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ORIGINAL RESEARCH

MicroRNA-766 targeting regulation of SOX6 expression promoted cell proliferation of human colorectal cancer

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Abstract: MicroRNAs (miRNAs) have emerged as important regulators of cancer-cell biological processes. Previous studies have shown that miR-766 plays an important role in a variety of biological processes in various human cancers. However, the underlying mechanism of miR-766 in colorectal cancer (CRC) cells remains unclear. In this study, we investigated miR-766's role in CRC cell proliferation. Polymerase chain reaction results showed that miR-766 expression was significantly upregulated in CRC tissues and cells. Ectopic expression of miR-766 promoted cell growth and anchorage-independent growth in CRC cells. Bioinformatic analysis predicted SOX6, a potential target of miR-766, acting as a tumor suppressor. Luciferase reporter assay results demonstrated that miR-766 directly bound to the 3′-untranslated region of SOX6. Overexpression of miR-766 suppressed SOX6 expression, resulting in the downregulation of p21 and upregulation of cyclin D1. In a further experiment, SOX6-silenced SW480 cells transfected with miR-766 promoted cell growth, suggesting that downregulation of SOX6 was required for miR-766-induced CRC cell proliferation. Taken together, these results suggested that miR-766 represents an onco-miRNA and participates in the development of CRC by modulating SOX6 expression.

Keywords: miR-766, colorectal cancer, SOX6, cell proliferation

Introduction

Colorectal cancer (CRC) is the third-leading cause of cancer-related deaths worldwide.[1] However, the molecular underpinnings of CRC proliferation remain incompletely understood. Recently, microRNAs (miRNAs) were discovered to be endogenous noncoding small RNAs (~22 nucleotides) that play an important role in regulating the diverse biological progression of multiple cancers, such as proliferation, apoptosis, differentiation, and metabolism.[2–4] In view of the close relationship between the biological processes of cancers and miRNAs, miRNAs are presently considered new therapeutic targets for the treatment of cancer.[5–8]

Growing evidence indicates that miR-766 acts as a tumor promoter or suppressor in multiple cancers.[9–11] However, the relationship between CRC and miR-766 remains unclear. In this study, we investigated the role of miR-766 in CRC. Our results demonstrated that overexpression of miR-766 effectively promoted cell proliferation by repressing SOX6 in CRC. Further experimentation demonstrated that SOX6 was a bona fide target of miR-766. Taken together, our data demonstrated that miR-766 indeed acted as a proliferation promoter through the downregulation of SOX6.

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Materials and methods

Clinical specimens

Eight paired human CRC tissue samples and matched tumor-adjacent tissue were obtained from CRC patients and histopathologically diagnosed at the China–Japan Union Hospital of Jilin University (Changchun, People's Republic of China). The study was approved by the ethics committee of the China–Japan Union Hospital of Jilin University. Written informed consent was obtained from all patients. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen, and stored until total RNAs or proteins were extracted.

Cell culture

Human CRC cell lines HT-29, COLO205, SW620, SW403, KM202L, SW480, and COLO320DM and normal colonic cell line FHC were purchased from National Rodent Laboratory Animal Resource (Shanghai, People's Republic of China). All CRC cell lines were grown in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co, St Louis, MO, USA), 100 U/mL of penicillin–streptomycin (Thermo Fisher Scientific), and normal colon FHC cells were grown in Dulbecco's Modified Eagle's Medium/F-12 medium with 10% fetal bovine serum, 10 ng/mL cholera toxin, 5 μg/mL transferrin, 5 μg/mL insulin, 100 ng/mL hydrocortisone, and an extra 10 mM HEPES. Cell lines were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. The protocols in this study in regards to human cell lines were approved by China-Japan Union Hospital of Jilin University (Changchun, China).

