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Long noncoding RNA ROR promotes proliferation and invasion of colorectal cancer by inhibiting tumor suppressor gene NF2 through interacting with miR-223-3p

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Abstract. – OBJECTIVE: Colorectal cancer is one of the most common cancers in the world. LncRNA ROR, is a tumor oncogene associated with various human cancers. However, the role of ROR in colorectal cancer cells still remains unknown. The aim of this study was to measure the expression level of ROR and clarify its biological functions in colorectal cancer cells.

MATERIALS AND METHODS: The expression level of ROR in colorectal cancer cells was detected using qRT-PCR. We performed CCK8 assay, colony formation assay, cell migration and invasion assays to evaluate the effects of ROR on cell proliferation, migration and invasion of colorectal cancer cells. Then, transfection of ROR, ROR inhibitor, miRNA-223-3p-mimics and miRNA-223-3p-inhibitor, qRT-PCR, and luciferase reporter assay were used to explore the molecular mechanisms.

RESULTS: In the present study, Lnc-ROR was highly expressed in colorectal cancers compared with adjacent non-cancerous normal tissues. And the expression level of ROR was also increased in colorectal cancer cells ($p < 0.05$). CCK8 assay and invasion assay suggested that ROR can promote cell proliferation and invasion. The luciferase reporter assay showed ROR acted as sponge and directly competed with miRNA-223-3p, then decreasing the expression of tumor suppressor gene NF2.

CONCLUSIONS: The findings of this study first revealed that ROR was upregulated in colorectal cancer cells and can promote cell proliferation and invasion by inhibiting tumor suppressor gene NF2 through interacting with miR-223-3p.

Key Words:
Long noncoding RNA ROR, MiR-223-3p, Colorectal cancer.

Introduction

Colorectal cancer is one of the most common cancers in the world and has been one of the leading causes of cancer mortality for both men and women[1]. It kills about 700,000 people every year, making it the world's fourth most deadly cancer after lung, liver and stomach cancer[2,3]. In recent years, the incidence rate of the disease has significantly decreased with the development of improvement of diagnostic and therapeutic options, but the five-year survival rate for colorectal cancer cells is still unsatisfactory[4,5]. So, it is important to find the applications and potential targets for the treatment of colorectal cancer cell.

A lot of researches that go into the mechanism of colorectal cancer cells proliferation and invasion are mainly about protein-coding genes[6-8]. However, more and more studies revealed that non-coding RNAs also play a vital role in the physiology function of colorectal cancer cells, especially in cell proliferation, invasion and migration[9-11]. Long non-coding RNAs (LncRNAs), a group of non-protein-coding RNAs which length is greater than 200 nucleotides[12], have emerged as one of the largest and significantly diverse RNA families[13]. Wang et al[14] showed that LncRNAs can take part in cellular process in colorectal cancer cells. However, most lncRNAs have not been functionally characterized, and it is still unclear how LncRNAs work to modulate cell function. Increasing evidence have demonstrated that lncRNAs regulate gene expression at epigenetic[15], transcriptional and post-transcriptional level

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with the participation of DNA, microRNAs, mRNAs, and proteins [16-18]. Similarly, microRNAs (miRNAs) are a class of small, non-coding RNA molecules[19]. These RNA molecules are around 20-22 nucleotides in length and have been showed to be strongly associated with pathogenesis of several human diseases, including cancer[20]. The use of microRNAs showed significant promise in the diagnosis and prognosis of colorectal cancer, owing to their unique expression profile associations with cancer types and malignancies[21,22]. The recently proposed competing endogenous RNA (ceRNA) regulatory network also indicates that lncRNAs may work as sponges by competitively binding to target microRNAs (miRNAs) and finally inhibiting their functions by blocking interactions with target mRNAs[23].

LncRNA ROR, is a tumor oncogene dysregulated in various human cancers such as breast cancer, prostate cancer, gastric cancer, pancreatic cancer and lung cancer[24,25]. It has been reported that ROR plays a vital role in various cellular processes, such as proliferation, apoptosis and invasion through targeting multiple genes[26]. Merlin, also known as neurofibromatosis type 2 (NF2), is a major tumor suppressor gene in colorectal cancer cells[27-29]. Our previous research showed that ROR was also highly expressed in colorectal cancer cells. However, the molecular biological function cancer has not been clearly elucidated. In the current research, we assessed the biological functions of ROR in colorectal cancer cells, and further explored the molecular mechanism. In this study, our results revealed that ROR can sponge miR-223-3p in colorectal cancer cells and promote cell proliferation and invasion via inhibiting NF2.

Materials and Methods

Patients and Cell culture

The human colorectal carcinoma tissues and adjacent normal tissues (>5 cm from the margin of the tumor) were obtained from patients by surgical resection in our Hospital. Informed consent was obtained from all individual participants included in the present study. All tissue specimens in this study were approved by The Medical Ethics Committee of our Hospital. The tissue samples were stored at -80°C and collected for further use. The human colorectal cancer cell line HTC116 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin and 2.0 g/L sodium bicarbonate. The SW620 human colorectal cancer cell line was purchased from ATCC and cultured in RPMI-1640 medium. All the cell lines were incubated at 37°C in an atmosphere of 5% CO_2.

