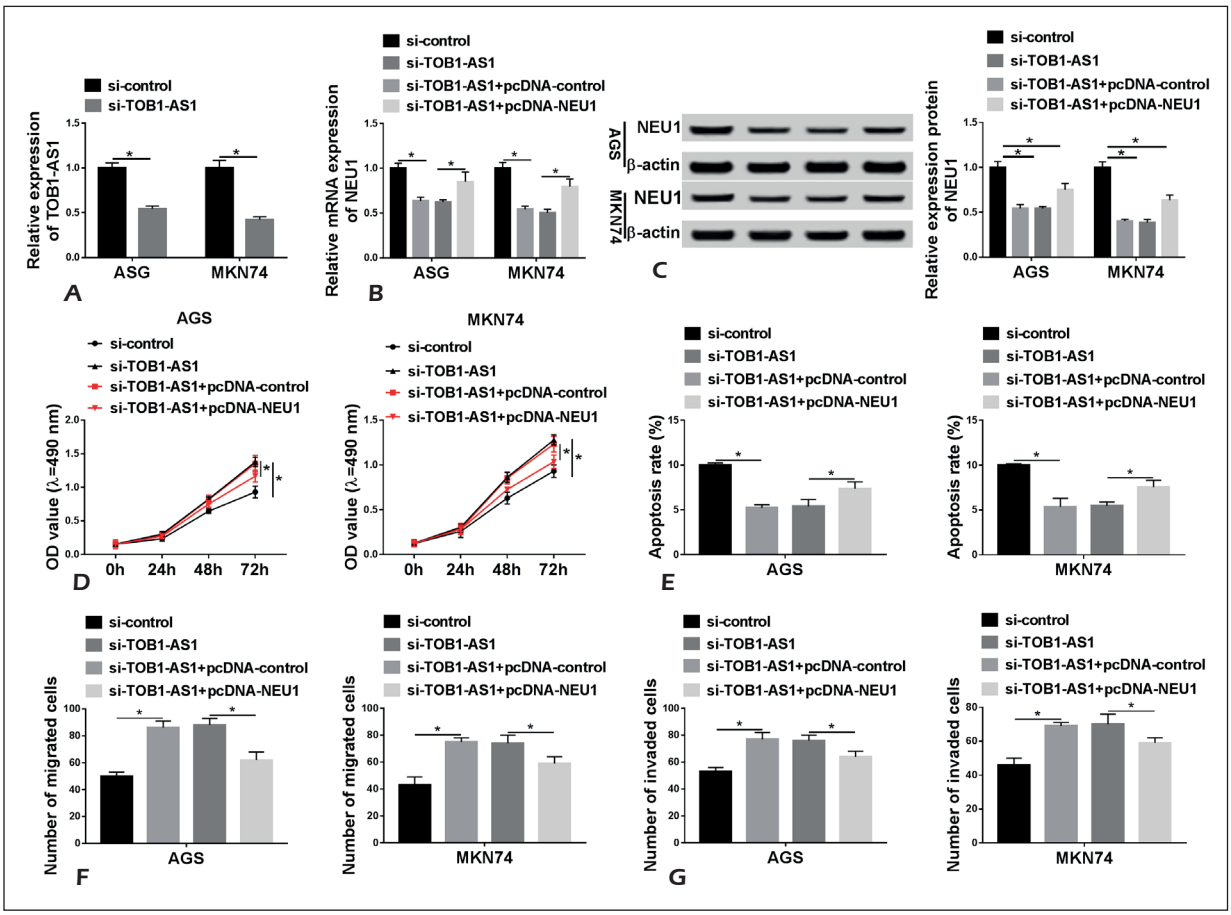

Figure 4. Impacts of TOB1-AS1 silencing on cell proliferation, apoptosis, migration, and invasion were totally reversed by NEU1 upregulation. Si-control, si-TOB1-AS1, si-TOB1-AS1+pcDNA-control or si-TOB1-AS1+pcDNA-NEU1 was transfected into AGS and MKN74 cells, (**A** and **B**) and levels of TOB1-AS1 and NEU1 were analyzed by qRT-PCR. **C**, Western blot was conducted to measure the protein level of NEU1. **D**, Cell proliferation ability was analyzed using MTT assay. **E**, Cell apoptotic rate was identified by flow cytometry. **F**, and **G**, Transwell assay was employed to determine cell migration and invasion in vitro. *$p<0.05$.

gered by TOB1-AS1 silencing, while this promoter influence was overturned by NEU1 upregulation *in vitro* (Figure 6A-6D). Thus, the role of TOB1-AS1 exerted its role through the miR-23a/NEU1 axis *via* Wnt/β-catenin signaling pathway.

