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MicroRNA-181 Functions as an Antioncogene and Mediates NF-κB Pathway by Targeting RTKN2 in Ovarian Cancers

Zilin Lin, MD[1], Dehao Li, MD[1], Wenjia Cheng, MD[2], Jiajia Wu, MD[1], Kun Wang, MD[1], and Yi Hu, MD[3]

Abstract

MicroRNA (miR)-181 has been reported to participate in carcinogenesis and tumor progression in several malignant cancers, but its expression and biological functions in ovarian cancer have remained largely unclarified. Here, we first measured miR-181 expression in clinical ovarian cancers and found the expression levels of miR-181 were significantly lower in ovarian cancer tissues than that in adjacent tissues. Next, we screened and identified a direct miR-181 target, Rhotekin2 (RTKN2). A correlation between miR-181 and RTKN2 expression was also confirmed in clinical samples of ovarian cancers. Upregulation of miR-181 would specifically and markedly suppress RTKN2 expression. The miR-181-overexpressing subclones showed significant cell growth inhibition by cell apoptosis induction and significant impairment of cell invasiveness in SKOV3 and HO8910 ovarian cancer cells. To identify the mechanisms, we investigated the NF-κB pathway and found that nuclear factor-kappa B (NF-κB), B-cell lymphoma-2 (Bcl-2), and vascular endothelial growth factor (VEGF) were suppressed, whereas IκBα was promoted in miR-181-overexpressing cells. These findings indicate that miR-181 functions as a tumor suppressor and plays a substantial role in inhibiting the tumorigenesis and reversing the metastasis of ovarian cancer through RTKN2-NF-κB signaling pathway in vitro. Taken together, we believe that miR-181 may be a promising therapeutic target for treating malignant ovarian cancers.

Keywords

miR-181, ovarian cancer, RTKN2, proliferation, apoptosis

Introduction

Approximately 239 000 women are anticipated to be diagnosed with ovarian cancer in 2012, and 152 000 deaths were associated with this disease.[1] It represents the seventh most common cancer and the eighth most common cause of cancer death in women.[1] Ovarian cancer has a relatively poor prognosis because of its early metastasis. More than 60% of patients presenting with ovarian cancer have stage III or stage IV cancer, when it has already spread beyond the ovaries.[2] Recently, focusing on known genes, such as BRCA1, for ovarian cancer has already unveiled new treatment possibilities.[3] However, previously unknown noncoding RNAs, such as microRNAs (miRNAs), may also lend insight into the biology of ovarian cancer.

MicroRNAs are small noncoding RNA molecules that play key roles in the regulation of gene expression.[4] These molecules lead to translational silencing or transcript degradation through sequence-specific binding to target messenger RNAs (mRNAs).[4] The miR-181 is known as a group of short RNA molecules with important functions in tumor developmental and physiological progressions, including cell proliferation, apoptosis, and invasiveness.[5,6] Recently, mounting evidence indicates that miR-181 can function as a tumor suppressor gene or as an oncogene depending on the cell type.[7-10] Previous studies have shown that miR-181 acts as a tumor suppressor in the pathogenesis of acute myeloid leukemia (AML) and exhibits a significant impact on the survival of patients with AML,[9] although it targets the tumor suppressor gene Wnt inhibitory factor 1 in colorectal cancers.[7] However, its expression and biological functions in ovarian cancer have remained largely unclarified.

In order to gain insights into the expression alteration of miR-181 in ovarian cancer, we measured its expression in

[1] Medical Department, General Hospital of the People's Liberation Army, Beijing, China
[2] Surgery of Nanlou Department, General Hospital of the People's Liberation Army, Beijing, China
[3] Department of Oncology, General Hospital of the People's Liberation Army, Beijing, China

Corresponding Author:
Yi Hu, Department of Oncology, General Hospital of the People's Liberation Army, 28 Fuxing Road, Haidian District, Beijing 100853, China.
Email: yihu301@163.com

patients with ovarian cancer and several ovarian cancer cell lines. We found that miR-181 was downregulated in ovarian cancer and miR-181 was able to function as a tumor suppressor in SKOV3 and HO8910 ovarian cancer cell lines. Furthermore, we identified that Rhotekin 2 (RTKN2), a critical regulator of the nuclear factor-kappa B (NF-κB) pathway, is a direct miR-217 target and demonstrated that miR-181-RTKN2-NF-κB cascade was supposed to play a critical role in regulating apoptosis and invasiveness of ovarian cancer cells.