Plasmids, small interfering RNA, and transfection

MiR-766 mimic, miR-766 inhibitor, and negative control were purchased from GeneCopoeia (Rockville, MD, USA). For depletion of SOX6, SOX6 small interfering RNA (siRNA) and negative control were purchased from Qiagen NV (Venlo, the Netherlands). Transfection of plasmids and siRNAs was performed using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from culture cells and patient samples using Trizol (Thermo Fisher Scientific) as recommended by the manufacturer's instructions, and quantitative real-time polymerase chain reaction (qRT-PCR) for miRNA was performed using the TaqMan miRNA reverse-transcription kit (Thermo Fisher Scientific). The relative miR-766 expression levels after normalization to U6 small nuclear RNA were calculated using $2^{-[(\text{CtofmiR}-766)-(\text{CtofU6})]}$. The qRT-PCR assays were performed using an SYBR kit (Qiagen) using a LightCycler system. The primers selected were as follows: cyclin D1 forward 5′-TCCTCTCCAAAATGC CAGAG-3′, cyclin D1 reverse 5′-GGCGGATTGGAAATGAACTT-3′; p21 forward 5′-CATGGGTTCTGACGGACAT-3′, p21 reverse 5′-AGTCAGTTCCTTGTGGAGCC-3′. Expression data were normalized to the geometric mean of GAPDH (forward primer 5′-GACGGCCGCATCTTCTTGT-3′, reverse primer 3′-CACACCGACCTTCACCATTTT-5′) to control the variability in expression levels and calculated as $2^{-[(\text{CtofCyclinD1andp21})-(\text{CtofGAPDH})]}$.

Western blotting

Protein lysates were lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, People's Republic of China); equal quantities (50 μg) of protein were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then gels were transferred onto polyvinylidene difluoride membranes for 2 hours at 4°C, run at a current of 125 mA, and blocked for 2 hours. The membrane was incubated overnight with anti-SOX6 (1:500; Abcam, Cambridge, MA, USA), anti-cyclin D1, anti-p21 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (1:2,000 dilution; Abcam) was used as a reference protein. Membranes were further incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature, and blots were visualized using an ECL Kit (Beyotime).

MTT assay and colony-formation assay

Cell growth was measured by MTT assay (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 5×10^3 transfected cells were seeded in 96-well plates and cultured for 1, 2, 3, and 4 days. Exactly 20 μL of 5 mg/mL MTT solution (Sigma-Aldrich) was added to the test well and incubated for 4 hours, and then the culture medium was removed and 150 μL dimethyl sulfoxide (Sigma-Aldrich) was added. Optical density at 490 nm was measured using a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

For the colony-formation assay, SW480 cells after transfection were plated in six-well plates (1,000 cells per well) and incubated for 10 days. Cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Anchorage-independent growth assay

Two milliliters complete medium supplemented with 0.5% agar (Sigma-Aldrich) was added to each well of the six-well plates to form a layer of base gel. Plates were incubated at

37°C in 5% CO_2 for 14 days. Colonies were observed and counted under microscope.

Luciferase assays

The PPP2R2A open reading frames with 3′-untranslated region (UTR) was cloned into pGL3. Vectors were purchased from GeneCopoeia. The primers selected were as follows: SOX6-3′UTR-wt-up: 5′-GCCCCGCGGTGGCTCC ACAATTACATCAGC-3′; SOX6-3′UTR-wt-down: 3′-GCCCTGCAGCATAAAATCACTATGTACA CAGGA-5′.

SW480 cells (5×10^4/well) were cultured in 24-well plates and cotransfected with pGL3-PPP2R2A-3′-UTR and 100 nM of miR-766 or miR-766-in or miR-766-mutant (mut) or control mimics using Lipofectamine 2000 reagent. Luciferase and *Renilla* activities were assayed 48 hours after transfection using the dual-luciferase assay kit to the manufacturer's protocol.

Statistical analysis

All statistical analyses except for microarray data were performed using SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Student's *t*-test was used to evaluate the significance of the differences between two groups of data in all the pertinent experiments. $P<0.05$ (using a two-tailed paired *t*-test) was thought to be significantly different for two groups of data.

Results

MiR-766 expression elevated in CRC tissues and CRC cell lines

To investigate the potential roles of miR-766 in CRC development, results of RT-PCR analysis revealed that compared with the matched tumor-adjacent tissue, miR-766 expression was differentially upregulated in the CRC tissues, and all eight tested CRC cell lines showed significantly upregulated expression of miR-766 compared to the normal colonic cell line FHC (Figure 1).

