

# MicroRNA-129 inhibits colorectal cancer cell proliferation, invasion and epithelial-to-mesenchymal transition by targeting SOX4

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**Abstract.** Colorectal cancer (CRC) is one of the most common digestive tract cancers and ~90% of CRC-related deaths are caused by metastasis. MicroRNA (miR)-129 has been reported to be involved in the metastasis of various malignant tumors. However, the role of miR-129 in CRC metastasis remains unclear. The purpose of the present study was to identify the potential functions and mechanisms of action of miR-129 in CRC progression. The expression of miR-129 and sex-determining region Y-related high-mobility group-box 4 (SOX4) was determined in CRC tissues or cell lines by reverse transcription-quantitative PCR, western blot or immunofluorescence assays. The mechanism underlying the role of miR-129 in CRC progression was assessed by MTT, wound healing, Transwell, western blot and dual-luciferase report assays. The results revealed that miR-129 was significantly decreased, whereas SOX4 was increased, in CRC tissues and cell lines. SW620 and SW480 cells exhibited a higher proliferation, migration and invasion capacity compared with NCM460 cells. miR-129 overexpression significantly inhibited cell proliferation, migration, invasion and epithelial-to-mesenchymal transition (EMT), and it activated the nuclear factor (NF)- $\kappa$ B signaling pathway in CRC cells, while the inhibition of miR-129 exerted opposite effects. Additionally, SOX4 was identified as a direct target gene

of miR-129. Taken together, the findings of the present study suggested that miR-129 may act as a tumor suppressor in CRC by inhibiting CRC cell proliferation, migration, invasion and EMT, in part through targeting the 3'-untranslated region of SOX4 mRNA, and the mechanism may involve activation of the NF- $\kappa$ B signaling pathway.

## Introduction

CRC is one of the most common malignancies of the gastrointestinal tract, ranking third among common causes of cancer-related mortality worldwide (1). Over the past decade, substantial improvements have been made in the diagnosis and management of CRC, achieving an annual decline of ~3% in CRC deaths (2). Unfortunately, CRC still causes ~600,000 deaths annually (3). Metastatic lesions, particularly liver metastases, are the main cause of CRC-related mortality (4). Similar to most solid tumors, CRC has a complex pathogenesis involving inactivation of tumor suppressor genes and activation of oncogenes (5). Although increasing evidence has partly uncovered the regulatory mechanism underlying CRC tumorigenesis, the exact mechanism underlying cancer metastasis remains elusive. Therefore, in-depth study of the molecules implicated in the metastasis and recurrence of CRC is crucial for improving the prognosis and treatment of the patients.

MicroRNAs (miRNAs), a class of small non-coding RNAs with a length of 19-24 nucleotides, mainly exert their biological effects by binding to the 3'-untranslated region (3'-UTR) of target mRNAs to cause mRNA degradation or translational repression at the post-transcriptional level (6,7). miRNAs have been identified as tumor suppressors or oncogenes in multiple cancers, including CRC (8-10). Mounting evidence has also revealed that dysregulation of miRNAs is associated with tumor growth, cell proliferation and migration (11,12). Recent studies have demonstrated that miR-129 is expressed at low levels in several cancers, including renal cell carcinoma (13), bladder cancer (14) and gastric cancer (15), in which miR-129 has been confirmed to act as a tumor suppressor. In addition, miR-129 has been reported to promote apoptosis and chemosensitivity to 5-fluorouracil in CRC (16). Those studies indicated that miR-129 is implicated in CRC progression, but its role and potential regulatory mechanisms in CRC metastasis has yet to be fully elucidated.

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**Abbreviations:** CRC, colorectal cancer; SOX4, sex-determining region Y-related high-mobility group-box 4; RT-qPCR, reverse transcription-quantitative PCR; EMT, epithelial-to-mesenchymal transition; NF- $\kappa$ B, nuclear factor- $\kappa$ B; 3'-UTR, 3'-untranslated region; miRNAs, microRNAs; NC, negative control; PE, plating efficiency; Mut, mutant; WT, wild-type

**Key words:** colorectal cancer, microRNA-129, sex-determining region Y-related high-mobility group-box 4, invasion, epithelial-to-mesenchymal transition

Sex-determining region Y-related high-mobility group-box 4 (SOX4), a member of the SOX transcription factor family, is involved in embryonic development and cell differentiation (17,18). SOX4 overexpression in CRC was previously demonstrated to be correlated with poor prognosis and recurrence (19), whereas knockdown of SOX4 was reported to suppress CRC cell proliferation and invasion (20). SOX4 is a potential target for multiple miRNAs in CRC, including miR-320 (21), miR-363-3p (22) and miR-539 (23). In addition, miR-129-5p was shown to suppress cell proliferation and invasion via regulating the SOX4/Wnt/ $\beta$ -catenin signaling pathway in chondrosarcoma (24). Kang *et al.* (25) reported that miR-129-2 inhibits cell proliferation and migration by targeting SOX4 in esophageal carcinoma. However, whether miR-129 can inhibit the malignant phenotypes of CRC by regulating SOX4 has not been reported to date.

Therefore, the aim of the present study was to elucidate the interaction between miR-129 and SOX4 in CRC. The expression of miR-129 and SOX4 was assessed in CRC tissues and cell lines, and it was investigated whether the effects of miR-129 on CRC cell proliferation, migration, invasion and epithelial-to-mesenchymal transition (EMT) were mediated through targeting SOX4 and activation of the nuclear factor (NF)- $\kappa$ B signaling pathway.

## Materials and methods

**Tissue samples.** A cohort of 60 CRC tissue samples and matched tumor-adjacent tissue samples were acquired from patients undergoing a surgical procedure between May 2017 and May 2018, according to the institutional guidelines. The clinical characteristics of the 60 patients with CRC are summarized in Table I. For all patients, original diagnosis and tumor grading were conducted in a blinded manner by two experienced pathologists according to the American Joint Committee on Cancer (AJCC) pathologic tumor-node metastasis (TNM) classification system (26). The protocol of the present study was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University. Informed consent was obtained from all the patients. The tissue samples were collected during surgery and immediately stored in liquid nitrogen.

**Cell culture.** The human CRC cell lines SW620 (CL-0225) and SW480 (CL-0223) were obtained from Procell Life Science & Technology Co., Ltd., and the normal colorectal cell line NCM460 (BNF-3068) was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM (HyClone, Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

**Transfection.** miR-129 mimic, inhibitor and negative control (NC) were purchased from Guangzhou RiboBio Co., Ltd. At 24 h before transfection, SW620 and SW480 cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells/well. The cells in each well were transfected with a solution of 50 pmol/ml miR-129 mimic or inhibitor using Lipofectamine™ 2000 (Invitrogen;

Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Fresh medium was replaced following transfection for 6 h. Transfection efficiency was determined using reverse transcription-quantitative PCR (RT-qPCR) assay following transfection for 48 h. The sequences of the oligonucleotides were as follows: miR-129 mimic: 5'-CUUUUUGCGGUC UGGGCUUGC-3'; miR-129 inhibitor: 5'-GCAAGCCCA GACCGCAAAAAG-3'; and miR-NC: 5'-AGUGCUAGUAGC UUUGCAUGU-3'.