Materials and Methods

Tissue Samples

The study protocol and acquisition of tissue specimens were approved by Specialty Committee on Ethics of Biomedicine Research, General Hospital of the People's Liberation Army. Tissue specimens were obtained from archived tissue samples from patients with ovarian cancer who underwent surgical treatment from February 2006 to December 2014.

Cell Culture, Constructs, and Transfection

The malignant ovarian cancer cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in RPMI1640 (Gibco, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS, PAA, Carlsbad, California). The RTKN2 inhibitor constructs were purchased from Life Technologies (Carlsbad, California) and transfected using the manufacturer's reagents and protocol. The miR-181 mimics were purchased from Ambion and transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, California), according to the manufacturer's instructions. The miR-NC includes scramble sequences that are predicted to have no interactions in cells. SKOV3 and HO8910 cells were transfected with 50 ng of luciferase reporter vectors and 150 ng of either miR-181 or miR-NC using FuGENE HD according to the manufacturer's instructions for luciferase reporter assays.

Cell Proliferation Assay

Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All cells were plated in 96-well plates at 4×10^3 cells/well and incubated with 0.2 mg/mL MTT for 4 hours at 37°C. The optical density of each sample was read using a microplate reader (BioRad, Hercules, California) at 570 nm.

Apoptosis Assay

Apoptosis assay was performed as previously described.[11] ApoScreen Annexin V Apoptosis Kit (Bender Med System, Burlingame, California) was used for labeling of apoptotic cells. For each experiment, 20 000 cells were analyzed using FACSCalibur flow cytometer (BD Biosciences, San Jose, California). Experiments were performed in triplicate.

Colony Formation Assay

Colony-forming capacity was assayed by seeding 1000 cells/well in 12-well plates. Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) plus 10% FBS and allowed to form colonies for 2 to 3 weeks in a 37°C humidified atmosphere containing 5% CO_2. After removing the media and washing the cells with cold PBS, colonies were fixed with glutaraldehyde (6.0% vol/vol), stained with crystal violet (0.5% wt/vol), and counted using a stereomicroscope. Only colonies containing >50 cells were scored. Results are reported as the mean number of colonies observed in 10 randomly chosen microscope fields by investigators blind to the cell transfection history.

Protein Extraction and Western Blotting

All experiments were completed on ice at 4°C. The primary antibodies used were a RTKN2 (Sigma Aldrich, 1:1000), B-cell lymphoma-2 (Bcl-2;Santa Cruz, 1:500), Bax (Santa Cruz, 1:1000), and β-actin (Santa Cruz, 1:1000) used as a gel loading control. Western blot data were quantified by normalizing the signal intensity of each sample to that of β-actin.

Invasion Assay

Equal numbers (1×10^5) of transfected cells were plated onto separate 24-well cell culture inserts coated with Matrigel with 8 μm pores. The invasion assay was described as previously.[12] The invasion rate was determined from 3 independent experiments.

Statistics

All data are presented as mean (standard deviation; SD) of 3 separate experiments. Statistical analysis was performed using Student *t* test. Kaplan-Meier survival analysis was applied to compare survival times in patients with colorectal cancer. *P* values <.05 were considered statistically significant. Analyses were performed with the SPSS 10.0 software (SPSS).

Results

MicroRNA-181 Expression in Ovarian Cancer Specimens

We first measured the expression levels of miR-181 in 24 human ovarian cancer specimens and corresponding adjacent normal tissues by quantitative reverse transcription polymerase chain reaction (qRT-PCR). We found miR-181 downregulation was detected in 20 (83.33%) of 24 of ovarian cancer tumors (Figure 1A), suggesting that the decrease of miR-181 is a common event in ovarian cancer. The results showed that miR-181 expression in the tumors was approximately 2.6-fold lower compared to that in the adjacent normal tissues (mean [SD]: 3.68 [1.15] vs 1.42 [0.12]; $P < .001$; Figure 1B). The mean miR-181 expression level of ovarian cancer tissues was 1.42, which is utilized to divide patients with ovarian cancer into low-expression group (≥ 1.42, $n = 58$)

