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MiR-411 inhibits gastric cancer proliferation and migration through targeting SETD6

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) affect almost all cell behaviors of human cancers including gastric cancer (GC). However, the biological role of miR-411 in GC remains to be elucidated.

MATERIALS AND METHODS: We determined the expression level of miR-411 in GC cell lines. After synthetic miRNAs or SET domain containing 6 (SETD6) expression vectors transfection, cell proliferation, colony formation, and migration were examined. Moreover, the target of miR-411 was identified by luciferase activity reporter assay and Western blot assay.

RESULTS: Results revealed that the miR-411 expression was significantly down-regulated in GC cell lines compared with normal colonic epithelial cells. Overexpression of miR-411 inhibits GC cell proliferation, colony formation, and migration, whereas the overexpression of SETD6 promotes cell proliferation, colony formation, and migration. Moreover, SETD6 was identified as a putative target of miR-411. In addition, we showed miR-411 regulates GC cell behaviors by targeting SETD6. The overexpression of SETD6 promoted the activation of the nuclear factor (NF)-κB signaling pathway.

CONCLUSIONS: These results suggested that miR-411 functions as a tumor suppressor in GC through targeting SETD6/NF-κB pathway. Targeting miR-411 may be a novel clue for GC treatment.

Key Words

MiR-411, SETD6, NF-κB, Gastric cancer, Tumor suppressive miRNA.

Introduction

Gastric cancer (GC) is currently the sixth most commonly diagnosed cancer type but the second most common cause of cancer-related death[1]. The infection of *Helicobacter pylori* is considered a vital risk factor for the initiation and progression of GC[2]. In recent years, significant improvements including surgery, radiotherapy and targeted therapy have been made in the treatment measures for GC, partially owing to the understanding of mechanisms underlying the progression of GC[3]. Hence, a better understanding of the factors involved in GC progression could help to improve the survival of cancer patients. The miRNAs are endogenous RNAs with the length of 18 to 24 nucleotides which can regulate target genes expression mainly by binding to the 3'-untranslated region (3'-UTR) of the targeted message RNA[4]. It is reported that both the oncogenes and tumor suppressive genes can be regulated by miRNAs, highlighted the importance of miRNAs in the development of human cancers[5,6]. MiR-411 is a miRNA reported to be abnormally expressed in several human cancers including bladder cancer, renal cell cancer, lung cancer, and osteosarcoma[7-10]. Of note, miR-411 has dual functions in human cancers[7-10]. For example, miR-411 functions as a tumor suppressive gene in bladder cancer and renal cell cancer, while acting as an oncogene in lung cancer and osteosarcoma[7-10]. MiR-411 is able to inhibit the growth and metastasis of bladder cancer via targeting zinc transporter 1[7]. On the contrary, it was shown that miR-411 could promote osteosarcoma cell proliferation and migration by targeting metastasis suppressor protein 1[10]. However, to date, whether miR-411 has a role in GC carcinogenesis is unknown. The structure of the SET domain containing 6 (SETD6), a protein consisting of the helical structures of i-SET and a C-terminal mainly made up by α-helices and a few β-strands, is similar to Rubisco large subunit methyltransferase originated from plant[11]. Increasing evidence has suggested that SETD6 can induce the progression of human cancers including bladder cancer and breast carcinoma[12,13]. In the present, work we aimed to investigate the role and biological function of miR-411 in GC progression. MiR-411 expression in GC cells was analyzed by quantitative Real Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR). The effects of miR-411 or SETD6 expression on GC cell proliferation, colony formation, and migration was analyzed by the cell counting kit-8 (CCK-8)

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assay, colony formation assay, and wound-healing assay. Associations of miR-411 and SETD6 were analyzed by luciferase activity reporter assay and Western blot assay. The involvement of nuclear factor (NF)-κB signaling pathway in the miR-411/SETD6 axis was analyzed through Western blot by investigating the expression levels of the key molecules in this pathway.