Real-time Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)

The cells were planted in 24-well plates and treated according to the experimental design. To extract RNA, cells were washed with PBS. Then the total RNA was isolated using TRIzol reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. And the reverse transcription was performed using SuperScript II (Invitrogen-Life Technologies, Carlsbad, CA, USA). qRT-PCR was performed with SYBR Prime Script RT-PCR Kits (TaKaRa, Otsu, Shiga, Japan) based on the manufacturer's instructions. For analysis, the expression level of mRNA was normalized against GAPDH mRNA measured per sample. Specific primers were shown in Table I.

CCK8 Assay

We use the CCK-8 kit to evaluate cell proliferation activity according to the experimental procedure instruction. In short, about 1×10^3 cells were seeded in 96-well plates uniformly. Then the cells were transfected with Lnc-ROR, si-ROR and negative control. The cell proliferation activity was measured at 0 h, 24 h, 48 h and 72 h after transfection. Before detection, the medium was removed, and cells were washed with PBS to remove dead cells. Next, the CCK8 dilution was added to 96-well plates (100 μL per well) and incubated at 37°C for about 1.5 hours. After incubation, the plate was taken out, and the absorption value of 450 nm was measured.

Colony Formation Assay

500 cells were seeded in 6-well plates and incubated with RPMI 1640 containing 10% FBS at 37°C. After incubating for 12 days, the plates were taken out and the cells were washed with PBS. Then the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of visible colonies was counted manually.

Cell Migration and Invasion Assays

Cell invasion was measured by Transwell chamber (8 um pore size, Corning, Costar, NY, USA). 48 h after transfection, 5×10^3 cells were cultured with serum-free media and seeded into the upper chamber. And the complete media containing 20% FBS was added into the lower chamber and incubated at 37°C for about 48 hours. After incubation, the cells were wiped off remaining in upper membrane, while cells that invaded were fixed and stained with 0.1% crystal violet. The number of invaded cells was counted under a microscope. As to cell migration, wound healing assay was performed. 1×10^5 cells were seeded in 6-well plates and cultured in 37°C incubation. When cells were cultured to 100% confluence, the cells were scrapped with a pipette tip (tips range in volume from 1 to 200 μL) in a cross pattern in the center of each well, washed with PBS, and immediately given fresh low-serum medium. After incubation for 48 hours, the migration distances of the cells were observed and photographed under a microscope.

Construction of Lentivirus and Cell Transfection

The full-length of human ROR cDNA was synthesized by Shanghai GeneChem Co., Ltd (Shanghai, China) and subcloned into a lentiviral vector (Lnc-RORR). A GFP-lentiviral vector (Lnc-NC) was used as a negative control. Transfections were performed using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's instructions. And for miRNA analysis, miR-223-3p, the miR-223-3p mimics, miR-223-3p inhibitor and the corresponding negative control were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). For the lentiviral transduction, HTC116 and SW620 cells were subcultured at $1x10^5$ cells/well in 6-well culture plates and then transduced with miRNA-223-3p mimics, miRNA-223-3p inhibitor and miRNA-NC lentivirus at a multiplicity of infection (MOI) of 50. The cells were collected 72 h after infection, and the transduction efficiency was evaluated by counting the percentage of GFP-positive cells.

Luciferase Assay

At 48 h post-transfection, luciferase activity was determined using the Dual-Luciferase® Reporter Assay system (Promega Corporation, Madison, WI, USA) and the GloMax 20/20 luminometer (Promega Corporation) following the manufacturer's instructions. Data are expressed as mean values of normalized firefly relative luciferase units (RLUs) per setup.

Statistical Analysis

Except for other indicated, the results obtained from this experience were presented as means ±standard deviation (SD). The statistical analysis was performed using unpaired two-tailed Student's *t*-test to detect differences between the two groups. Analysis of variance with post-hoc Tukey's honestly significant difference (HSD) was used to test significant differences between multiple setups. In all tests, $p < 0.05$ and $p < 0.01$ were defined as significance value.

Results

Lnc-ROR is Upregulated in Human Colorectal Cancer Tissues

Firstly, we performed RNA-sequencing on 15 human colorectal carcinoma and adjacent non-cancerous normal tissues. Heatmap of differentiated expressed LncRNA showed that Lnc-ROR was highly expressed in colorectal cancers compared with normal tissue (Figure 1A). To further confirm the expression level in colorectal cancers, we detected ROR expression in 12 pairs of colorectal cancer tissues and pair-matched adjacent non-cancerous normal tissues by qRT-PCR. The results revealed that ROR was highly expressed in cancerous tissues ($p < 0.01$) compared with normal tissues (Figure 1B).

Expression Levels of ROR and NF2 in Colorectal Cancers Cells

To further understand the biological functionality of ROR in colorectal, we detected ROR expression in human colorectal cancer cells HTC116 and SW620 using qRT-PCR. The results showed that ROR expression was significantly upregulated in colorectal cancer cells compared with human epithelia cells HEK293 ($p < 0.05$) (Figure 2A). We then performed qRT-PCR to evaluate the expression of NF2 in colorectal cancer cells. The results revealed that expression levels of NF2 was down-regulated in both cancer cells ($p < 0.05$) (Figure 2B). Taken together, ROR is significantly upregulated in colorectal cancer cells.