**RT-qPCR analysis.** Total RNA was extracted from CRC cell lines, CRC tissues and matched tumor-adjacent tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for miRNA or mRNA analysis. To quantify mRNA expression, cDNA was synthesized using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol at 37°C for 15 min and 85°C for 5 sec. SYBR® Premix Ex Taq II (Takara Biotechnology Co., Ltd.) was used to perform RT-qPCR in an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 3 min at 95°C; 40 cycles at 95°C for 5 sec and 60°C for 30 sec, followed by 72°C for 30 sec. To quantify miRNA expression, reverse transcription and qPCR were performed using Bulge-Loop™ miRNA RT-qPCR Primer and Bulge-Loop™ miRNA RT-qPCR Starter kit (Guangzhou RiboBio Co., Ltd.). The thermocycling conditions of qPCR were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 2 sec, 60°C for 20 sec and 70°C for 10 sec. The 2<sup>- $\Delta\Delta C_q$</sup>  method was used for comparative quantitation (27). GAPDH and U6 small nuclear RNA were used as internal normalization controls.

**Western blot analysis.** Total protein was extracted from CRC cells using radioimmunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology, Ltd.) containing protease inhibitors (Roche Diagnostics) according to the manufacturer's protocol. Total protein concentrations were measured using a bicinchoninic acid protein assay kit (Wuhan Boster Biological Technology, Ltd.). Equal amounts (20  $\mu$ g) of protein were separated with 10% SDS-PAGE and then transferred onto PVDF membranes (EMD Millipore). The membranes were blocked by 5% skimmed milk in TBST for 1 h at room temperature, followed by incubation at 4°C overnight with primary antibodies against SOX4 (ab80261; 1:500), P21 (ab227443; 1:1,000), P27 (ab215434; 1:1,000), cyclin D1 (ab226977; 1:1,000), matrix metalloproteinase (MMP)-2 (ab37150; 1:1,000), MMP-9 (ab38898; 1:1,000), E-cadherin (ab15148; 1:500), N-cadherin (ab18203; 1:500), vimentin (ab137321; 1:1,000), ataxia telangiectasia-mutated (ATM; ab17995; 1:5,000), P50 (ab131546; 1:1,000), NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ; ab97783; 1:2,000), p-I $\kappa$ B $\alpha$  (ab24784; 1:2,000), NF- $\kappa$ Bp65 (ab16502; 1:1,000), p-NF- $\kappa$ Bp65 (ab86299; 1:2,000) and GAPDH (ab9485; 1:2,000), all purchased from Abcam. Subsequently, the membranes were washed with Tris-buffered saline with Tween-20 and incubated with HRP-conjugated goat anti-rabbit immunoglobulin G secondary antibody (BA1054; Wuhan Boster Biological Technology, Ltd.) at a dilution of 1:5,000 for 1 h at room temperature. Enhanced chemiluminescence reagent (EMD Millipore) was used to detect the signals on the membranes using Bio-Rad ChemiDoc™ TMMP system

Table I. Clinical characteristics of 60 patients with colorectal cancer.

Variables	No.
Age, years	
<60	39
≥60	21
Sex	
Male	33
Female	27
Tumor stage	
I-II	29
III-IV	31
Lymph node metastasis	
Yes	35
No	25

(Bio-Rad Laboratories, Inc.). Image-ProPlus software v6.0 (Media Cybernetics, Inc.) was used to quantify bands, and GAPDH was used as a loading control.

**Immunofluorescence assay.** NCM460, SW620 and SW480 cells were plated in 12-well plates and incubated overnight for adherence. Briefly, the cells were fixed in 100% methanol for 5 min at room temperature and then permeabilized with 0.1% Triton X-100 and incubated in 5% BSA (Thermo Fisher Scientific, Inc.) to block non-specific protein-protein interactions. The cells were then incubated with primary rabbit anti-SOX4 antibody (ab80261; 1:500; Abcam) overnight at 4°C. The cells were washed twice with PBS/0.1% Tween-20 and incubated with a secondary antibody (green) of DyLight®488 goat anti-rabbit IgG-H&L (ab96899; 1:250) for 1 h at room temperature. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) staining solution. All images were captured using an Olympus FV-1000 confocal microscope (Olympus Corporation) at a magnification of x200, and adjusted for brightness using ImageJ software v6.0 (National Institutes of Health).

**Cell proliferation and colony formation assay.** To evaluate cell viability, NCM460, SW620 or SW480 cells ( $6 \times 10^3$ /well) were seeded in 96-well culture plates and incubated in an atmosphere containing 5% CO<sub>2</sub> at 37°C. After the cells had reached 70% confluence, they were transfected with miR-129 mimic or inhibitor and maintained for 24, 48 and 72 h. Subsequently, 10 µl MTT diluted in fresh medium (90 µl) was added to each well and incubated for an additional 4 h at 37°C. Subsequently, the culture medium was removed and 110 µl formazan was added into each well to dissolve the formed crystals with agitation for 10 min. The absorbance of each well was measured at 490 nm using a microplate reader (Thermo Fisher Scientific, Inc.). All experiments were performed in triplicate.

To determine colony formation ability, NCM460, SW620 or SW480 cells (1,000 cells/dish) were seeded in 3.5-cm cell culture dishes and incubated in complete DMEM at 37°C for 2 weeks. Subsequently, the colonies were fixed with 20%

methanol for 10 min, followed by staining with 0.1% crystal violet solution (Beyotime Institute of Biotechnology) for 5 min at room temperature. The number of formed colonies (>50 cells) was counted under an inverted microscope (Nikon Eclipse TE2000; Nikon Corporation) at a magnification of x40. Plating efficiency (PE) was calculated as follows: PE=(number of colonies formed/number of plated cells) x100%. Three independent experiments were performed.

**Wound healing assay.** To assess the tumor cell migration ability, a wound healing assay was performed. Briefly, cells were evenly plated in 6-well culture plates and allowed to reach 70% confluence. An artificial wound across the cell monolayer was made using a 200-µl sterile pipette tip. The cells were then washed with PBS thrice and incubated in serum-free DMEM. To visualize migrating cells and wound healing, images were captured at 0 and 48 h using an inverted microscope (Nikon Eclipse TE2000; Nikon Corporation). at a magnification of x200.

**Transwell migration and invasion assays.** Cell migration assay was performed using a 24-well Transwell chamber (Corning, Inc.) with 8.0-µm pore membranes. Briefly, NCM460, SW620 or SW480 cells ( $1 \times 10^5$ ) were suspended in a serum-free DMEM and then plated in the upper chamber (200 µl). The lower chamber was filled with 600 µl DMEM supplemented with 10% FBS. After being incubated for 48 h at 37°C under 5% CO<sub>2</sub>, non-migrated cells were wiped with a cotton swab, and the migrated cells on the lower surface were fixed with 70% ethanol for 20 min at room temperature and stained with 0.1% crystal violet solution for 15 min at room temperature. Finally, the migrated cells were photographed under an inverted microscope (Nikon Eclipse TE2000; Nikon Corporation) in five randomly selected fields at a magnification of x200 and counted using ImageJ software v6.0 (National Institutes of Health). The invasion assay was performed in a similar manner, except that the Transwell chambers were coated with Matrigel (100 µg/ml at 37°C; BD Biosciences).