TOB1-AS1 Overexpression Suppressed the Progression of GC In Vivo

Concluding our investigation, we focused on the function of TOB1-AS1 in the progression of GC *in vivo*. After transfected AGS cells were injected into mice, the tumor volumes were measured every 7 days, and the results exhibited that the overexpression of TOB1-AS1 repressed tumor volumes compared with the control (Figure 7A). At 28-day post-injection, tumor weight was also measured, and the tumor weight of the pcDNA-TOB1-AS1 group was lower than that of the blank control groups (Figure 7B). The mRNA levels of TOB1-AS1, miR-23a, and NEU1 were evaluated by qRT-PCR. The results demonstrated that TOB1-AS1 and NEU1 levels were prominently increased, but miR-23a expression was evidently decreased in pcDNA-TOB1-AS1-mediated tumor samples (Figure 7C-7E). What's more, the protein expression of NEU1 was also enhanced in the TOB1-AS1 upregulation group (Figure 7F). From the above descriptions, we uncovered that TOB1-AS1 upregulation suppressed the GC process.

Discussion

LncRNAs have been recorded to be involved in the diverse cellular processes of cancer[26,27]. The role of TOB1-AS1 in the pathogenesis of GC, as a lncRNA, although it has been found overexpressed in cervical cancer[13], is still not clearly

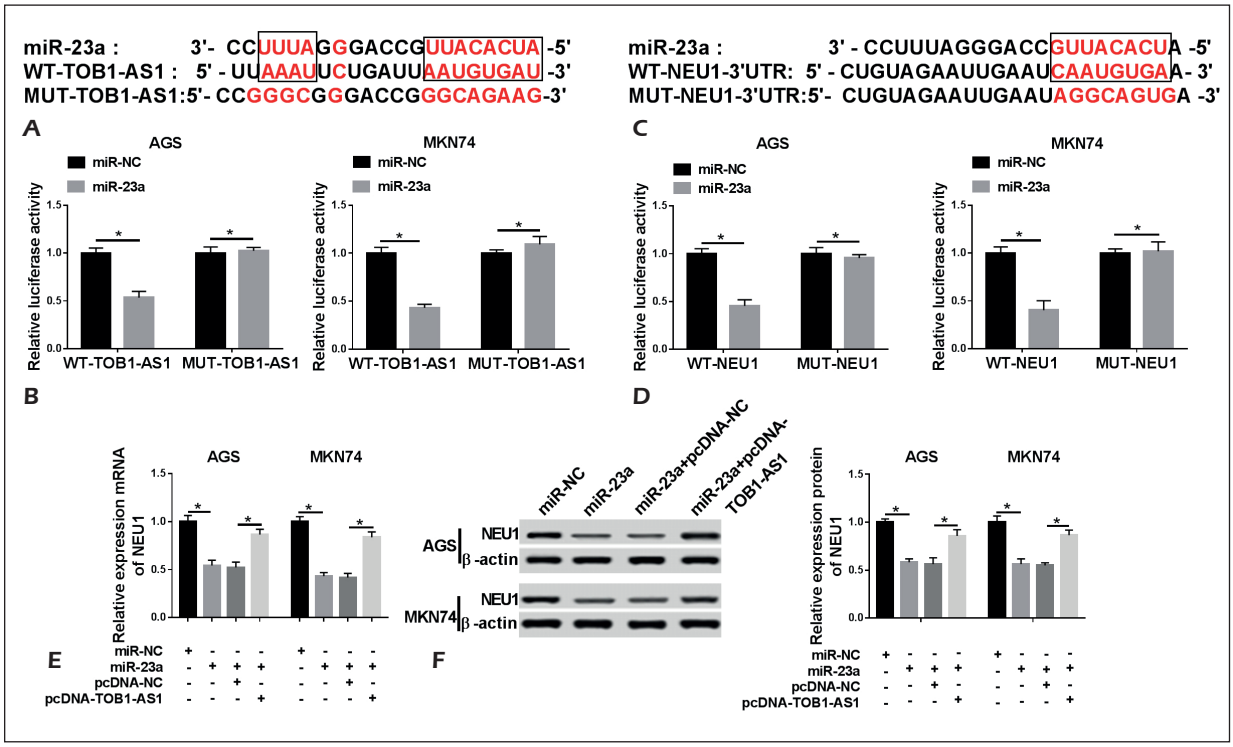

Figure 5. MiR-23a was targeted by TOB1-AS1, but directly targeted NEU1. **A**, and **C**, Common domain prediction between miR-23a and TOB1-AS1 (**A**) or NEU1 (**C**) was predicted via starBase software. **B**, and **D**, Dual-Luciferase reporter assay was conducted to indicate the relationship between miR-23a and TOB1-AS1 (**B**) or NEU1 (**D**). **E**, and **F**, MRNA and the protein levels of NEU1 were estimated using qRT-PCR and Western blot assays, severally. **p*<0.05.