MiR-766 promoted CRC cell proliferation

To investigate the role of miR-766 in CRC cell proliferation further, SW480 cells were transfected with miR-766 mimics, miR-766 inhibitor, or the relative controls. Relative miR-766 expression was verified using RT-PCR (Figures 2A and 3A). Ectopic expression of miR-766 significantly increased the growth rate of SW480 cells from day 2 of the experiment (Figure 2B). Colony-formation assay showed that upregulation of miR-766 promoted the colony-formation capacity of SW480 cells (Figure 2C). Strikingly, we found that overexpression of miR-766 in SW480 cells significantly enhanced their anchorage-independent growth ability (Figure 2D). In contrast, miR-766-in showed the opposite effect (Figure 3B–D). Collectively, these results showed that miR-766 enhanced the proliferation of SW480 cells in vitro.

MiR-766 directly targeted SOX6 by binding to its 3′-UTR

As SOX6 was a binding target of miR-766 predicted by TargetScan (Figure 4A), SW480 cells were transfected with miR-766 mimic, miR-766-in, or the respective controls. The results of Western blots revealed that the expression of SOX6 on protein level was remarkably decreased after the overexpression of miR-766 (Figure 4B), while miR-766-in

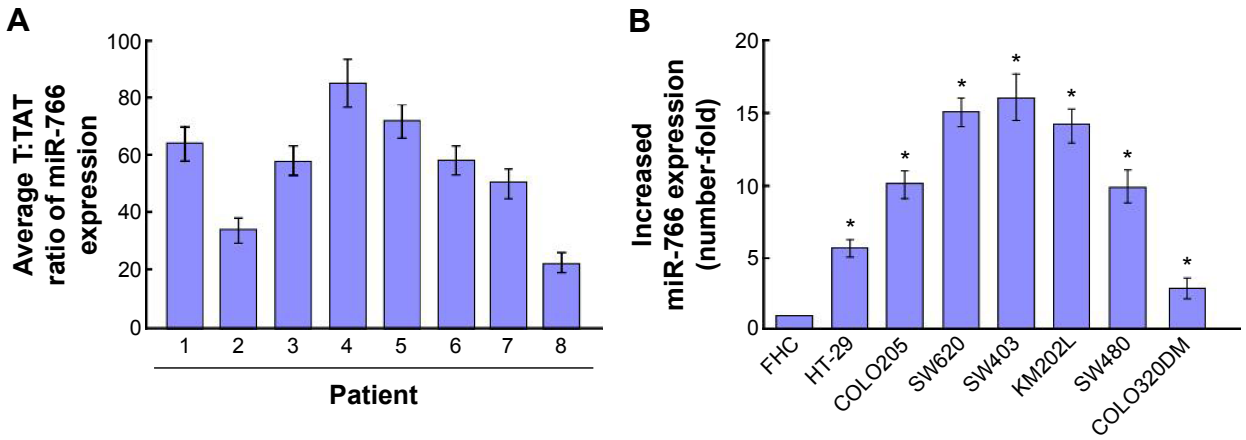


Figure 1 Expression of miR-766 in human colorectal cancer (CRC) tissues and cell lines.

Notes: (**A**) Relative miR-766 messenger RNA-expression levels in eight paired primary CRC tissue (T) samples and matched tumor-adjacent tissue (TAT) from the same patient were detected by polymerase chain reaction (PCR) analysis. (**B**) Real-time PCR analysis of miR-766 expression in FHC cells and CRC cell lines, including HT-29, COLO205, SW620, SW403, KM202L, SW480, and COLO320DM. Experiments were repeated at least three times. Each bar represents the mean of three independent experiments. *$P<0.05$.