ROR Expression is Associated with Cell Proliferation and invasion.

In order to further evaluate the role of ROR in colorectal cancer cells, we constructed ROR over-expressed plasmid and transfected into HTC116 and SW620 cells. In addition, si-ROR was also synthesized and transfected into both cells. First the

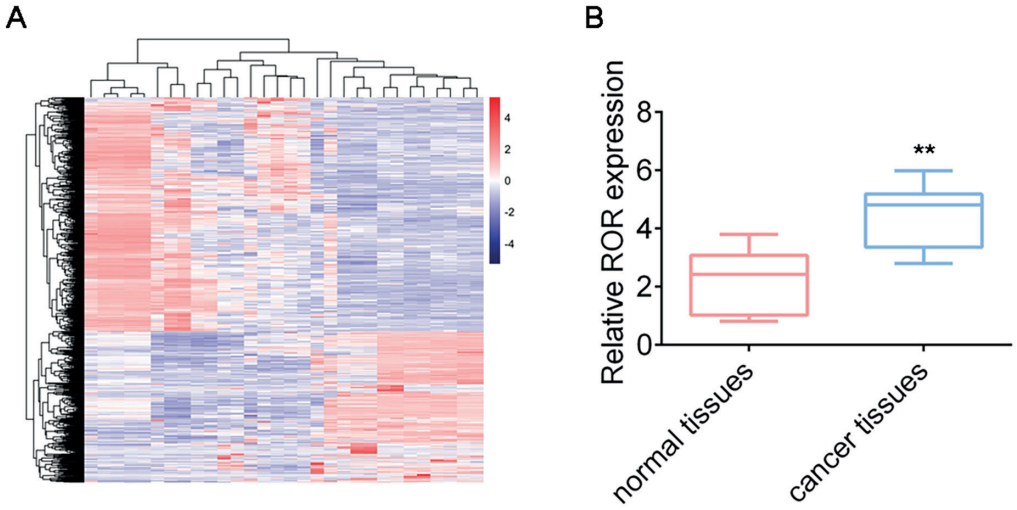

Figure 1. Lnc-ROR is upregulated in human colorectal cancer tissues. **(A)** Heatmap of differentiated expressed LncRNA in 15 human colorectal carcinoma and adjacent non-cancerous normal tissues. **(B)** ROR expression levels between colorectal cancer tissues and pair-matched adjacent non-cancerous normal tissues. ROR expression levels were detected with qRT-PCR. Statistical differences between samples were analyzed with paired samples *t*-test (n = 12, $p < 0.01$).

expression of ROR was verified by qRT-PCR. The results showed that the expression of ROR in the Lnc-ROR expression plasmid-transfected group was significantly increased compared with the vector1 group in both cell lines ($p < 0.01$) and the relative expression levels of ROR was reduced in the si-ROR group compared with the negative control vector2 group ($p < 0.01$) (Figure 3A). To verify the role of ROR on cell proliferation, CCK8 assay was performed on HTC116 cells and SW620 cells. The results showed that ROR overexpression significantly promote cell proliferation ability, compared to the control. And cell proliferation ability was decreased when transfected with si-ROR compared with the negative control ($p < 0.01$) (Figure 3B, 3C). Subsequently, a colony formation assay was performed on HTC116 and SW620 cells at 12 days after transfection. It was demonstrated that the colony number of HTC116 and SW620 cells was decreased upon transfection with the si-ROR expression plasmid (Figure 3D, 3E), while Lnc-ROR significantly elevated the colony forming ability of HTC116 and SW620 cells (Figure 3F, 3G).

Furthermore, to investigate the role of ROR on cell migration and invasion, we used wounding healing and transwell assays. The migration

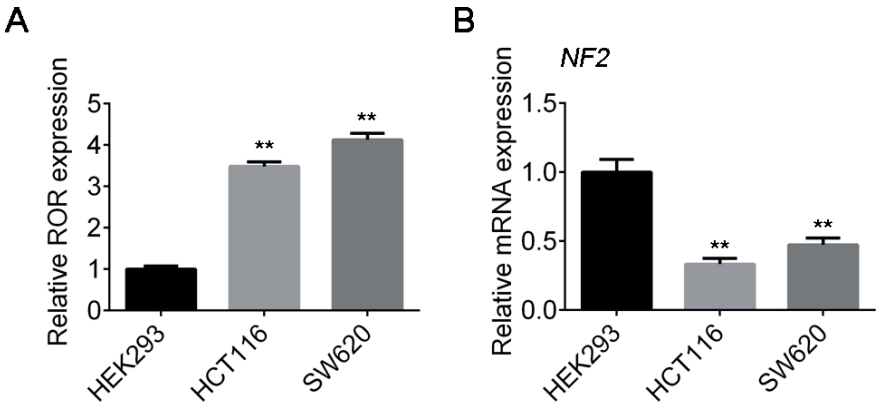

Figure 2. Expression levels of ROR and NF2 in colorectal cancers cells. **(A)** Relative ROR expression in human epithelia cells HEK293 and colorectal cancer cells HCT116 and SW620. **(B)** Relative mRNA expression levels of NF2 in HEK293, HCT116 and SW620 cells. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