understood. In the current study, we found consistent evidence exhibiting downregulated TOB1-AS1 in the GC patient samples compared with a matched control. Based on the established criteria, GC contains two types of cancers, including intestinal-type and diffuse-type adenocarcinoma[28]. After measuring the levels of TOB1-AS1 in 21 paired GC and non-tumor tissues, we also further understood the role of TOB1-AS1 in GC cell behaviors *in vitro*. Firstly, two GC cell lines of AGS and MKN74; the results displayed that the overexpression of TOB1-AS1 could improve cell apoptosis, restrain proliferation, migration, and invasion in GC cells. The tumor suppressor role was also confirmed *in vivo*. The subsequent assays were applied to understand the potential regulatory mechanism of TOB1-AS1 in GC.

So far, different groups revealed that miRNAs interact with lncRNAs, thereby exerting their roles in regulating the target gene expression. Besides, earlier works also attract the "ceRNA" role of lncRNA[29], and we speculated that TOB1-AS1 might serve as the ceRNA of miR-23a. MiR-23a, as one of the miRNAs, has been found to be dysregulated in multiple tumor diseases and participate in diverse biological progress, such as cell behaviors of proliferation, migration, and invasion, and metabolism by modifying various signaling pathway[30,31]. In our study, we uncovered that TOB1-AS1 acted the "ceRNA" to editing miR-23a by direct reciprocal action. Previously, miR-23a has been identified to be a tumor promoter in GC, such as miR-23a upregulation, contributing to GC progression and leading to poor prognosis[32]. It also boosts tumor growth and impedes cell apoptosis by modulating programmed cell death 4 in GC[33]. However, miR-23a has been indicated to suppress tumor process in osteosarcoma[34]; but the opposite roles of miR-23a in GC and osteosarcoma may be because of the different organs, or impact different regulatory mechanisms. The above evidence proved that miR-23a functions with different characters in diverse organs or diseases.

Besides, we also verified the relationship between miR-23a and NEU1 exploiting Dual-Luciferase reporter assay. The results emerged that miR-23a directly targets NEU1, and sequentially regulates GC cell behaviors. In the present research, the repression roles of NEU1 in GC cell