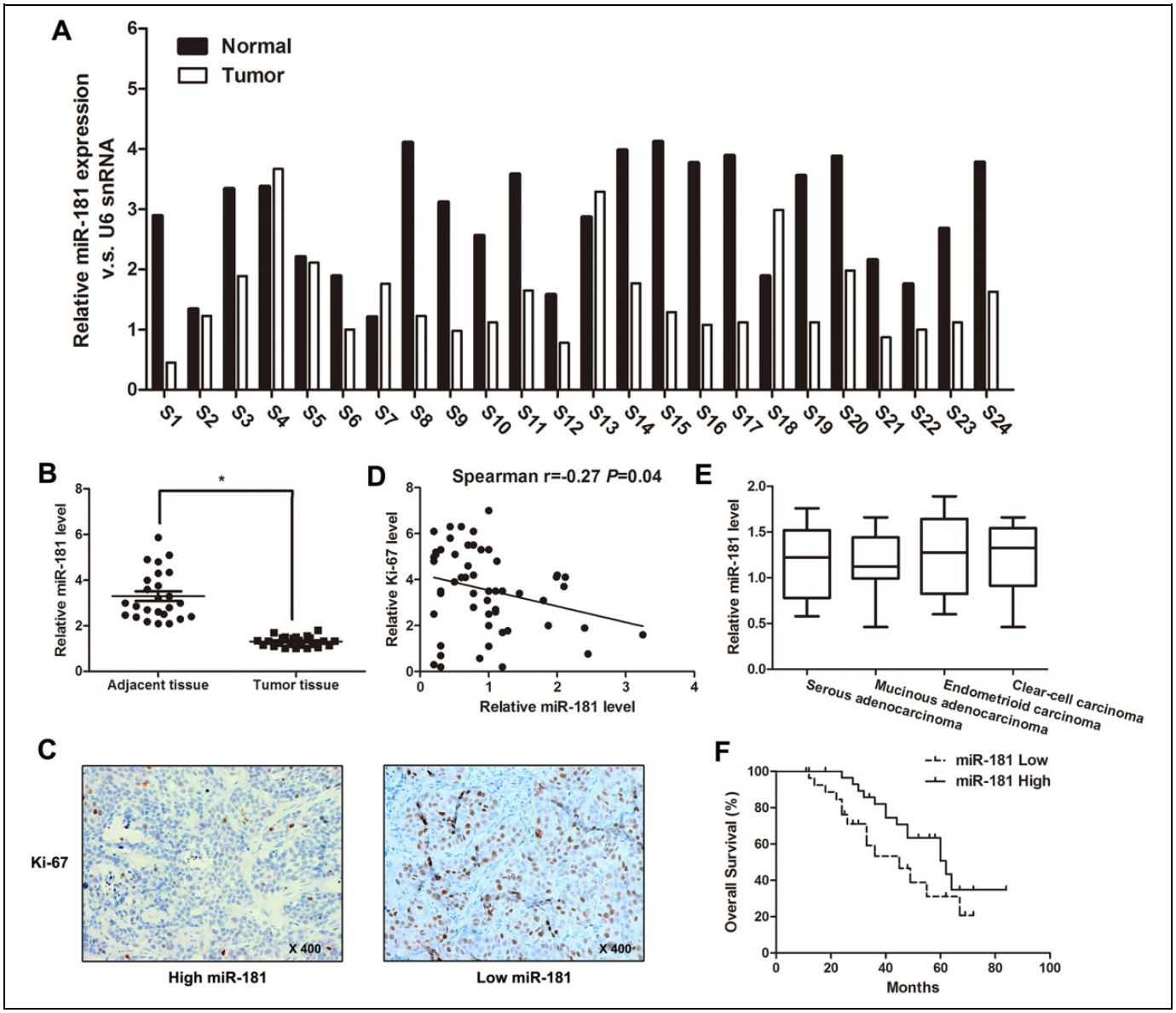

Figure 1. A, The miR-181 expression in 24 ovarian cancer tissues and corresponding adjacent normal tissues as determined by qRT-PCR analysis. B, The miR-181 expression was measured by qRT-PCR in adjacent normal tissues and ovarian cancer tissues. All expression was normalized to the level of U6 small nuclear RNA expression. *$P < .05$. C, Representative immunostaining showed low expression of Ki-67 in miR-181 high-expressing ovarian cancer tissue and high expression of Ki-67 in miR-181 low-expressing tumor. D, A significant inverse correlation between the mRNA levels of miR-181 and Ki-67 was observed in ovarian cancer tissues. $r = -0.27$, $P = .04$. E, The miR-181 expression in different types of ovarian cancer tissues as determined by qRT-PCR analysis. F, Kaplan-Meier survival curves according to miR-181 expression ($P < .001$). qRT-PCR indicates quantitative reverse transcription polymerase chain reaction; miR-181, microRNA-181; mRNA, messenger RNA.