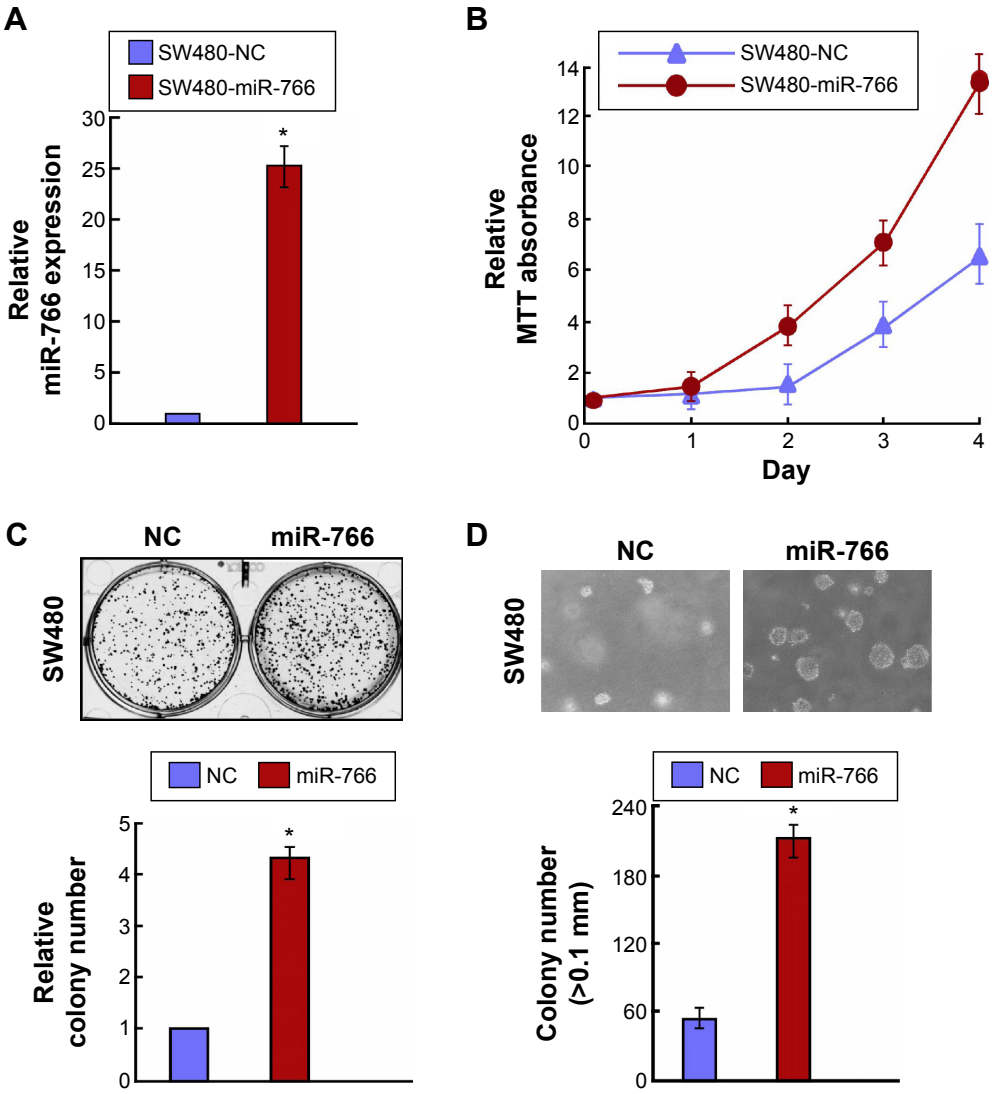


Figure 2 miR-766 upregulation promoted CRC cell proliferation.
Notes: (**A**) Validation of miR-766 expression levels after transfection by PCR analysis. (**B**) MTT assays revealed that upregulation of miR-766 promoted growth of SW480 cells. (**C**) Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. (**D**) Upregulation of miR-766 promoted the anchorage-independent growth of SW480 cells. Representative micrographs (left) and quantification of colonies that were >0.1 mm (right). Each bar represents the mean of three independent experiments. *$P<0.05$.
Abbreviations: CRC, colorectal cancer; PCR, polymerase chain reaction; NC, negative control.