**Cell cycle assay.** SW620 or SW480 cells were harvested at 48 h post-transfection by centrifugation at 800 x g at room temperature for 5 min. The collected cells were suspended in 1 ml PBS ( $1 \times 10^6$ /ml) and then centrifuged at 800 x g at room temperature for 2 min to remove the supernatant. Subsequently, the cells were fixed with 70% ethanol (500 µl) at 4°C overnight. Prior to staining, the cells were washed with PBS and then suspended in 100 µl RNase A solution (Beijing Solarbio Science & Technology Co., Ltd.) and bathed in 37°C for 30 min, then stained with propidium iodide (PI; 400 µl; Beijing Solarbio Science & Technology Co., Ltd.) for 5 min at 4°C in the dark. Alterations in DNA content were analyzed using a FACSCalibur™ flow cytometer (BD Biosciences) within 1 h and the data were analyzed using FlowJo 10.07 software (Tree Star, Inc.).

**Luciferase reporter assay.** Bioinformatics software, including TargetScan (<http://www.targetscan.org/>), mirDB (<http://mirdb.org/>) and DIANA TOOLS (<http://diana.imis.athena-innovation.gr>), was used to predict the putative target genes of miR-129 in SW620 or SW480 cells. The SOX4 3'-UTR encompassing



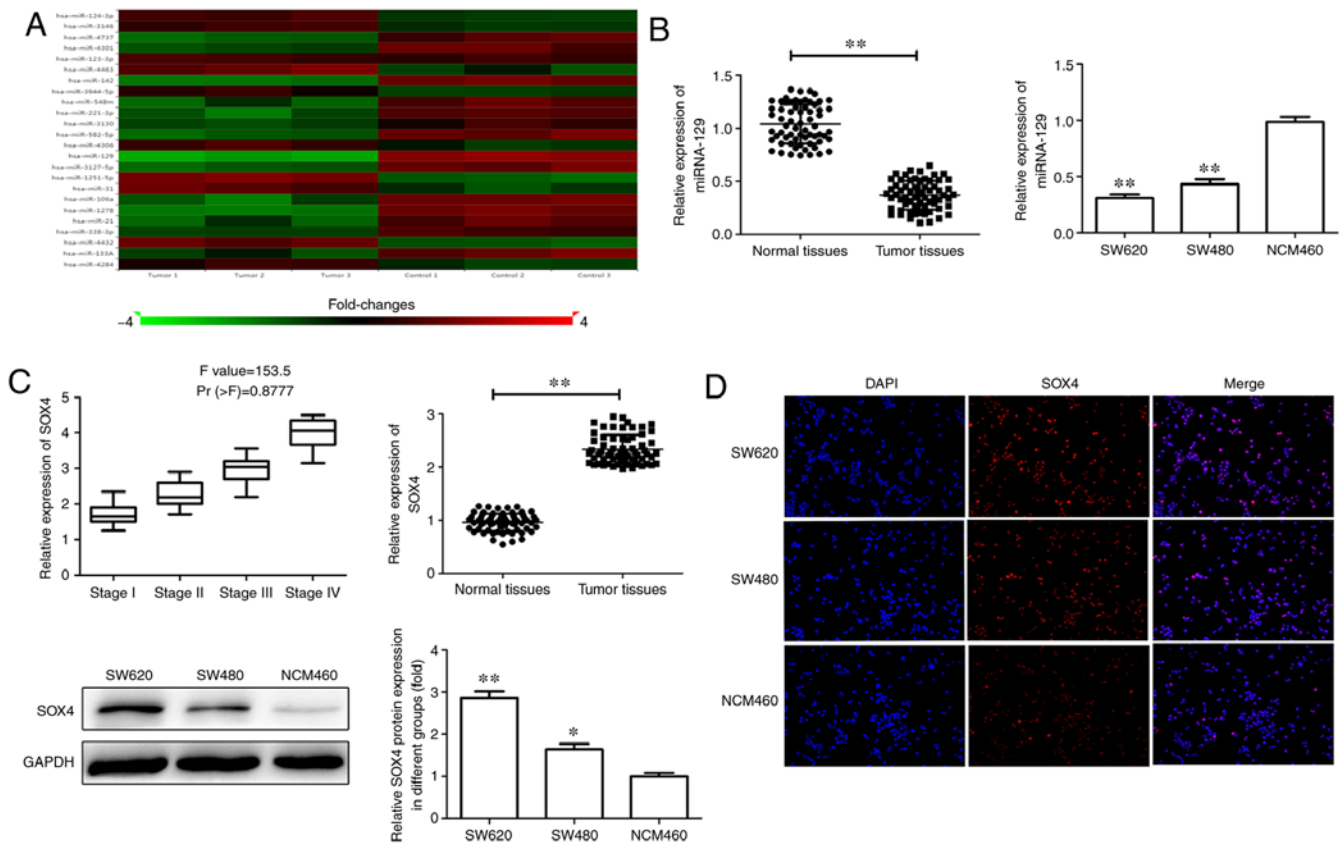


Figure 1. Expression of miR-129 and SOX4 in CRC tissues and cell lines. (A) Heatmap of CRC-related microRNA expression profiles. (B) RT-qPCR was applied to quantify the expression of miR-129 in CRC tissues and adjacent tissues (n=60).  $^{**}P<0.01$ . miR-129 and SOX4 expressions were determined using RT-qPCR in CRC cell lines and a normal colorectal cell line. (C) SOX4 was upregulated in CRC patients with advanced TNM stage; RT-qPCR was applied to quantify the expression of SOX4 in CRC tissues and adjacent tissues (n=60).  $^{**}P<0.01$ ; SOX4 protein expression was measured using western blotting in CRC cell lines and a normal colorectal cell line. (D) SOX4 protein expression was examined by immunofluorescence assay. Cell nuclei were labeled by DAPI and images were observed by fluorescence microscopy (magnification, x400). The experiment was repeated three times and results are presented as means  $\pm$  standard deviation.  $^{*}P<0.05$  and  $^{**}P<0.01$  vs. NCM460 cell line. SOX4, sex-determining region Y-related high-mobility group-box 4; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR.