Materials and Methods

Cell Lines

GC cell lines (HGC-27 and SGC-7901) and normal gastric epithelial cells (GES-1) were purchased at the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell lines were incubated in Roswell Park Memorial Institute-1640 medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% of fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at a 37°C in a humidified incubator containing 5% of CO_2.

Cell Transfection

The miR-411 mimic and negative control (NC-miR) were synthesized by RiboBio (Guangzhou, China). The pcDNA3.1 containing the open reading frame of SETD6 (pcSETD6) and the empty pcDNA3.1 vector was purchased from GenScript (Nanjing, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. After transfection for 48 h, cells were collected for further analyses.

RNA Extraction and QRT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen, Carlsbad, CA, USA). The complementary DNA was synthesized from the extracted RNA with PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). QRT-PCR was carried out to measure the relative expression level of miR-411 using SYBRGreen Master Mix (TaKaRa, Dalian, China) at ABI 7500 instrument (Bio-Rad, Hercules, CA, USA). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method with U6 small nuclear RNA (U6 snRNA) as internal control. The primers we used were listed as follows: miR-411: forward, 5'-CCATGUAUGUAACACGGUCCAC-3', reverse, 5'-GGUUAGUGGACCGGTCACC-3'; U6 snRNA: forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Protein Extraction and Western Blot

The total protein was isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. After concentration quantified by the BCA method, equal amounts of protein samples (40 μg) were separated at 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Beyotime, Shanghai, China). After blocked with skimmed milk, membranes were incubated with the primary antibodies (IKKβ: ab124957, p-IKKβ: ab59195, NF-κB p65: ab32536, or Histone H3: ab176842, all purchased from Abcam (Cambridge, MA, USA) at 4°C overnight. The membranes were incubated with HRP-conjugated secondary antibodies (ab6721, Abcam, Cambridge, MA, USA) at room temperature for 4 h after washed with Tris-Buffered Saline and Tween (TBST). Lastly, membranes were incubated with BeyoECL (Beyotime, Shanghai, China) and then detected at the Gel Imaging system.

Prediction of MiR-411 Target Genes

The target genes of miR-411 were predicted using the following algorithms: TargetScan and miRDB.

Dual-Luciferase Reporter Assay

The 3'-UTR of SETD6 was amplified from the genome and cloned into a pmiR-REPORT vector (Promega, Madison, WI, USA) and named wt-SETD6. Site-directed mutagenesis kit (TaKaRa, Otsu, Shiga, Japan) was used to generate the 3'-UTR mutation type of SETD6 and named mt-SETD6. Cells were transfected with wt-SETD6 or mt-SETD6 and miR-411 mimic and NC-miR with Lipofectamine 2000. After transfection for 48 h, luciferase activity was analyzed with the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) using Renilla luciferase activity as a reference control.

CCK-8 Assay

The CCK-8 assay was conducted to analyze cell proliferation. Briefly, 1,000 cells were seeded in a 96-well plate and incubated in the above-mentioned conditions. At the indicated time points, 10 μl of CCK-8 reagent (Beyotime, Shanghai, China) was added to the well and further incubated for 2 h. The optical density was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 450 nm.

Colony Formation Assay

The cells were seeded into a 6-well plate. After 10 days of incubation, the colonies in the plate were fixed with methanol, followed by the staining with crystal violet. Images were captured using the inverted microscope (Olympus, Tokyo, Japan). Colonies numbers were calculated from five independent fields.

Wound-Healing Assay

Cell motility was evaluated by the wound-healing assay. Cells were seeded in 24-well plate at the density of 2×10^4 cells/well and incubated until approximately 100% confluence. Then, a wound was generated on the cell surface using a pipette tip. The cell debris was removed by washing three times with phosphate-buffered saline (PBS). Cell images were captured at 0 and 24 h after wound creation to analyze cell migration at Image J 1.42 software (NIH, Bethesda, MD, USA).