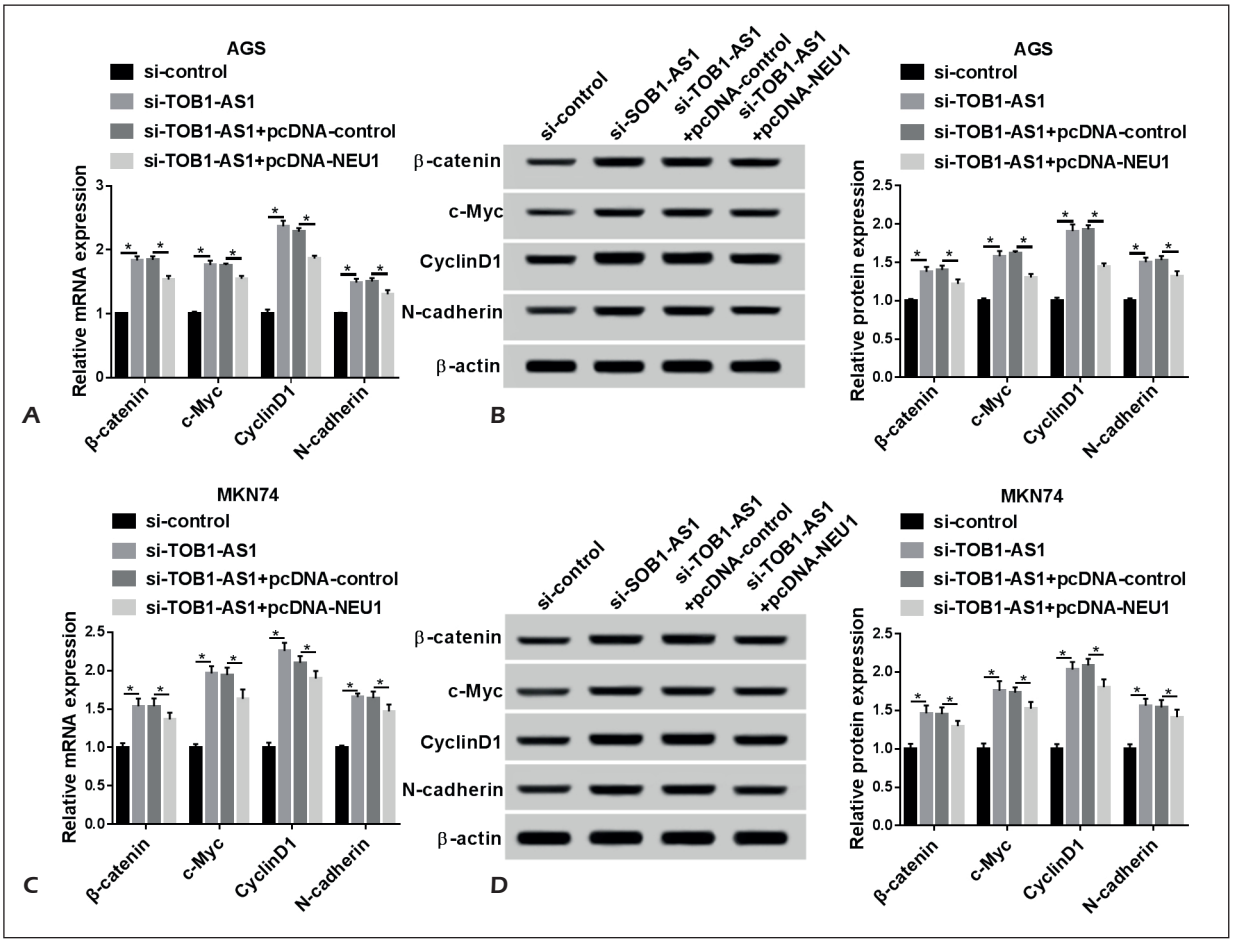

Figure 6. TOB1-AS1 and NEU1 exerted their roles via Wnt/β-catenin signaling pathway. AGS and MKN74 cells were transfected with si-control, si-TOB1-AS1 si-TOB1-AS1+pcDNA-control or si-TOB1-AS1+pcDNA-NEU1, (**A** and **C**) the mRNA levels of β-catenin, c-Myc, Cyclin D1, and N-cadherin were measured by qRT-PCR. **B**, and **D**, Western blot assay was performed to examine the protein expression, including β-catenin, c-Myc, Cyclin D1, and N-cadherin. *$p<0.05$.

proliferation, migration, and invasion were verified, similarly with previous evidence of NEU1 in colon cancer[35]. Furthermore, the levels of NEU1 in GC tissues and cell lines are both notably decreased, which were contrary to the expression level of NEU1 in ovarian cancer[36]. This adverse function of NEU1 was possible because of the different environments of GC and ovarian cancer. Subsequently, the roles of NEU1 overexpression in cell proliferation, apoptosis, migration, and invasion were detected, and the results revealed that the upregulation of NEU1 restrained the progression of GC cell lines *in vitro*. Additionally, the effects of TOB1-AS1 detection on cell behaviors were abolished by NEU1 upregulation. NEU1 plays a vital role in the process of GC, and its role was exerted *via* interacting with TOB1-AS1 and miR-23a. Besides, we also found that NEU1 plays its role *via* the Wnt/β-catenin signaling pathway, of which the classical Wnt/β-catenin pathway is involved in multiple regulatory mechanisms. For example, the Wnt/β-catenin signaling pathway is related to gastrointestinal stromal tumor malignancy[37]. From the above descriptions, the partial molecular mechanism of TOB1-AS1, miR-23a, and NEU1 are explored in GC.

Conclusions

MRNA and protein levels of TOB1-AS1 and NEU1 were both reduced in GC tissues and cell lines. The role of TOB1-AS1 was clarified in suppressing the progression of GC for the first time. The upregulation of TOB1-AS1 could promote cell apoptosis, hinder proliferation, migration, and invasion in AGS and MKN74 cells, while the TOB1-AS1 siRNA had the opposite roles on