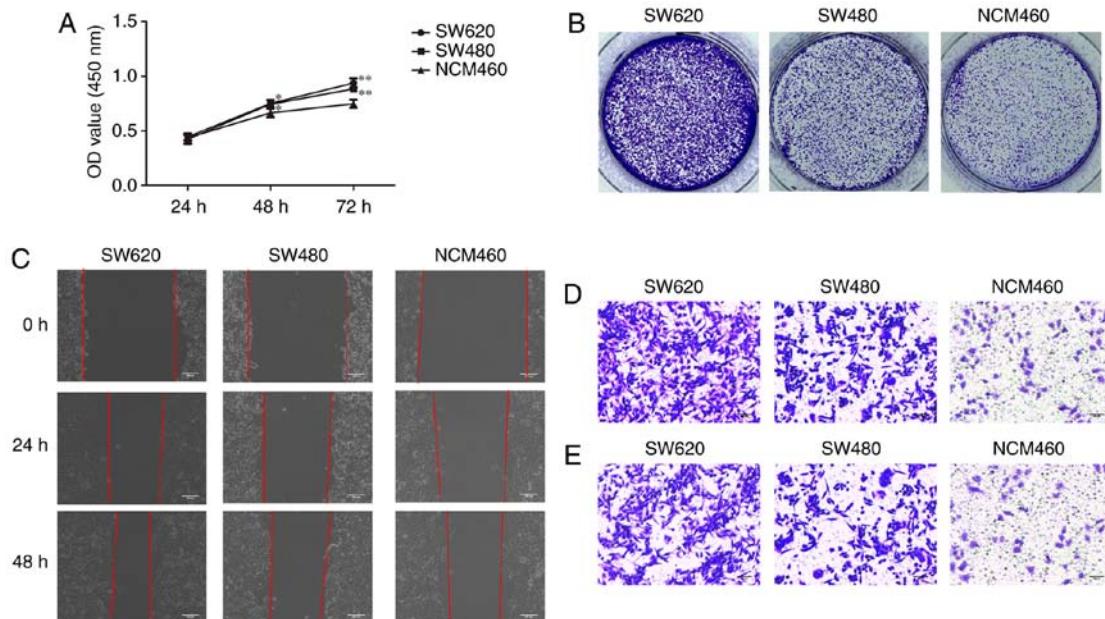


Figure 2. Cell proliferation, migration and invasion were increased in CRC cell lines. (A) Cell viability was evaluated by MTT assay in NCM460, SW620 and SW480 cells. (B) Cell proliferation was evaluated by colony formation assay in NCM460, SW620 and SW480 cells. (C) Cell migration was assessed using wound healing assay in NCM460, SW620 and SW480 cells. Scale bar, 200  $\mu$ m. (D and E) Cell migration and invasion were determined by Transwell assay in NCM460, SW620 and SW480 cells. The experiment was repeated three times and results are shown as means  $\pm$  standard deviation.  $^{*}P<0.05$  and  $^{**}P<0.01$  vs. NCM460 cell line. CRC, colorectal cancer.

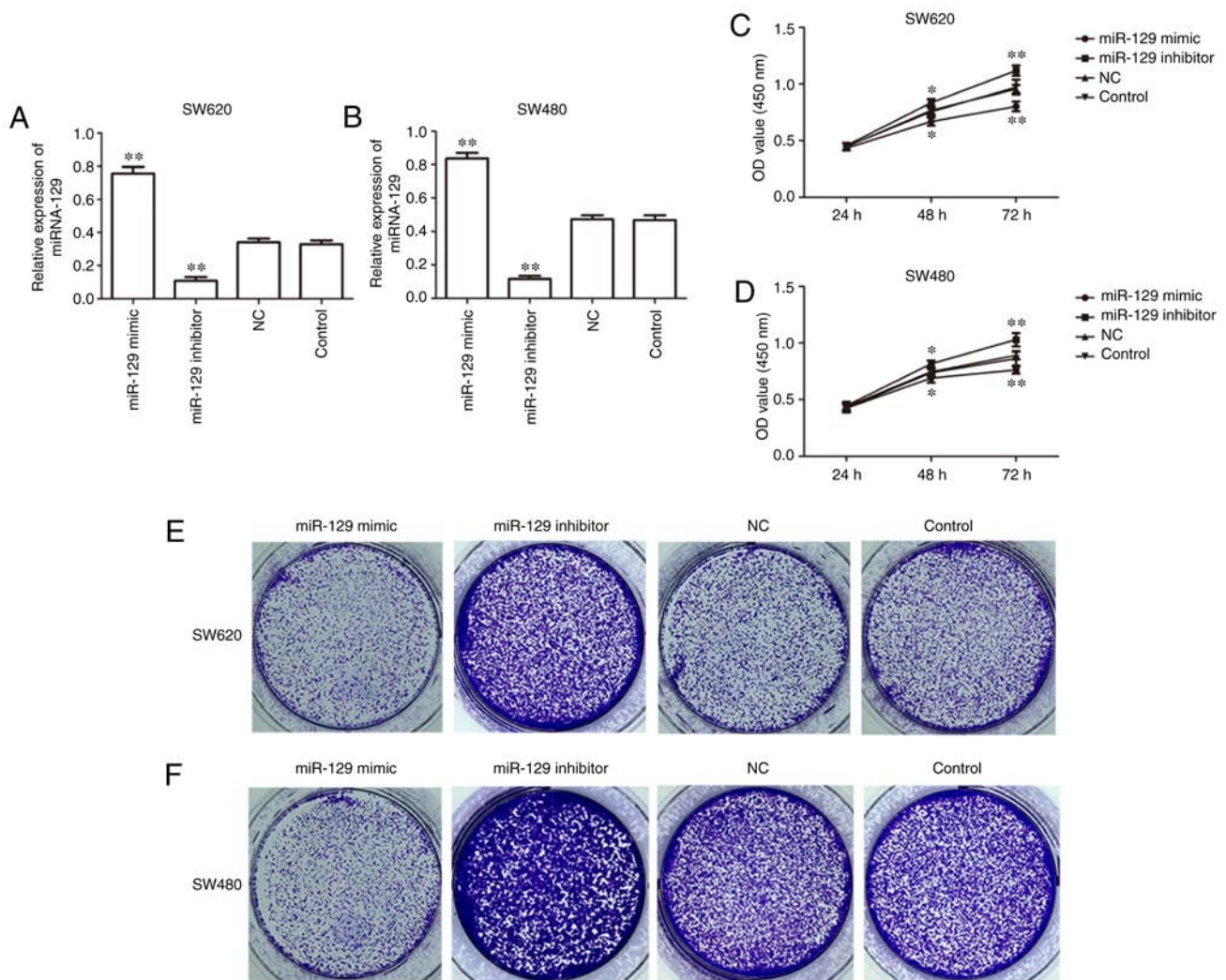


Figure 3. Effect of miR-129 overexpression or inhibition on cell proliferation *in vitro*. SW620 and SW480 cells were transfected with miR-129 mimic or inhibitor for 48 h. (A and B) Reverse transcription-quantitative PCR analysis was used to quantify the expression of miR-129. (C and D) Cell viability was evaluated by the MTT assay. (E and F) Cell proliferation was evaluated by the colony formation assay. The experiment was repeated three times and results are shown as means  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$  vs. NC group. NC, negative control.

the wild-type (WT) or mutant (Mut) fragments of the miR-129 binding site was amplified by PCR and then cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega Corporation) to form the reporter vector, namely SOX4 WT and SOX4 Mut, respectively.

For luciferase reporter assays, SW620 or SW480 cells were plated in 24-well plates ( $5 \times 10^4$  cells/well) and allowed to reach 50% confluence. Then, the cells were co-transfected with 2  $\mu$ g/ml of SOX4 WT or SOX4 Mut, together with 100 ng/ml of Renilla luciferase pRL-TK plasmid (Promega Corporation) and 50 pmol/ml of miR-129 mimic or corresponding NC using Lipofectamine<sup>TM</sup> 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The Dual-Luciferase Reporter Assay kit (Promega Corporation) was used to calculate the luciferase activity at 48 h post-transfection. Firefly luciferase activity was normalized to Renilla luciferase activity for each tested well.

**Microarray analysis.** Microarray analysis was conducted to detect the expression profiles of miRNAs in CRC using CRC tissue samples and matched tumor-adjacent tissue samples. miRNAs isolated from clinical specimens were labeled by Cy3 and

hybridized with Agilent Human miRNA Microarrays (Agilent Technologies, Inc.) following the manufacturer's instructions. Microarrays were scanned by an Agilent SureScan Microarray Scanner and then analyzed with GeneSpringGX software 11.0 (Agilent Technologies, Inc.). The differentially expressed miRNAs were screened with fold change  $> 2$  and  $P < 0.05$ .