Statistical Analysis

GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Data were presented as mean ± SD. A one-way analysis of variance (ANOVA) and the Tukey's post-hoc test or the two-tailed Student's *t*-test was used to compare differences in groups. $p < 0.05$ was considered statistically significant.

Results

MiR-411 was Downregulated and SETD6 Was Upregulated in GC Cells

QRT-PCR was conducted to analyze the miR-411 expression in GC cell lines (HGC-27 and SGC-7901) and normal gastric epithelial cells (GES-1). We found that in the studied GC cell lines, miR-411 expression exhibited significantly low expression levels compared with the GES-1 cell line (Figure 1A). On the contrary, SETD6 protein expression levels were significantly increased in GC cell lines (Figure 1B).

MiR-411 Overexpression Inhibited GC Cell Proliferation, Colony Formation, and Migration

To investigate the role of miR-411 in GC, synthetic miRNAs were transfected into the GC cell lines. MiR-411 mimic transfection markedly increased the expression levels of miR-411 (Figure 2A). Next, we found that cell proliferation in GC cells was significantly inhibited by miR-411 mimic transfection (Figure 2B). The colony formation assay showed that miR-411 mimic introduction decreased the colony formation ability in GC cells (Figure 2C). In addition, the wound-healing test showed that cell migration was markedly suppressed by miR-411 mimic (Figure 2D). These results suggested that overexpression of miR-411 can hinder the progression of GC *in vitro*.

SETD6 was a Direct Target of MiR-411

Bioinformatic analyses were conducted to predict the target of miR-411 with the purpose to further understand the role of miR-411 in GC. SETD6 was predicted as a possible target of miR-411 as shown in Figure 3A. Furthermore, the wt-SETD6 and mt-SETD6 luciferase reporter vectors were designed and co-transfected into GC cells with synthetic miRNAs. It was observed that miR-411 mimic transfection significantly inhibited the luciferase activity in cells transfected with wt-SETD6, while it did not change the luciferase activity in cells transfected with mt-SETD6 (Figure 3B). Besides that, we found that SETD6 expression could be repressed in GC cells transfected with miR-411 mimic compared with the NC-miR group (Figure 3C). These results indicated that SETD6 was a direct target of miR-411.

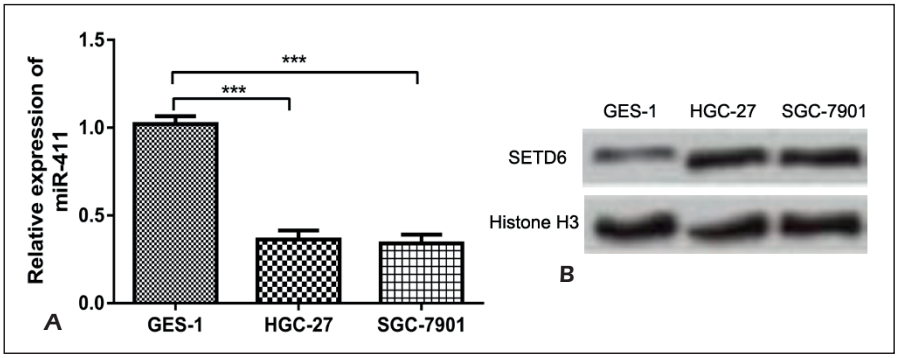

Figure 1. MiR-411 was significantly down-regulated while SETD6 was up-regulated in GC cell lines. (**A**) miR-411 expression and (**B**) SETD6 expression in GC cell lines (HGC-27 and SGC-7901) and normal gastric epithelial cells (GES-1). (***$p<0.001$) miR-411: microRNA-411; SETD6: SET domain containing 6; GC: gastric cancer.