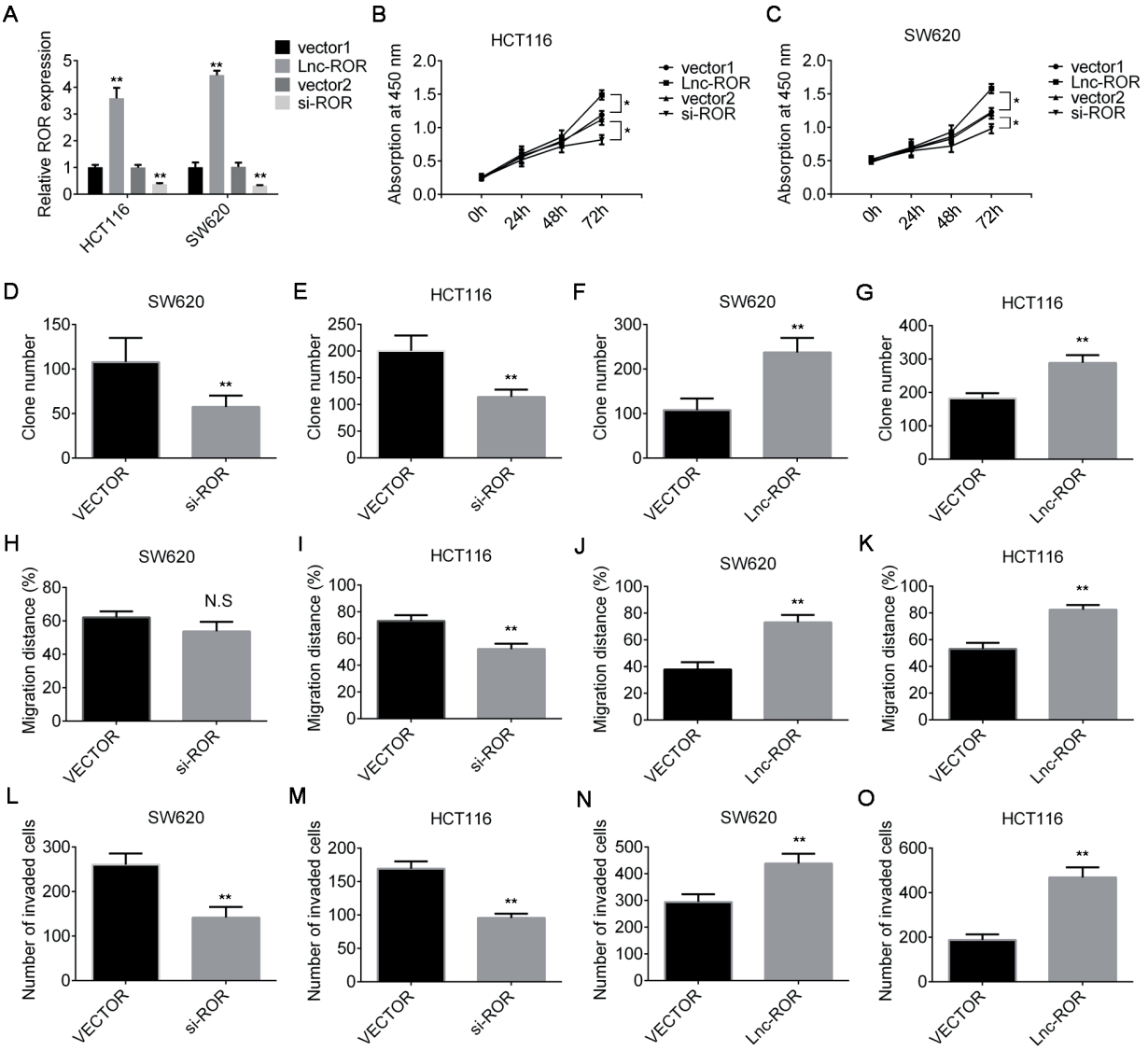

Figure 3. ROR expression is associated with cell proliferation and invasion. **(A)** ROR expression plasmid was synthesized and transfected into HCT116 and SW620 cells. ROR specific siRNA was constructed and transfected in HCT116 and SW620 cells. Relative expression of ROR in both cell lines was determined by qRT-PCR. **(B)** CCK8 assay was used to determine cell proliferation activity of HTC116 cells after transfection. **(C)** CCK8 assay was used to determine cell proliferation activity of SW620 cells after transfection. **(D-E)** Colony formation ability was detected in HCT116 and SW620 cells at 14 days after transfection with si-ROR. **(F-G)** Clone number was detected to evaluate colony formation ability of both cells transfected with ROR-overexpressed plasmid. **(H-I)** Wound healing assay was performed to evaluate cell migration. Migration distance was measured of HCT116 and SW620 cells transfected with si-ROR. **(J-K)** Migration distance of both cells transfected with ROR-overexpressed plasmid. **(L-M)** The invasion of cells transfected with si-ROR was determined by transwell invasion assays. The cell invasion number was measured. **(N-O)** The invasion of cells transfected with ROR-overexpressed plasmid was determined by transwell invasion assays. The cell invasion number was measured. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