**Statistical analysis.** Statistical analyses were performed using SPSS 20.0 software (IBM Corp.). Values are presented as mean  $\pm$  standard deviation (SD) from three separate experiments. Differences among multiple groups were compared by one-way ANOVA followed by Tukey's post hoc test, and differences between tumor specimens and matched tumor-adjacent tissue samples were compared by paired Student's t-test. \* $P < 0.05$  and \*\* $P < 0.01$  were considered to indicate statistically significant differences.

## Results

**miR-129 is downregulated and SOX4 is increased in CRC tissues and cell lines.** To identify miRNAs potentially

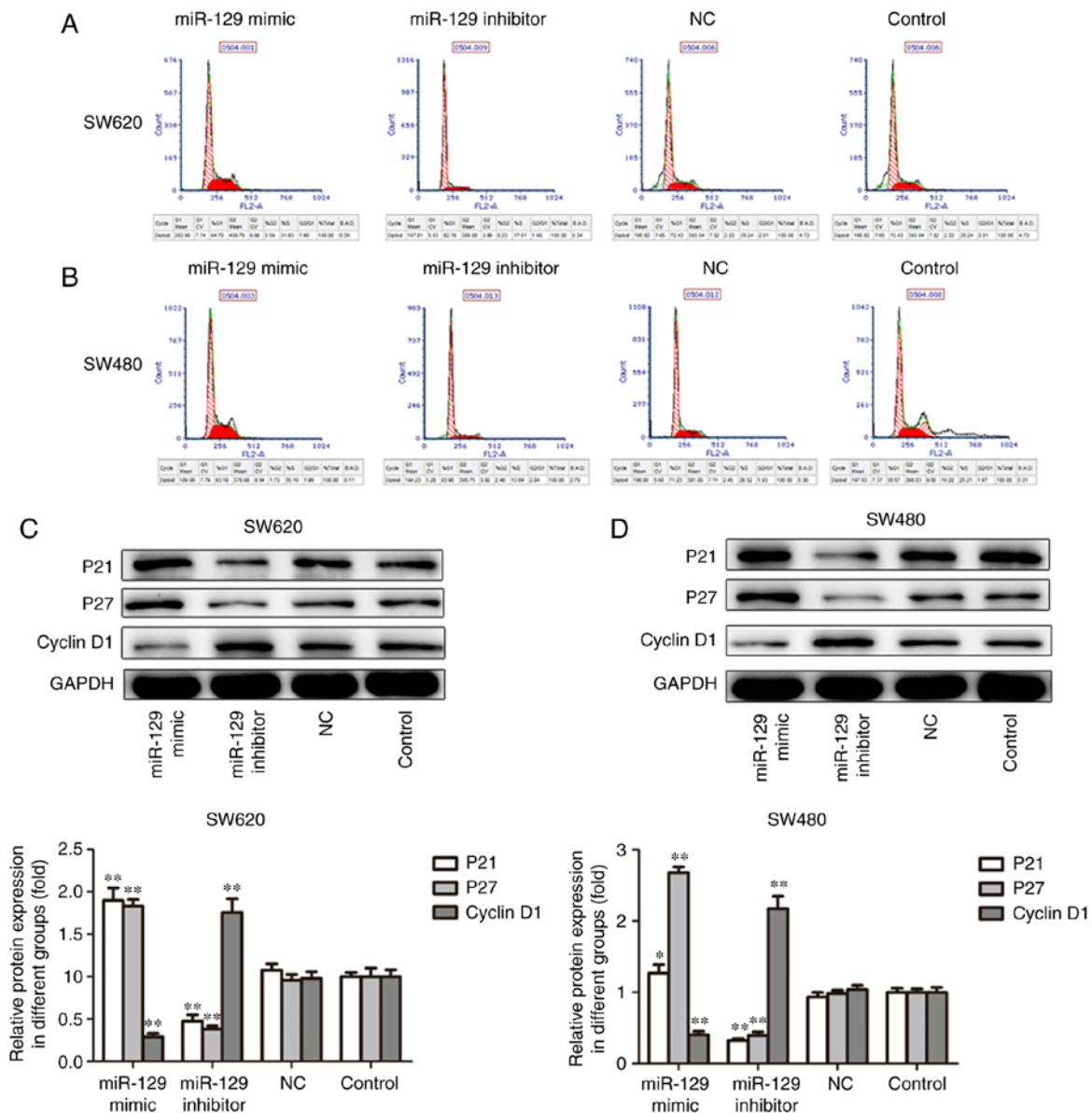


Figure 4. Effects of miR-129 on cell cycle *in vitro*. SW620 and SW480 cells were transfected with miR-129 mimic or inhibitor for 48 h. (A and B) Cell cycle distribution was examined by flow cytometric analysis. (C and D) The expression of cell cycle-related proteins was examined by western blotting. The experiment was repeated three times and results are shown as means  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$  vs. NC group. NC, negative control.

implicated in CRC, microarray expression profiles were analyzed. Three human CRC microarray datasets were selected and analyzed for miRNAs consistently aberrantly expressed between CRC and normal tissues. miR-129 was found to be significantly decreased in CRC. RT-qPCR, western blot or immunofluorescence assays were performed to evaluate the changes in the expression levels of miR-129 and SOX4 in CRC tissues and cell lines. As shown in Fig. 1A, miR-129 expression was significantly decreased in CRC tissues compared with that in adjacent tissues. Similarly, SW620 and SW480 cells also exhibited significantly lower expression of miR-129 compared with that in NCM460 cells (Fig. 1B). Furthermore, SOX4 expression was measured in CRC tissues and cell lines and was found to be significantly upregulated at both the mRNA and protein levels in CRC tissues and cell lines. Moreover, elevated SOX4 expression was positively correlated with advanced TNM stage (Fig. 1B-D). These data demonstrated

miR-129 expression was decreased whereas SOX4 expression was increased in CRC, suggesting that miR-129 and SOX4 may play a key role in CRC tumorigenesis and progression.

**Proliferation, migration and invasion of CRC cells.** To investigate the malignant behavior of CRC cells, the proliferation, migration and invasion of CRC cell lines and a normal colorectal cell line were examined. As shown in Fig. 2, the proliferation, migration and invasion capacity of SW620 and SW480 cells was significantly higher compared with that of NCM460 cells. In addition, compared with SW480 cells, SW620 cells exhibited a significantly higher proliferation, migration and invasion capacity, indicating that SW620 cells display a more malignant behavior.