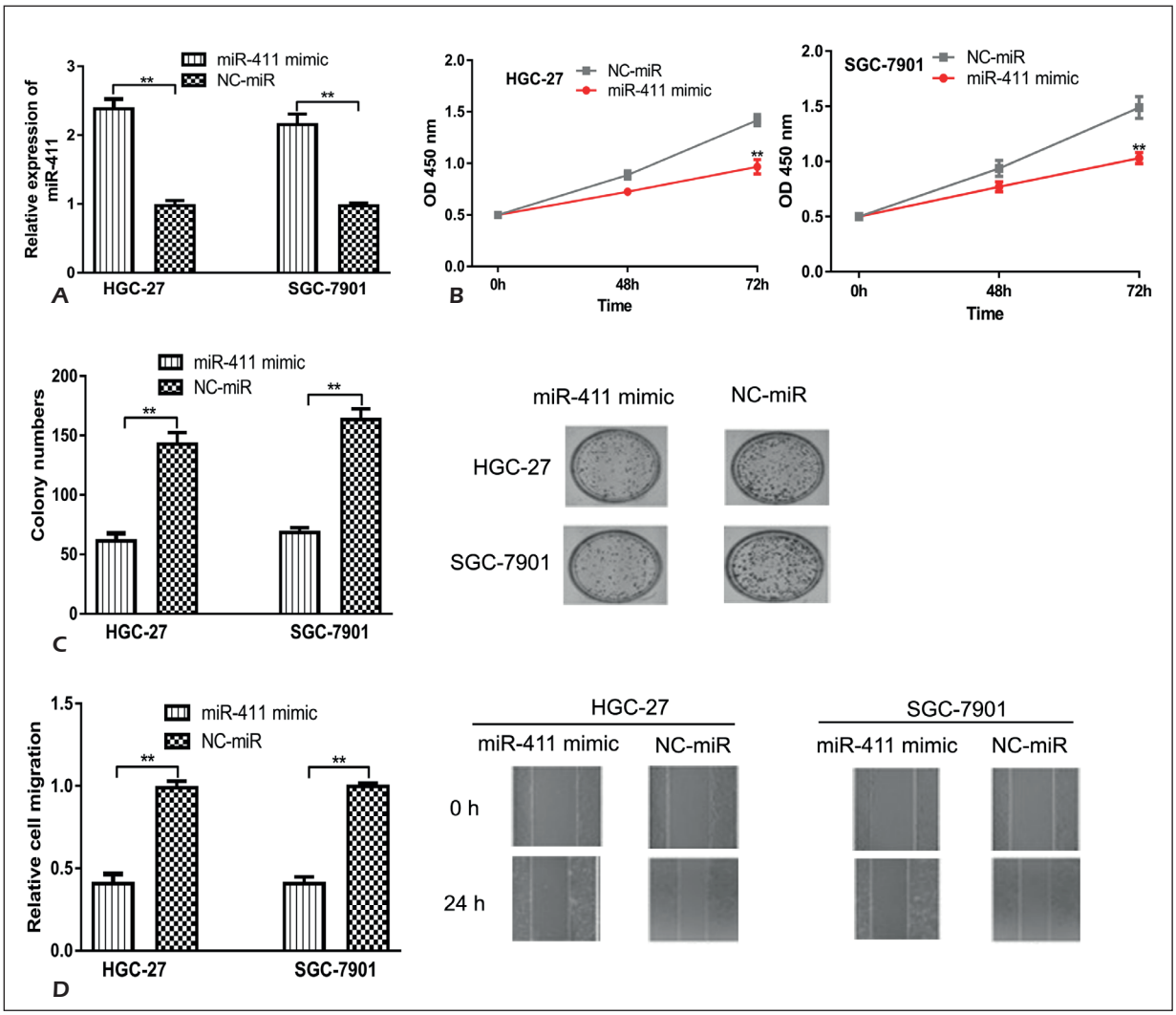

Figure 2. MiR-411 inhibits GC cell proliferation and migration. Effects of miR-411 mimic or NC-miR transfection on the (**A**) miR-411 expression, (**B**) Cell proliferation, (**C**) Colony formation, and (**D**) Cell migration in GC cell lines. (***p<0.001) miR-411: microRNA-411; NC-miR: negative control miRNA; GC: gastric cancer.