distance showed no significant change in SW620 cells transfected with si-ROR, compared with negative control (Figure 3H). While in HTC116 cells, the migration ability was significantly weakened in si-ROR group (Figure 3I). And overexpressing of ROR enhanced the cell migration capacity in HTC116 and SW620 cells (Figure 3J, 3K). Afterward, the transwell assay revealed the same result. It was showed that the invasion capability of both si-ROR HTC116 and SW620 cells significantly reduced (Figure 3L, 3M), whereas overexpression of ROR significantly promoted cell invasion (Fig-

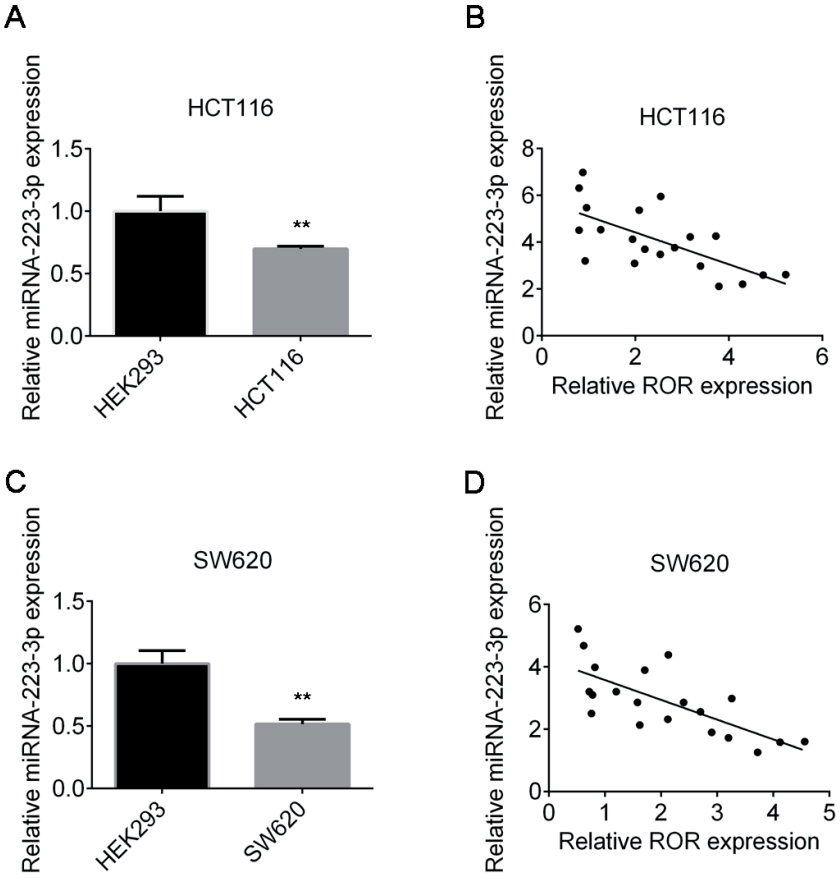

Figure 4. miRNA-223-3p was downregulated in colorectal cancer cells and negatively correlated with ROR. **(A)** Relative miRNA-223-3p expression in HTC116 cells compared with control HEK293 cells. **(B)** Correlation analysis was performed to determine the relationship between ROR and miRNA-223-3p. **(C)** Relative miRNA-223-3p expression of SW620 cells detected by qRT-PCR. **(D)** Correlation analysis between ROR and miRNA-223-3p in SW620 cells. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

ure 3N, 3O). Together, these results demonstrated that ROR can promote cell proliferation and invasion, indicating that ROR may serve as a key regulator of colorectal cancer cells.

MiRNA-223-3p Was Downregulated in Colorectal Cancer Cells and Negatively Correlated with ROR

LncRNAs could act as miRNA sponges and regulate miRNAs available for binding their target mRNAs. To further explore whether ROR correlated with miRNA, we used TargetScan and starBase to predict the relative target miRNA of ROR. And the bioinformatics prediction suggested that miRNA-223-3p can bind with ROR. Then we performed qRT-PCR to evaluate miRNA-223-3p expression in colorectal cancer cells. The results revealed that the expression of miRNA-223-3p was decreased in both HTC116 and SW620 cells, compared with HEK293 cells (Figure 4A, 4C). Then we further detected the relationship between ROR and miRNA-223-3p by using correlation analysis. The results showed that miRNA-223-3p was negatively correlated with ROR in both cells (Figure 4B, 4D), suggesting that miRNA-223-3p may be regulated by ROR.