**Cell proliferation is suppressed by miR-129 overexpression and promoted by miR-129 inhibition.** To investigate the



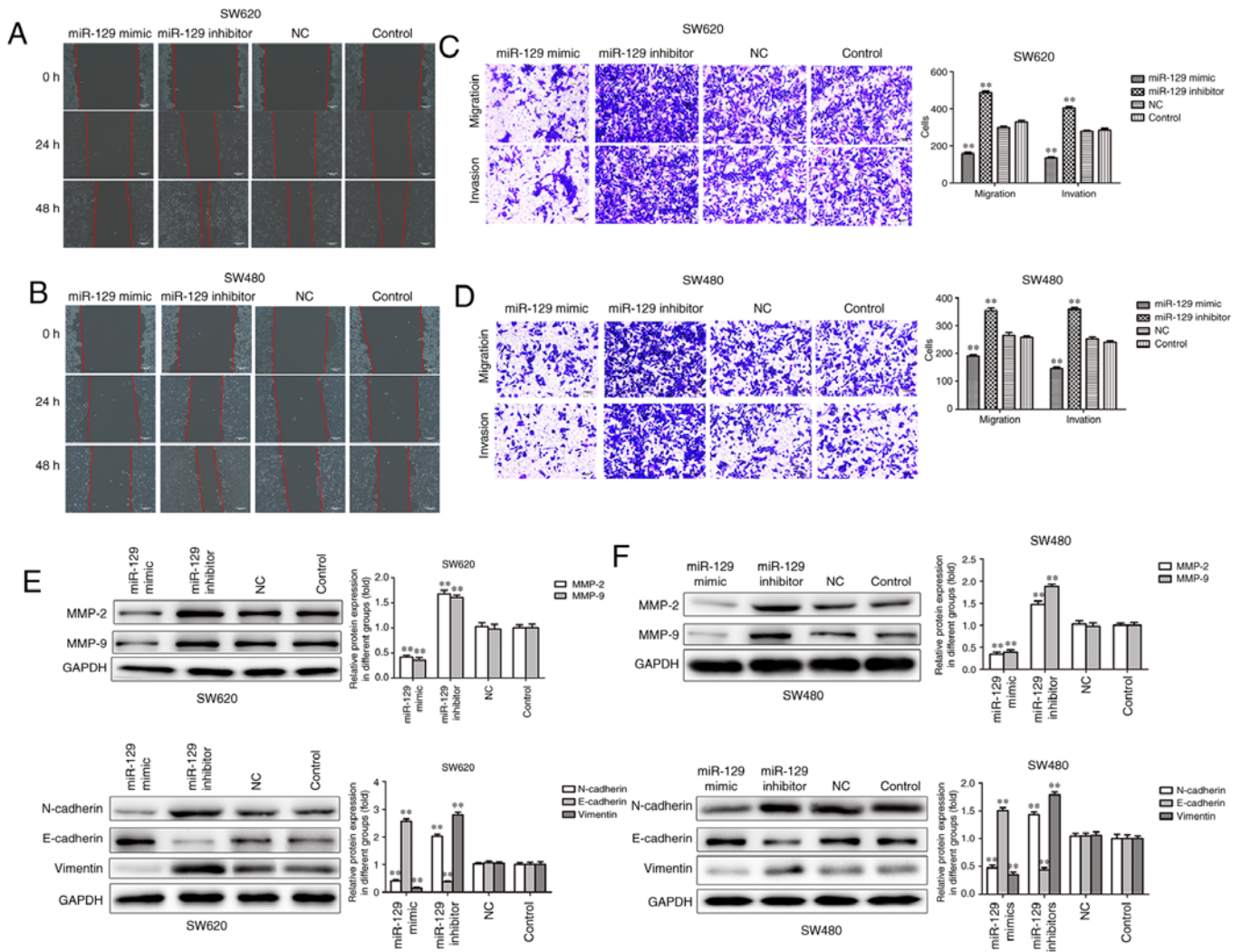


Figure 5. Effects of miR-129 on cell migration, invasion and EMT *in vitro*. SW620 and SW480 cells were transfected with miR-129 mimic or inhibitor for 48 h. (A and B) Cell migration was assessed using wound healing assay. Scale bar, 200  $\mu$ m. (C and D) Cell migration and invasion was determined by Transwell assay. (E and F) Migration and EMT-related proteins were examined by western blotting. The experiment was repeated three times and results are shown as means  $\pm$  standard deviation. \*\*P<0.01 vs. NC group. EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; NC, negative control.

effect of miR-129 on human CRC cells, gain-of-function and loss-of-function experiments were performed to verify the biological role of miR-129. miR-129 expression was shown to be markedly upregulated following transfection with miR-129 mimic and downregulated following transfection with miR-129 inhibitor in SW620 and SW480 cells (Fig. 3A and B). RT-qPCR analysis was performed to evaluate the transfection efficiency and the result indicated a high transfection efficiency in SW620 and SW480 cells. Furthermore, MTT assay revealed that miR-129 overexpression inhibited whereas miR-129 inhibition promoted the proliferation of SW620 and SW480 cells (Fig. 3C and D). In addition, colony formation assay demonstrated that miR-129 overexpression decreased the number of colonies formed, while miR-129 inhibition increased the colony number in SW620 and SW480 cells (Fig. 3E and F).

In order to further confirm the aforementioned results, the present study investigated the effects of miR-129 on cell cycle and associated proteins. As shown in Fig. 4A and B, miR-129 overexpression increased the percentage of cells in the S phase, whereas miR-129 inhibition increased the percentages of

SW620 and SW480 cells in the S phase. In addition, miR-129 overexpression decreased the expression of cyclin D1 and increased the expression of P21 and P27 proteins, while the inhibition of miR-129 exerted the opposite effects on the expression of cell cycle-related proteins in SW620 and SW480 cells. Taken together, these data suggest that miR-129 inhibits the CRC cell proliferation ability *in vitro*.

**Overexpression of miR-129 suppresses migration, invasion and EMT of CRC cells.** Sequentially, wound healing and Transwell assays were performed to elucidate the role of miR-129 in cell migration and invasion. The wound healing and Transwell assays revealed that the migration and invasion of SW620 and SW480 CRC cells were inhibited by miR-129 mimic and increased by miR-129 inhibitor (Fig. 5A-D). In addition, MMP-2 and MMP-9 protein levels were significantly decreased by miR-129 mimic and increased by miR-129 inhibitor (Fig. 5E and F).

EMT is considered to be an essential part of the process of tumor cell metastatic dissemination (28). Thus, it was herein