significantly increased SOX6 protein expression. For the purpose of identifying SOX6 as a direct target of miR-766, SW480 cells were transfected with SOX6 3′-UTR (wild or mutant) vector and miR-766 mimic, miR-766-in, or miRNA negative controls. Dual-luciferase reporter assay results showed that reduction in luciferase activity was observed in miR-766 -transfected SW480 cells, whereas the repressive effect of miR-766-in increased wild-type SOX6 luciferase activity. Meanwhile, the luciferase activities of cells cotransfected with miR-766 and SOX6 3′-UTR-mut vector were unaffected. Our data indicated that SOX6 might be involved in cellular proliferation of CRC. We detected that the SOX6 downstream genes, messenger RNA, and protein expression of p21 were remarkably downregulated and cyclin D1 expression was remarkably upregulated by miR-766 (Figure 4D). In contrast, the expression level of p21 was upregulated and cyclin D1 downregulated in SW480 cells transfected with miR-766-in (Figure 4E). Collectively, these results revealed that SOX6 was a bona fide target of miR-766.

SOX6 suppression required for miR-766-induced cell proliferation in CRC

To investigate the role of SOX6 reduction in CRC proliferation further, we first examined the effects of SOX6

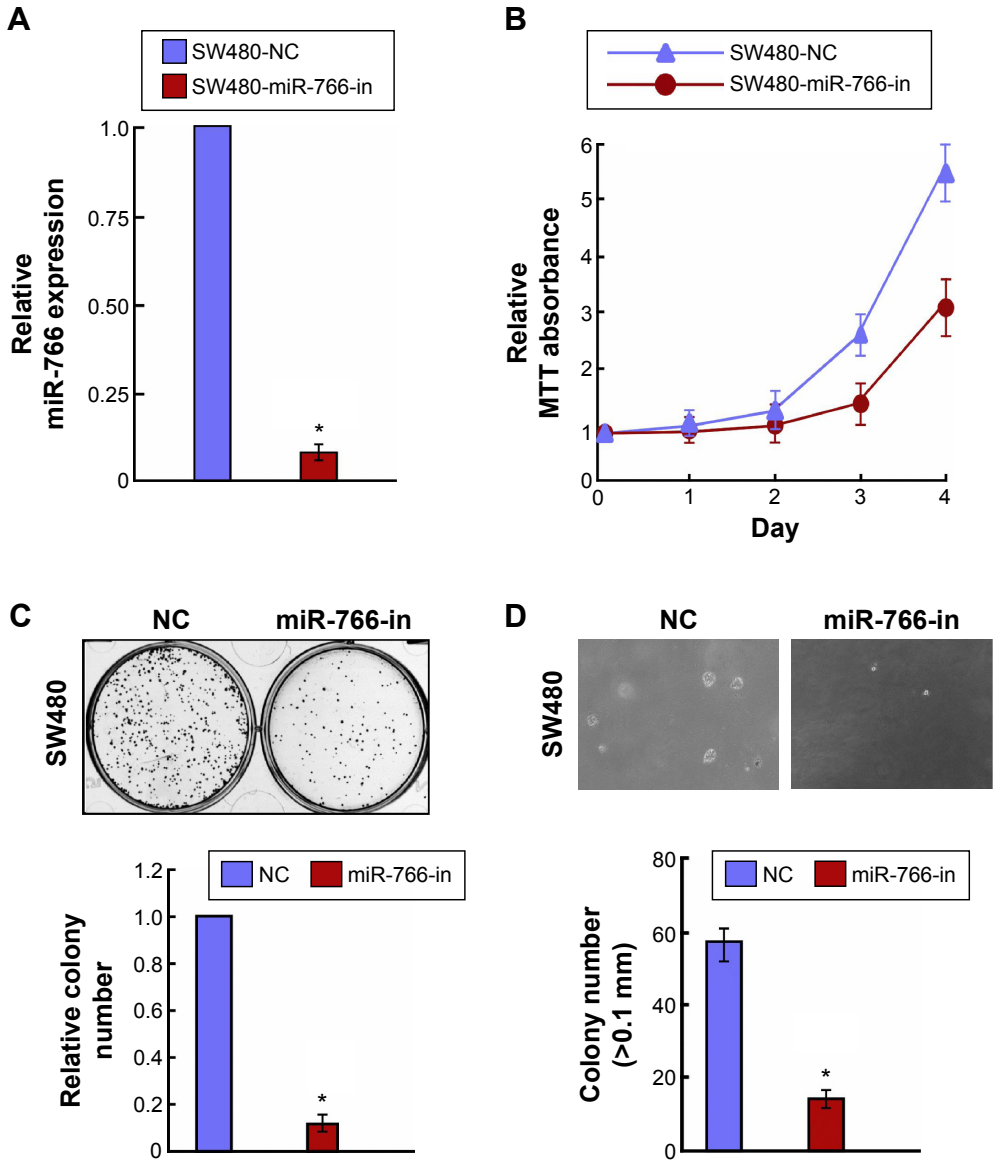


Figure 3 Inhibition of miR-766 inhibited CRC cell proliferation.