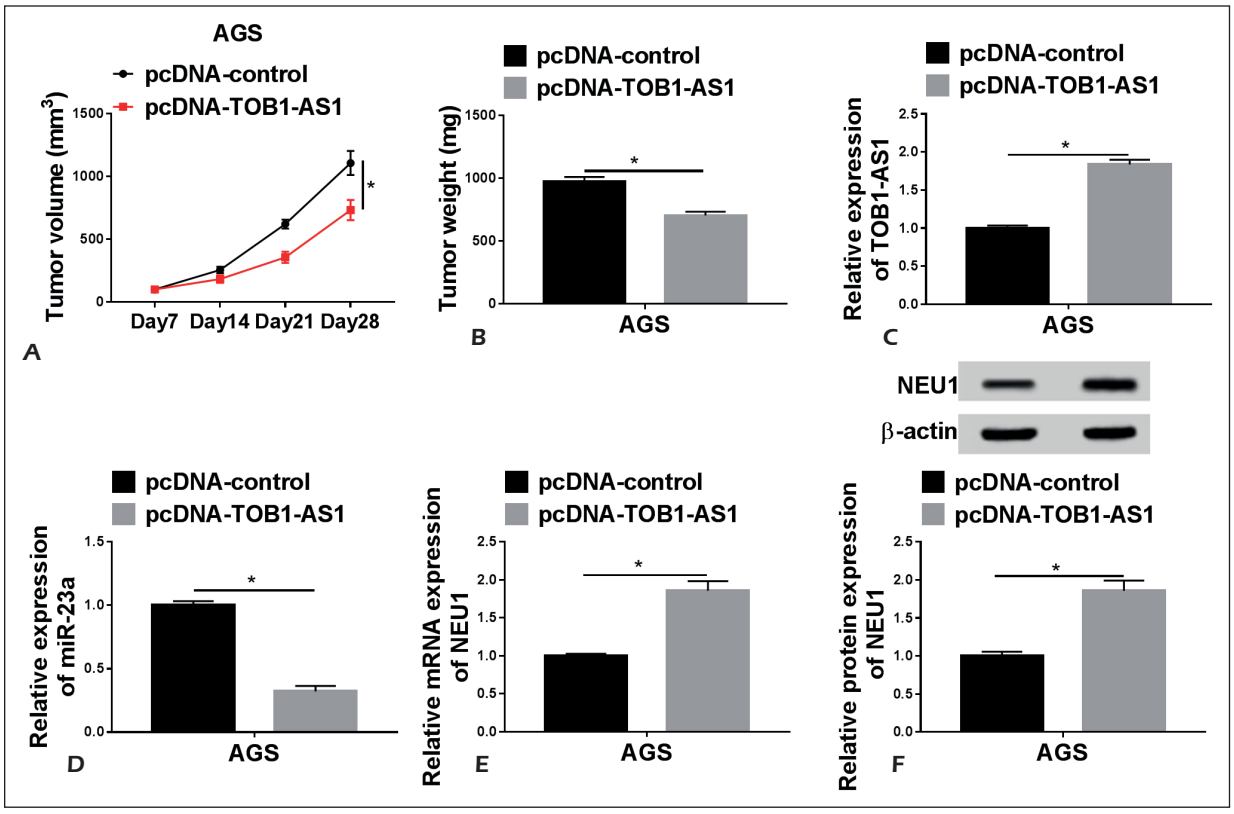

Figure 7. TOB1-AS1 overexpression suppressed the progression of GC *in vivo*. PcDNA-TOB1-AS1 or pcDNA-control was transfected into AGS cells, then, these transfected cells were subcutaneously injected into nude mice, respectively. **A**, The tumor volume was measured at 7 day, 14 day, 21 day, and 28 day post-injection and then, the tendency was drew according to the above data. **B**, The tumor weight was measured at day 28. **C-E**, QRT-PCR was applied to assess the levels of TOB1-AS1, miR-23a and NEU1, respectively. **F**, The protein expression of NEU1 was determined utilizing Western blot assay. *$p<0.05$.

the above cell behaviors, inferring the possibility that TOB1-AS1 acted as one of the therapeutic targets. However, these effects of TOB1-AS1 knockdown on cell behaviors were abolished by introducing NEU1 overexpression in GC cells. Additionally, TOB1-AS1 exerted its roles through miR-23a/NEU1 axis in regulating the progression of GC. However, the current research supplied little information on the regulatory mechanism involved in the role of TOB1-AS1 even though we exhibited the interaction between TOB1-AS1 and Wnt/β-catenin signaling pathway. For further understanding of the effect of TOB1-AS1 in the progression and initiation of human GC, more comprehensive studies are necessary to investigate TOB1-AS1 impacts in GC pathogenesis.

Conflict of Interests

The authors declare that they have no financial conflicts of interest.

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