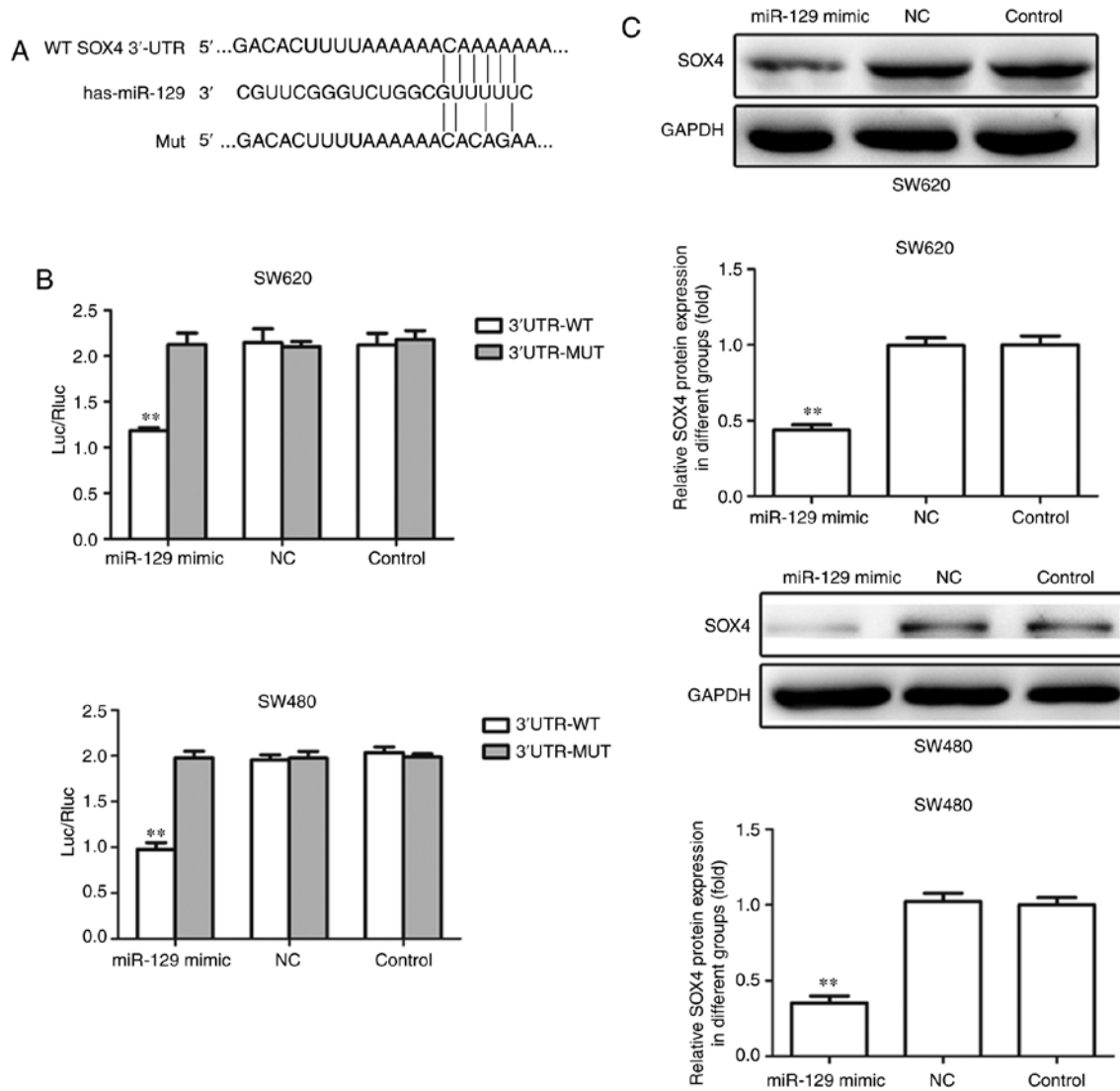


Figure 6. miR-129 targets SOX4 in colorectal cancer cell lines. (A) Predicted miR-129 binding sites on SOX4. (B) The relative Luciferase activities were determined using a Dual-Luciferase Reporter Assay Kit following co-transfection with SOX4-WT or SOX4-Mut and miR-129 mimic or mimic NC in SW620 and SW480 cells. (C) Western blotting was applied to quantify the expression level of SOX4 following transfection with miR-129 mimic or mimic NC in SW620 and SW480 cells. The experiment was repeated three times and results are shown as means  $\pm$  standard deviation. \*\* $P < 0.01$  vs. NC group. SOX4, sex-determining region Y-related high-mobility group-box 4; NC, negative control; WT, wild-type; Mut, mutant; UTR, untranslated region; NC, negative control.

investigated whether miR-129 is involved in EMT. As shown in Fig. 5E and F, miR-129 overexpression was able to significantly increase the expression of the epithelial marker E-cadherin and decrease the expression of the mesenchymal markers N-cadherin and vimentin, while the inhibition of miR-129 exerted the opposite effects on the expression of EMT-related proteins in SW620 and SW480 cells. Taken together, these results indicate that miR-129 may act as a tumor suppressor through inhibiting CRC cell migration, invasion and EMT.

*SOX4 is a direct target of miR-129 in CRC cell lines.* To further investigate the molecular mechanism underlying the regulatory role of miR-129 in the tumorigenesis and progression of CRC, bioinformatics databases were employed. As a result, the 3'-UTR of SOX4 was found to contain the complementary binding sequences for miR-129 (Fig. 6A). To investigate whether miR-129 directly binds to the 3'-UTR of SOX4 mRNA, a luciferase reporter vector containing this

region with miR-129 binding sites was constructed. As shown in Fig. 6B, luciferase reporter assays indicated that transfection of miR-129 mimic indeed suppressed the activity of SOX4 3'-UTR region, whereas the luciferase activity of the mutant type of SOX4 3'-UTR exhibited no obvious change. Furthermore, the protein expression level of SOX4 in SW620 and SW480 cells decreased significantly upon transfection of miR-129 mimic (Fig. 6C). These data suggest that miR-129 targets SOX4 and suppresses its expression in CRC cell lines.

*miR-129 overexpression may inhibit CRC cell proliferation, invasion and EMT by activating the NF- $\kappa$ B signaling pathway.* RT-qPCR and western blot assays were conducted to further investigate the potential signaling pathway associated with the regulatory effect of miR-129 on CRC cell proliferation, invasion and EMT. The results demonstrated that miR-129 overexpression resulted in an upregulation of ATM and P50 mRNA expression, while the inhibition of miR-129 resulted