ROR Functions as miR-223-3p Sponge in Colorectal Cancer Cells

As previous results shown, miRNA-223-3p was negatively correlated with ROR. To further confirm whether ROR acted as endogenous sponge RNA to interact with miR-223-3p, we conducted luciferase reports (Figure 5A). We synthesized luciferase reporters containing ROR-WT and ROR-MUT of miRNA-223-3p binding

sites and transfected with miRNA-223-3p into HTC116 and SW620 cells. Relative luciferase activity was detected by luciferase reporter assay. The miR-223-3p mimic obviously down-regulated the luciferase activities of the ROR WT but not the ROR MUT, compared with the control ($p < 0.01$) (Figure 5B, 5C). These results demonstrated that miRNA-223-3p could directly bind to ROR. Moreover, to further elucidate whether or not miR-223-3p was regulated by ROR, expression levels of miR-223-3p in HTC116 and SW620 cells that were transfected with si-NC, si-ROR or ROR over-expressed plasmid, were detected. The results showed that miRNA-223-3p expression was significantly elevated in cells transfected with si-ROR, compared with cells transfected with si-NC ($p < 0.01$) (Figure 5D, 5E). And Lnc-ROR group showed lower expression of miRNA-223-3p in both HTC116 and SW620 cells, compared with negative control group ($p < 0.01$) (Figure 5F, 5G). In addition, we also tested ROR expression of cells transfected with miR-223-3p NC, miR-223-3p mimics or miR-223-3p inhibitors. The results suggested that miRNA-223-3p mimic inhibited ROR expression and miRNA-1897 inhibitor enhanced MIRG expression in HTC116 and SW620 cells (Figure 5H-5K). Taken together, these results showed that ROR directly bound to miRNA-223-3p at the specific sites and may function as miR-223-3p sponge in colorectal cells.

ROR Promotes Cell Proliferation and Invasion via Inhibiting NF2

To further evaluate the mechanism that ROR regulates cell proliferation and invasion, we detected the NF2 expression in colorectal cells transfected with miRNA-NC and miRNA-223-3p mimics. The results showed that the expression level of NF2 was significantly increased in HTC116 and SW620 cells with the transfection of miRNA-223-3p, compared with the negative control (miRNA-NC) (Figure 6A, 6B). Then we explored whether ROR could regulate NF2 expression via competition for miRNA-223-3p binding. The results suggested that the expression level of NF2 reduced significantly in HTC116 and SW620 cells transfected with Lnc-ROR, whereas the expression was increased after miRNA-223-3p transfection (Figure 6C, 6D). Furthermore, as to biological function, CCK8 assay was performed to evaluate cell proliferation with the transfection of Lnc-NC, Lnc-ROR and Lnc-ROR+ miRNA-223-3p. We found that Lnc-ROR can significantly enhance the cell proliferation, while the effect was abrogated when transfected with miRNA-223-3p (Figure 6E). In addition, overexpression of ROR significantly increased cell invasion ability, whereas invasion ability was repressed with the transfection of miRNA-223-3p (Figure 6F). These results demonstrated that ROR plays a vital role in cell proliferation and invasion via inhibiting NF2.

Discussion

In our present study, we evaluated the role of Lnc-ROR in the cell proliferation and invasion of human colorectal cancer cells. From the bioinformatics analysis, we investigated that ROR was specifically expressed in human colorectal carcinoma compared with adjacent non-cancerous normal tissues. We also verified that ROR was overexpressed in colorectal cancer cells HTC116 and SW620. Furthermore, we explored the molecular mechanism and found out ROR can promote cell proliferation and invasion by inhibiting tumor suppressor gene NF2 through interacting with miR-223-3p.

Colorectal cancer, which affects more than 1.2 million people annually around the world, is one of the most common diseases worldwide[30]. Currently, symptoms of cancer are detected at an advanced stage, leading to poor prognosis[31,32]. Moreover, standard treatments of colorectal cancer cells still have side effect and drug resistance[33,34]. Consequently, there is an urgent need to explore the pathogenesis of colorectal cancers and find novel detection targets and treatment approaches. Recently increasing evidence indicates that LncRNAs are not transcriptional noise but serve as vital factors in cell biology[35]. LncRNAs dysregulation is associated with various human diseases, especially cancers[36]. Lnc-ROR, a characterized cancer-related LncRNA, can alter cellular functions such as proliferation, migration and invasion in various human cancers[37]. Peng et al[24] discovered that Lnc-ROR promotes estrogen-independent growth and activation of MAPK/ERK pathway of breast cancer cells by regulating the ERK-specific phosphatase DUSP7. Another research found out that ROR can promote breast cancer progression and metastasis through regulation of miRNAs[38]. Besides, in esophageal squamous cell carcinoma, it has been clarified that ROR can promote esophageal squamous cell carcinoma progression through the derepression of SOX9[39]. However, the role of ROR in human colorectal cancers remains unknown. In our pres-