Notes: (**A**) Validation of miR-766 expression levels after transfection by PCR analysis. (**B**) MTT assays revealed that inhibition of miR-766 suppressed the growth of SW480 cells. (**C**) Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. (**D**) Inhibition of miR-766 inhibited the anchorage-independent growth of SW480 cells. Representative micrographs (left) and quantification of colonies that were >0.1 mm (right). Each bar represents the mean of three independent experiments. *$P<0.05$.

Abbreviations: CRC, colorectal cancer; PCR, polymerase chain reaction; NC, negative control.

downregulation on CRC cell proliferation. Results of Western blot analysis indicated that silencing SOX6 effectively decreased SOX6 protein expression in SW480 cells transfected with miR-766-in (Figure 5A). Results of colony formation and anchorage-independent growth assays demonstrated that knockdown of SOX6 effectively counteracted the proliferation arrest by miR-766 (Figure 5B and C). Taken together, these results indicated that SOX6 was an important target of miR-766 and was involved in miR-766-regulated CRC cell proliferation.

Discussion

In the present study, we noted that miR-766 expression was significantly upregulated in CRC tissues and cells. Moreover, overexpression of miR-766 enhanced the cell growth of CRC cells, whereas miR-766-in showed the opposite effect, probably through repressing SOX6 expression. The downregulation of SOX6 by miR-766 led to upregulation of p21 and downregulation of cyclin D1. Our results showed that miR-766 might play an essential role via the SOX6-mediated pathway in the progress of CRC.