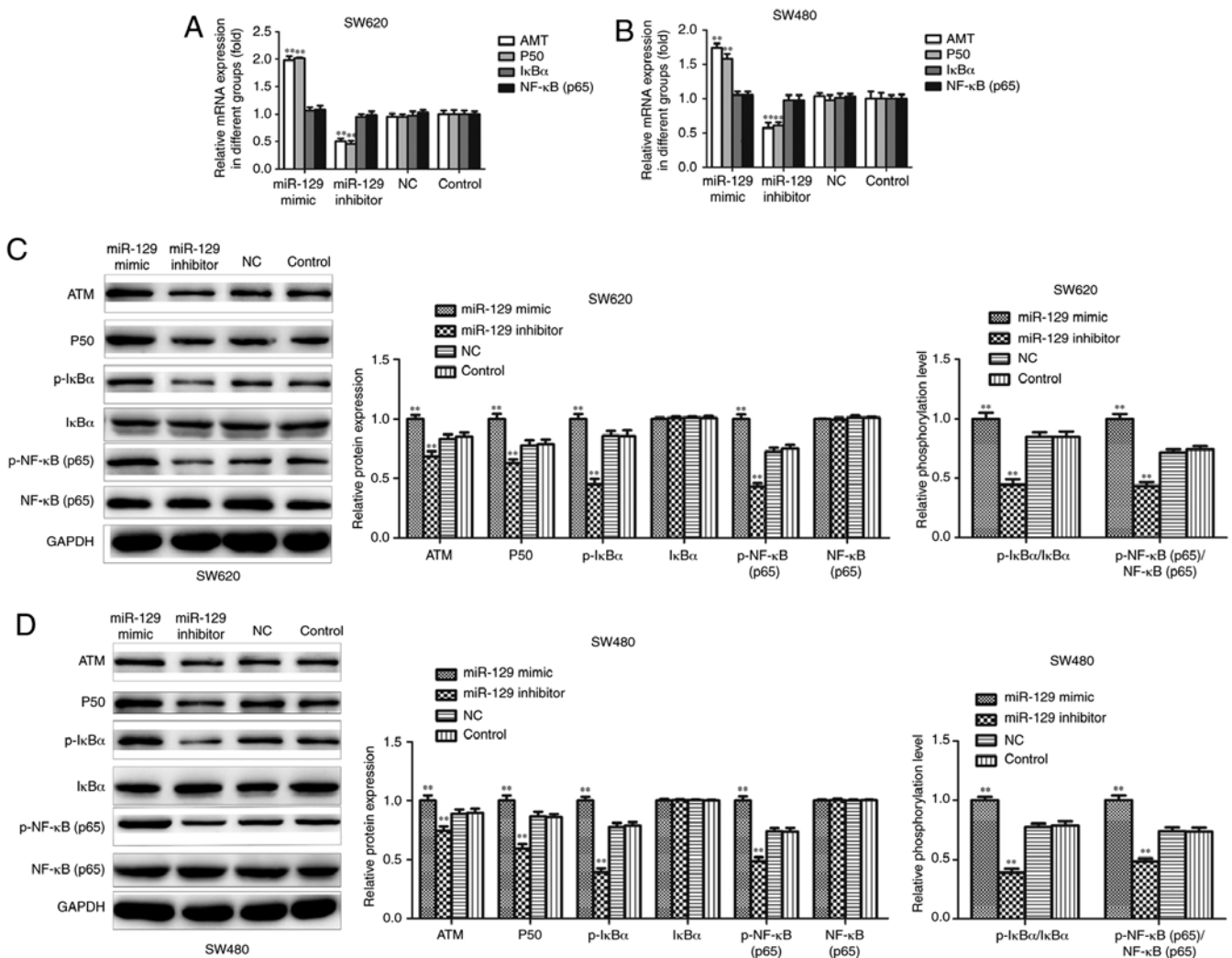


Figure 7. Effects of miR-129 on NF-κB signaling pathway in CRC cell lines. SW620 and SW480 cells were transfected with miR-129 mimic or inhibitor for 48 h. (A and B) Expressions of ATM, P50, IκBα and NF-κB mRNA were determined using reverse transcription-quantitative PCR assay. (C and D) Expressions of ATM, P50, p-IκBα, IκBα, p-NF-κB (p65) and NF-κB (p65) proteins were detected by western blotting assay. The experiment was repeated three times, results were shown as means ± standard deviation. \*P<0.01 vs. NC group. CRC, colorectal cancer; NF-κB, nuclear factor-κB; ATM, ataxia telangiectasia, mutated; IκBα, NF-κB inhibitor α; NC, negative control.

in a downregulation of ATM and P50 mRNA expression, while the expression of IκBα and NF-κB mRNA exhibited no obvious change in either group in SW620 and SW480 cells (Fig. 7A and B). Western blotting revealed that miR-129 mimic increased the expression of the ATM, P50, p-IκBα and p-NF-κBp65 proteins, while miR-129 inhibitor significantly decreased the expression of these proteins. Although the expression of IκBα and NF-κBp65 exhibited no obvious change in either group, the p-IκBα/IκBα and p-NF-κBp65/NF-κBp65 ratios were significantly increased following miR-129 overexpression, while miR-129 inhibition exerted the opposite effects in SW620 and SW480 cells (Fig. 7C and D).

## Discussion

CRC remains one of the most common malignant tumors of the gastrointestinal tract, despite significant advances in the diagnosis and treatment (29). Local recurrence and distant metastasis are the main causes of poor survival in patients with advanced CRC (30). Therefore, it is urgent to elucidate

the mechanisms underlying the occurrence and metastasis of CRC in order to design effective treatments. Several miRNAs were recently identified as important regulators of tumorigenesis, angiogenesis, invasion and metastasis (31). In addition, miRNAs have been considered as potential therapeutic targets and biomarkers for a number of human cancers, including CRC (32,33). The focus of the present study was the abnormal expression of miR-129 and the regulatory function of miR-129 in cell proliferation, invasion and EMT via the SOX4/NF-κB pathway.

Previous studies have demonstrated that miR-129 is abnormally regulated in various malignant tumors, including hepatocellular carcinoma (34), glioma (35), non-small cell lung cancer (36), and gastric cancer (15), and participates in the pathogenic processes. Recently, miR-129 was reported to be increased in CRC tissues and blood samples, and the high expression level of miR-129 contributes to cell proliferation and migration via targeting estrogen receptor beta (37). Inversely, Karaayvaz *et al* (16) reported that miR-129 was specifically decreased in CRC and functions as a tumor

suppressor to promote cell apoptosis and chemosensitivity. Thus, further investigation is required to identify the expression and mechanisms of action of miR-129 in CRC. In the present study, it was observed that miR-129 was downregulated in CRC clinical tissues and cell lines, and these results were consistent with those of other large-sample studies (38). Moreover, gain-of-function experiments demonstrated that miR-129 overexpression suppressed cell proliferation and induced cell cycle arrest at the S phase. Increased metastatic ability is a hallmark of human cancers (31). The present study revealed that the overexpression of miR-129 suppressed cell migration and invasion, while miR-129 knockdown increased the metastatic ability of CRC cells. EMT is closely associated with the occurrence of tumor metastasis, which is accompanied by the loss of membrane E-cadherin expression and increased levels of mesenchymal markers, such as vimentin (39). The present study demonstrated that the overexpression of miR-129 reduced N-cadherin and vimentin but increased E-cadherin expression in CRC cells, while miR-129 knockdown exerted the opposite effects. Taken together, these data demonstrated that miR-129 inhibited the progression of CRC by suppressing the proliferation, migration, invasion and EMT of CRC cells.

It is well known that miRNAs regulate cell biological behavior via directly regulating target genes. A number of target genes of miR-129 have been identified (33,34,39,40). SOX4, a critical oncogenic protein, has been shown to be involved in the progression and metastasis of various malignant tumors (41,42). Previous studies have indicated that the overexpression of SOX4 in CRC is regulated via miRNA-mediated post-transcriptional mechanisms that prevent cell proliferation, migration and invasion (43,44). However, the association between miR-129 and SOX4 in CRC remains unclear. In the present study, it was demonstrated that SOX4 is a direct target of miR-129 in CRC cells, which was supported by data from the luciferase and western blot assays. These data indicated that miR-129 may regulate cell migration, invasion and EMT through targeting SOX4 in CRC.

The NF- $\kappa$ B pathway, a critical regulator of cell apoptosis, mediates important biological processes, including tumorigenesis (45). Lan *et al* (46) reported that miR-15a/16 enhanced radiation sensitivity by targeting the TLR1/NF- $\kappa$ B signaling pathway in non-small cell lung cancer. Ren *et al* (47) demonstrated that miR-210-3p promoted prostate cancer cell EMT and bone metastasis by activating the NF- $\kappa$ B signaling pathway. The results of the present study revealed that miR-129 overexpression promoted the expression of NF- $\kappa$ B pathway-related proteins, while the inhibition of miR-129 exerted the opposite effect. These results indicated that miR-129 inhibited the malignant phenotype of CRC, possibly by activating the NF- $\kappa$ B signaling pathway. Thus, it may be inferred that SOX4 is involved in the activation of the NF- $\kappa$ B pathway through miR-129, which will be investigated further in future studies.

In summary, the present study demonstrated that miR-129 expression was downregulated while SOX2 expression was upregulated in CRC. The findings further revealed that miR-129 may function as a tumor suppressor by inhibiting cell proliferation, migration, invasion and

EMT in CRC cells via targeting SOX4, and the potential mechanism may involve activation of the NF- $\kappa$ B signaling pathway. These data may provide novel insight into the molecular mechanism underlying CRC progression, and the upregulation of miR-129 may be a promising strategy for the treatment of CRC.

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### Availability of data and materials

The datasets used or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

LW designed the experiments. ZC and TZ were the major contributors to the writing of the manuscript. JZ, YT and BL performed the experiments. All authors have read and approved the final version of the manuscript.

### Ethics approval and consent to participate

The protocol of the present study was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University. Informed consent was obtained from all the patients.

### Patient consent for publication

Not applicable.

### Competing interests

All the authors declare that they have no competing interests.

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