MiR-411 Regulates GC Cell Behaviors Through SETD6/NF-κB Signaling Pathway

To investigate the role of SETD6 in GC and validate the involvement of SETD6 in the miR-411 induced GC cell behaviors inhibition, we co-transfected pcSETD6 and miR-411 mimic in studied GC cell lines. We showed cell proliferation, colony formation, and migration were enhanced by SETD6 overexpression (Figure 4A-4C). In addition, the suppression effects of miR-411 mimic on GC cell proliferation, colony formation, and migration were found partially reversed by the pcSETD6 (Figure 4A-4C). Moreover, SETD6 protein expression was analyzed in these groups. As expected, the introduction of pcSETD6 increased the levels of SETD6 and partially attenuated the effects of miR-411 mimic on SETD6 expression (Figure 4D). To verify the involvement of the NF-κB signaling pathway, the expression levels of the key molecules involved in this pathway were detected by Western blot. Overexpression of SETD6 resulted in increased levels of p-IKKβ, and NF-κB p65, indicating the activation of the NF-κB pathway in GC cells (Figure 4D). Hence, miR-411 may regulate GC cell proliferation and migration through regulating the SETD6/NF-κB signaling pathway.

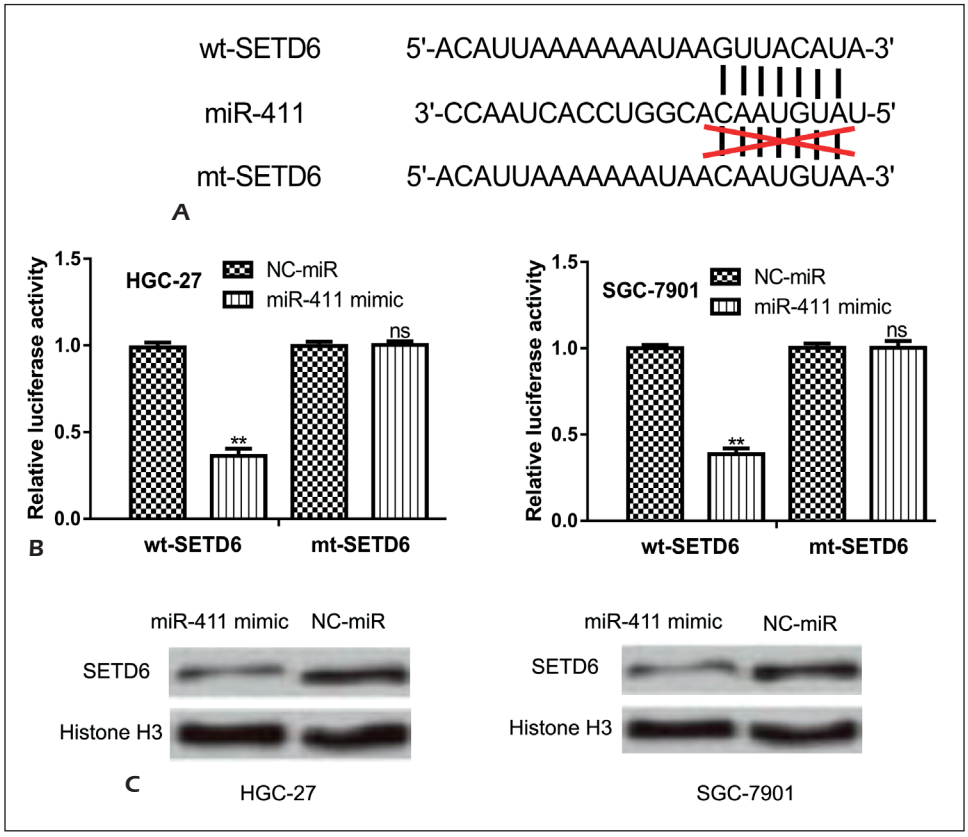

Figure 3. SETD6 was a direct target of miR-411. **A**, Predicted binding model for miR-411 and 3'-UTR of SETD6. **B**, Luciferase activity in cells transfected with miRNAs and luciferase vectors. **C**, SETD6 expression in cells transfected with miR-411 mimic or NC-miR. (ns not significant, ***p<0.001) miR-411: microRNA-411; SETD6: SET domain containing 6; UTR: untranslated region; wt: wild-type; mt: mutant; NC-miR: negative control miRNA.