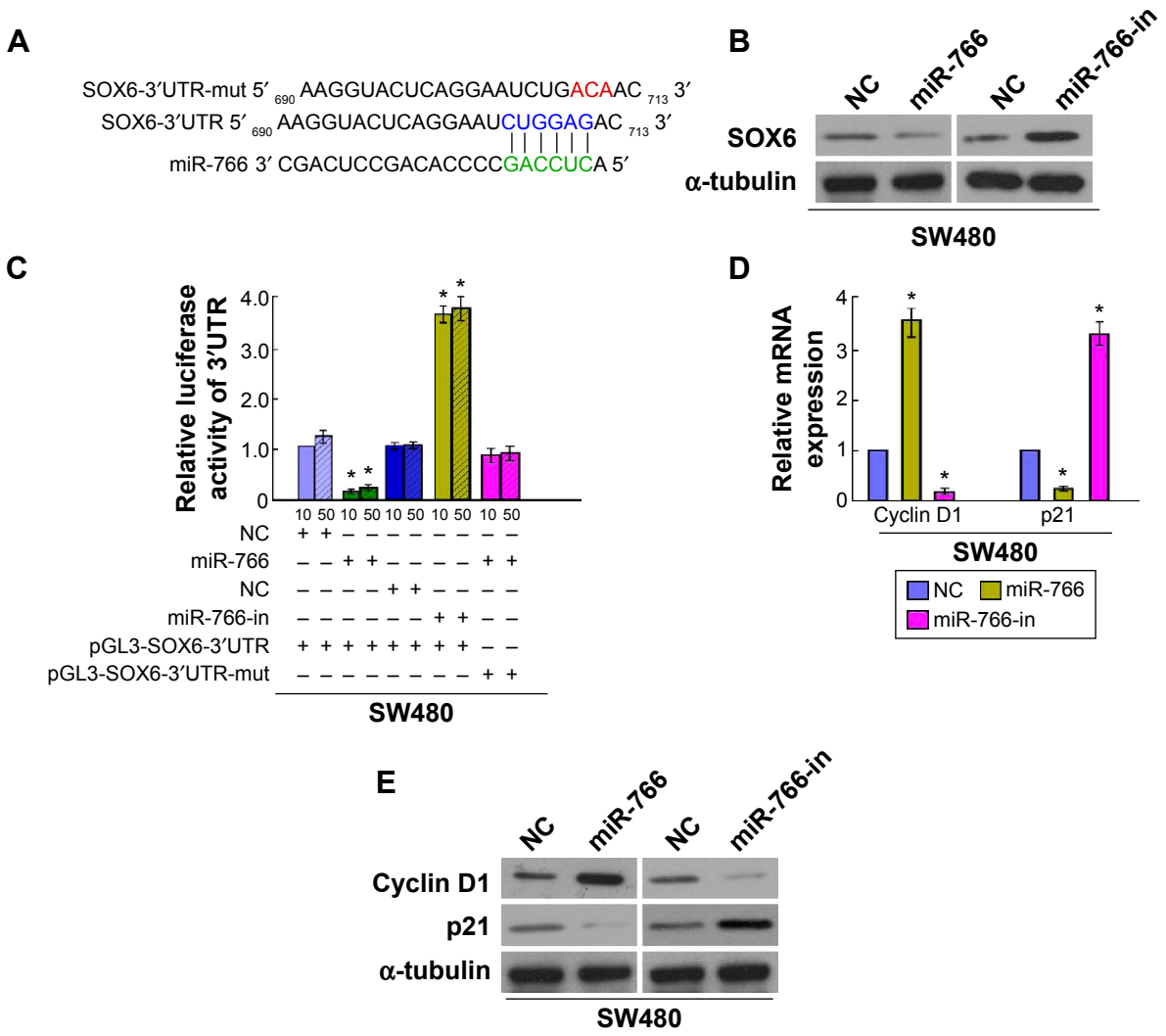


Figure 4 miR-766 suppressed SOX6 expression by directly targeting the SOX6 3′-UTR and altered levels of proteins related to proliferation in SW480 cells.
Notes: (**A**) Predicted miR-766 target sequence in the 3′-UTR of SOX6 (SOX6-3′-UTR) and positions of three mutated nucleotides (red) in SOX6-3′-UTR-mutant (mut). (**B**) SOX6 protein expression in SW480 cells transfected with miR-766 or the miR-766 inhibitor were detected by Western blotting analysis. α-tubulin served as the loading control. (**C**) Luciferase reporter assay of SW480 cells transfected with the pGL3-SOX6-3′-UTR reporter or pGL3-SOX6-3′-UTR-mut reporter and miR-766 or miR-766-in with increasing amounts (10 and 50 nM) of oligonucleotides. (**D**) Real-time PCR analysis of expression of cyclin D1 and p21 in SW480 cells. (**E**) Western blotting analysis of protein expression of cyclin D1 and p21 in SW480 cells. α-tubulin served as the loading control. *$P<0.05$.
Abbreviations: UTR, untranslated region; PCR, polymerase chain reaction; NC, negative control; mRNA, messenger RNA.

Growing evidence suggests that miRNAs play essential roles in a broad range of biological processes, including cell proliferation, differentiation, angiogenesis, migration, and invasion.[12–15] Recently, aberrant expressions of miRNAs have been increasingly investigated. However, it was uncertain whether miR-766 was associated with the development of CRC. In this study, we found that miR-766 negatively regulated SOX6 expression by targeting the SOX6 3′-UTR. SOX6, a member of the Sox transcription-factor family, plays critical roles in cell differentiation and proliferation.[16,17] SOX6 is reported to have a tumor-suppressive function in cancers.[18,19] Qin et al indicated that SOX6 inhibited cell proliferation by upregulating expressions of p53 and p21 and downregulating expressions of cyclin D1/CDK4, cyclin A, and β-catenin in esophageal cancer.[20] Cyclin D1 and p21 are crucial in the regulation of cell proliferation, differentiation, apoptosis, and other cellular functions.[21–23] In line with these reports, in our study, ectopic expression of miR-766 led to downregulation of SOX6, which resulted in the downregulation of p21, upregulation of cyclin D1, and thus the promotion of CRC cell proliferation.