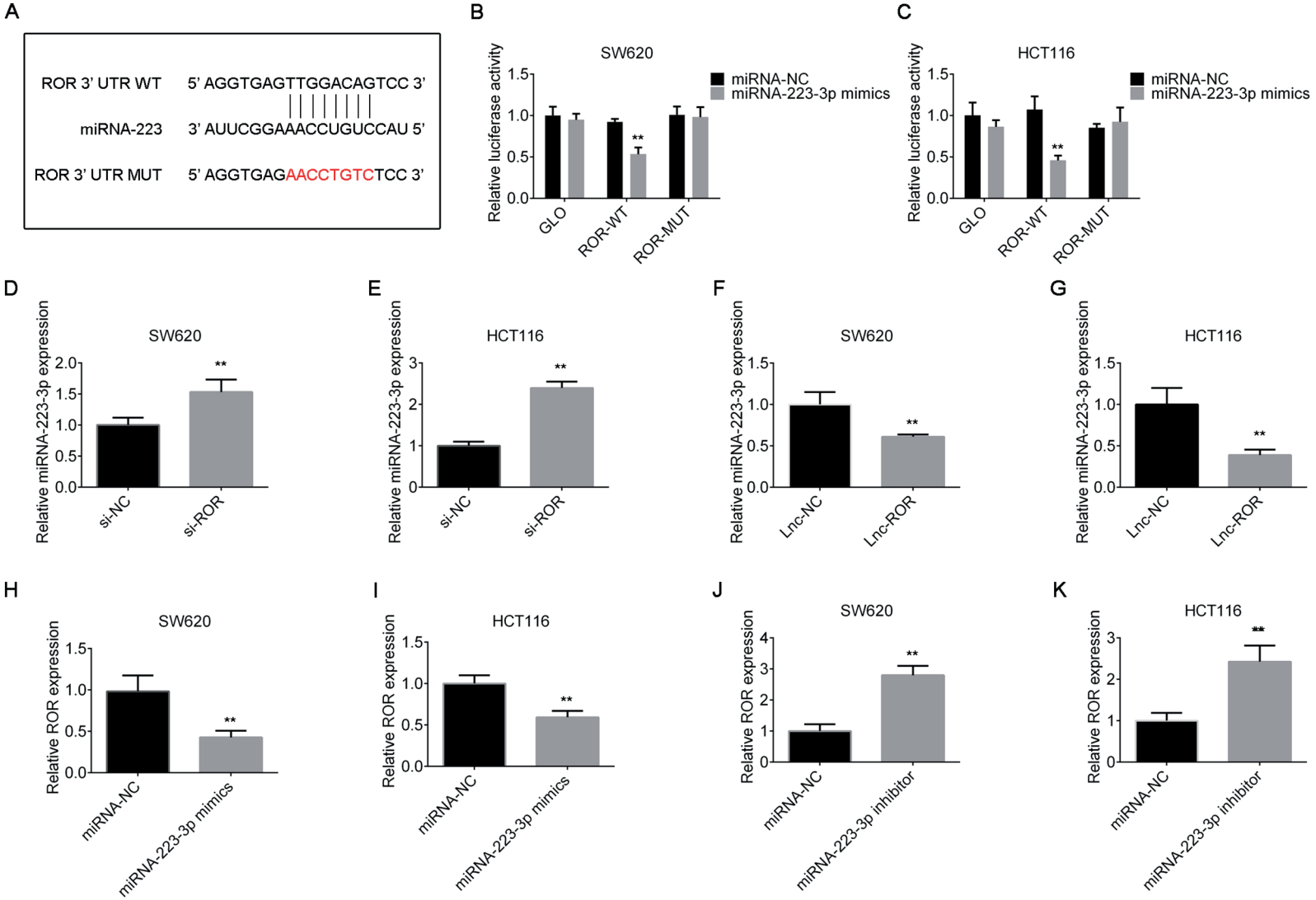

Figure 5. ROR functions as miR-223-3p sponge in colorectal cancer cells**. (A)** Schematic diagram of the presumed miR-223 binding sites and mutant sites in ROR. **(B-C)** Luciferase activity was detected using the dual luciferase assay. **(D-E)** Relative miRNA-223-3p expression in HTC116 and SW620 cells transfected with si-ROR. **(F-G)** Relative miRNA-223-3p expression in HTC116 and SW620 cells transfected with Lnc-ROR. **(H-I)** ROR expression was detected for both cells after treated with miR-223-3p mimic and miR-NC by RT-PCR. **(J-K)** Relative expression level of ROR in colorectal cancer cells treated with miR-223-3p inhibitor and miR-NC by RT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