Discussion

The importance of non-coding RNAs (ncRNAs) including circular RNA, long non-coding RNA, microRNA (miRNA) in the regulation of carcinogenesis of human cancers has been appreciated[14,15]. Therefore, the targeting of these ncRNAs has been widely studied for novel targeted therapy methods development[16]. The strategy for transferring these RNA-based molecules is still a challenge nowadays, but these novel treatment strategies have shed new hope for cancer patients[16]. Several miRNA-targeted therapeutic strategies have been developed and reached into preclinical development, even though no miRNA-based treatment methods have been put into clinical[17]. In this study, we showed that the miR-411 expression levels were significantly decreased in GC cell lines compared with the normal cell line. After overexpression the levels of miR-411, we found that GC cell proliferation, colony formation, and migration were significantly inhibited compared with NC-miR. These results suggested that miR-411 functions as a tumor suppressive miRNA in GC, which is consistent with its biological role in bladder cancer and renal cell cancer[7,8]. SETD6 was reported overexpressed in cancers including bladder cancer and breast cancer[12,13]. However, the role of SETD6 in human cancers remains not well defined. The finding of miRNA targets has advanced our understanding regarding the biological functions of miRNA and the carcinogenesis of human cancers[7-10]. In our current study, we found that SETD6 was a potential target of miR-411 by TargetScan and miRDB. The luciferase activity reporter assay revealed that miR-411 mimic could decrease the luciferase activity in GC cells transfected with wt-SETD6 but not mt-SETD6. Also, the levels of SETD6 were significantly reduced by miR-411 mimic in GC cell lines. Hence, these