In sum, our data demonstrated that miR-766 is a proto-oncogenic miRNA in CRC cells and that SOX6 is a novel and critical miRNA-766 target. Therefore, all the results indicated that miR-766 might serve as a potential therapeutic target for treating CRC.

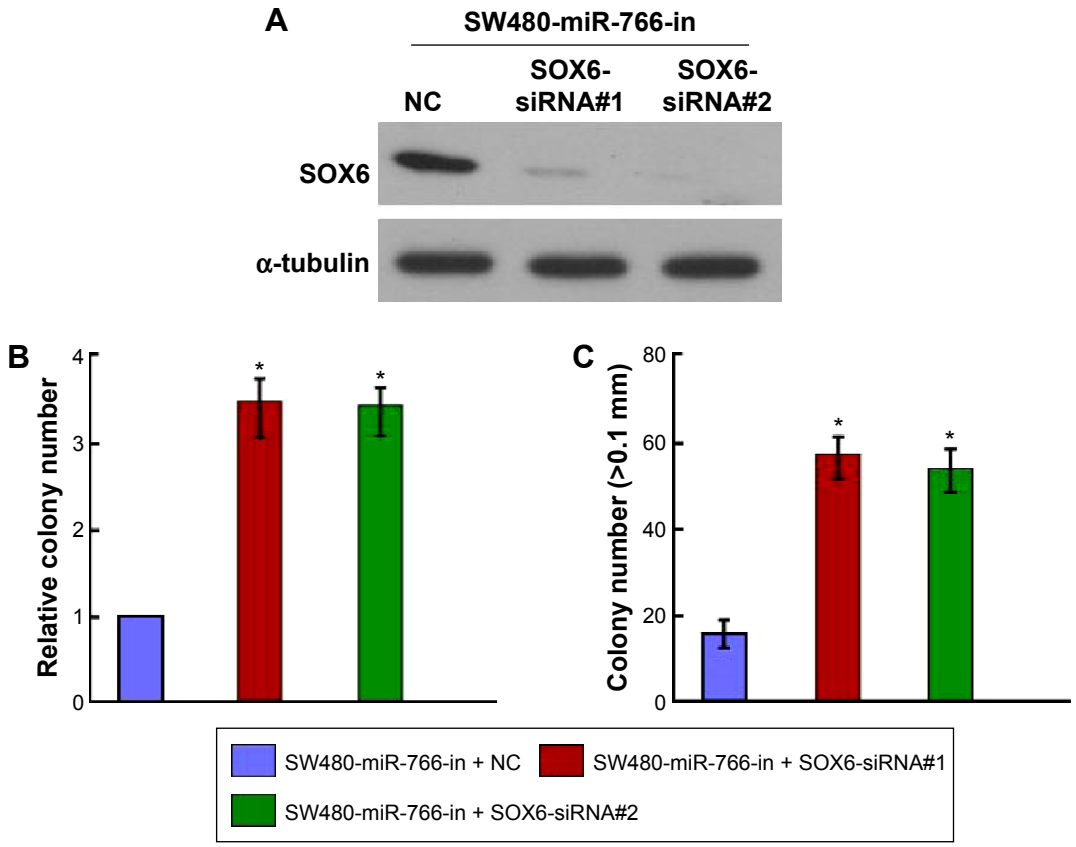


Figure 5 SOX6 downregulation was required for miR-766-induced proliferation of CRC cells.
Notes: (**A**) Western blot analysis verified that silencing SOX6 effectively decreased the expression of SOX6 in miR-766-in-transfected SW480 cells. (**B**) miR-766-in-transfected SW480 cells after transfection with SOX6 siRNAs promoted cell-colony formation. (**C**) miR-766-in-transfected SW480 cells after transfection with SOX6 siRNAs promoted anchorage-independent growth. Representative quantification of colonies that were >0.1 mm. Each bar represents the mean of three independent experiments. *$P<0.05$.
Abbreviations: CRC, colorectal cancer; siRNAs, small interfering RNAs; NC, negative control.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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