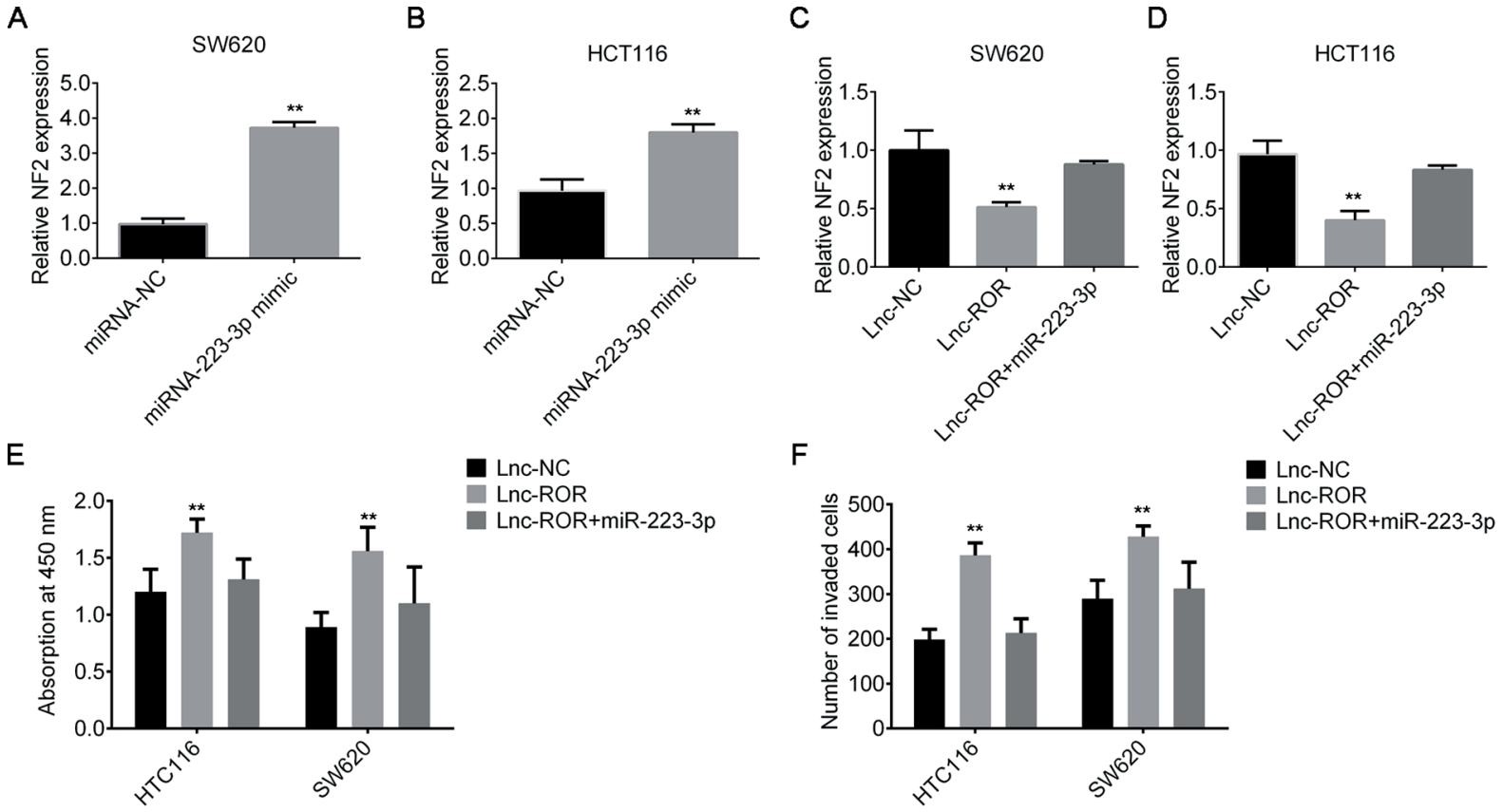

Figure 6. ROR promotes cell proliferation and invasion via inhibiting NF2**. (A-B)** Relative expression level of NF2 in HTC116 and SW620 cells transfected with miR-223-3p mimic and miR-NC. **(C-D)** qRT-PCR analysis of NF2 mRNA expression level in both cells treated with the Lnc-ROR, Lnc-ROR and miRNA-223-3p mimics. **(E)** Absorption at 450 nm of both cells treated with Lnc-NC, Lnc-ROR, Lnc-ROR+miRNA-223-3p mimics detected by CCK-8 assay. **(F)** The invasion of cells transfected with Lnc-NC, Lnc-ROR, Lnc-ROR+miRNA-223-3p mimics was determined by transwell invasion assays. The cell invasion number was measured. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

ent study, we discovered that ROR was highly expressed in colorectal cancer cells and can promote cell proliferation, migration and invasion.

The ceRNA regulatory network is a major mechanism of LncRNA in cancer development[40]. According to the ceRNA hypothesis, lncRNAs may act as endogenous decoy for miRNAs, in turn affecting the binding of miRNAs to their mRNA targets[41]. The present study suggested that inhibition of ROR promoted NF2 expression. Thus, we hypothesized that ROR regulates NF2 as a ceRNA. Then we performed dual luciferase reports. The results showed that ROR can directly bind with miRNA-223-3p and act as miR-223-3p sponge in colorectal cells. It is known that miRNA-223 can function as either an oncogene or a tumor suppressor gene, which is achieved by targeting a wide range of genes and regulating downstream signal transduction[42]. In our research, we demonstrated that miRNA-223-3p can regulate the expression of NF2, a tumor suppressor gene.

Conclusions

The findings of the present study first demonstrated that the expression level of ROR was higher in colorectal cancer tissues compared with normal tissues. In addition, ROR can act as a ceRNA to promote cell proliferation and invasion in colorectal cancers by competing bounding with miRNA-223-3p to inhibit tumor suppressor gene NF2. Thus, targeting ROR may have a promising effect in colorectal cancers treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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