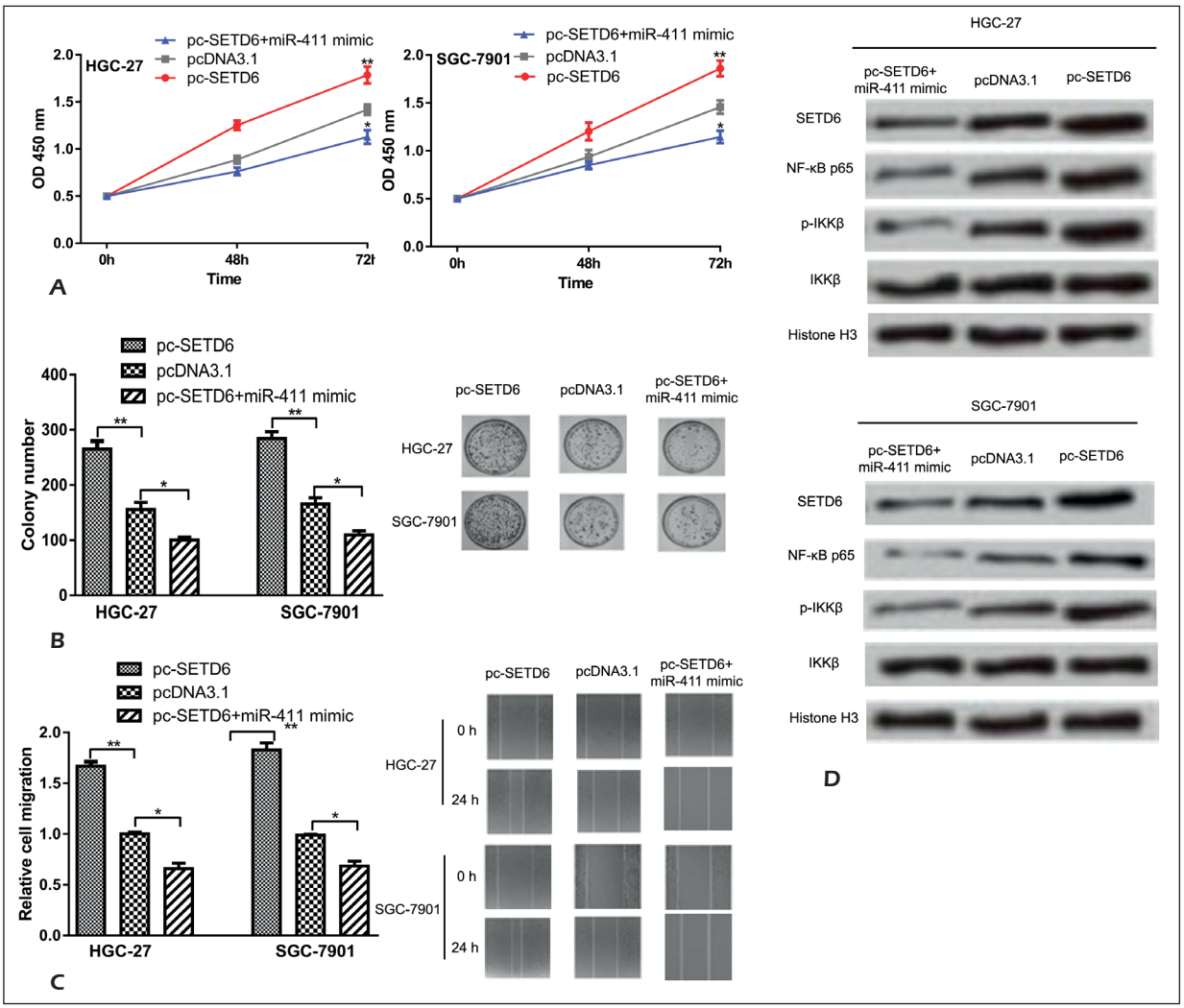

Figure 4. MiR-411 regulates GC cell behaviors through targeting SETD6/NF-κB signal. Effects of pcSETD6, pcDNA3.1 or miR-411 mimic and pcSETD6 co-transfection on (**A**) Cell proliferation, (**B**) Cell migration, (**C**) Colony formation, (**D**) SETD6 and key molecules in NF-κB pathway in GC cell lines. (*p<0.05, ***p<0.001) miR-411: microRNA-411; SETD6: SET domain containing 6; NF-κB: nuclear factor-kappa B; GC: gastric cancer.

data illustrated that SETD6 was a direct target of miR-411 in GC.

The NF-κB pathway could be triggered by cytokines, growth factors and tyrosine kinases[18-20]. In human cancers, it was suggested that NF-κB pathway activation could regulate cell proliferation, angiogenesis, invasion, and metastasis[21,22]. As it was previously reported that the NF-κB pathway could be regulated by SETD6 in bladder cancer, hence we are interested in investigating the involvement of the NF-κB pathway in the miR-411/SETD6 regulated GC cell behaviors. We found the expression levels of p-IKKβ and NF-κB p65 were enhanced by SETD6 overexpression. Therefore, these results indicated the involvement of the NF-κB signaling pathway.

Conclusions

We found that miR-411 expression was decreased in GC cells while SETD6 was increased. Overexpression of miR-411 was able to inhibit GC proliferation, colony formation and migration via targeting SETD6. In addition, SETD6 was identified as a downstream target of miR-411. Moreover, we should SETD6 could trigger the activation of the NF-κB pathway in GC. Therefore, the involvement of miR-411/SETD6/NF-κB axis in GC progression was speculated.

Conflict of Interest

The authors declare that they have no conflicts of interests.

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