and high-expression group (<1.42, n = 50). Ovarian cancer with low miR-181 expression exhibited greater immunoreactivity for Ki-67 proteins compared to those with high miR-181 expression (Figure 1C). Statistical analyses revealed that miR-181 expression inversely correlated with immunostaining for Ki-67, a nuclear protein highly expressed by proliferating cells ($r = -0.27$, $P = .04$ by Spearman correlation coefficient; Figure 1D). To further determine whether miR-181 expression correlates with tumor type, ovarian cancer tissues (n = 108; Table 1) were grouped into serous (n = 42), mucinous (n = 22), clear cell (n = 18), and endometrioid cancers (n = 26). The qRT-PCR revealed that miR-181 expression did not appear to differ between groups ($P > .05$; Figure 1E). We then investigated the association between miR-181 expression and clinicopathologic parameters in 108 patients with ovarian cancer (Table 1). The expression level of miR-181 was significantly correlated with TNM stage ($P < .01$), distant metastasis ($P = .02$), and lymph node metastasis ($P < .01$). No significant correlation was found between miR-181 expression and other clinical features.

Table 1. Association Between miR-181 Expression and Clinicopathological Variables of Patients With Ovarian Cancer.

Characteristics	Value	MiR-181 Expression		P
		High	Low	
No. of patients	108	50	58	
Age, years				.15
<50	64	30	34	
≥50	44	20	24	
MTD, cm				.18
<5 cm	77	35	42	
≥5 cm	31	15	16	
Distant metastasis				.02
Present	41	24	17	
Absent	67	26	41	
Lymph node metastasis				<.01
Present	31	6	25	
Absent	77	54	23	
TNM stage				<.01
II	43	31	12	
III/IV	65	19	46	
Tumor type				.96
Serous adenocarcinoma	42	20	22	
Mucinous adenocarcinoma	22	10	12	
Endometrioid carcinoma	18	8	10	
Clear cell carcinoma	26	12	14	

Abbreviations: M category describes the presence or otherwise of distant metastatic spread; MTD, mean tumor diameter; N category describes the regional lymph node involvement; TNM, T category describes the primary tumor site.

We further used the Kaplan-Meier survival curve method to assess the prognostic value of miR-181 between low- and high-expression groups. The results showed that the patients with high miR-181 expression had a significantly prolonged overall survival (OS) than those with low miR-181 expression (Figure 1F). Univariate proportional hazards regression model analysis indicated that low miR-181 expression, tumor stage, lymph node metastasis, and distant metastasis were independent prognostic parameters indicating poor prognosis for patients with ovarian cancer (Table 2). Moreover, in the Cox multivariate analysis, the statistical analysis showed that miR-181 expression is an independent feature for OS (Table 2).

Table 2. Univariate and Multivariate Analysis of Different Prognostic Parameters in Patients With Ovarian Cancer by Cox Regression Analysis.

Variable	Univariate Log-Rank Test (p)	Cox Multivariable Analysis (p)	Relative Risk
Age	0.154	0.131	0.921
MTD, cm	0.799	0.618	0.965
Distant metastasis	**0.031**	**0.042**	3.112
Lymph node metastasis	**0.021**	0.067	1.421
TNM stage	**0.039**	**0.041**	3.862
miR-181 expression	**0.004**	**0.021**	5.332

Abbreviations: M category describes the presence or otherwise of distant metastatic spread; miR-181, microRNA-181; MTD, mean tumor diameter; N category describes the regional lymph node involvement; TNM, T category describes the primary tumor site.
Bold values signify $P < .05$.

MicroRNA-181 Represses the Proliferation, Invasiveness, and Colony Formation Ability and Promoted the Apoptosis of Ovarian Cancer Cells in Vitro

Considering miR-181 was downregulated in ovarian cancer cell lines, we next investigated the role of miR-181 on the behaviors of ovarian cancer cells. First, we examined the expression level of miR-181 in a series of ovarian cancer cell lines, including 3AO, SKOV3, HO8910, and TYK by using qRT-PCR analysis. The miR-181 was dramatically downregulated in SKOV3 and HO8910 cells compared to that in 3AO and TYK cells (Figure 2A). So SKOV3 and HO8910 cells with relatively low miR-181 expression were selected for the next experiments in vitro. We next established SKOV3 and HO8910 cell line transfected with miR-181 mimics, noneffective control (miR-NC), or mock (control) to explore the effects of miR-181 on ovarian cancer cell proliferation. Transfection of SKOV3 and HO8910 ovarian cancer cell lines with a miR-181 mimics resulted in a dramatic increase in miR-181 expression in ovarian cancer cells compared to untransfected wild-type parental cells and cells transfected with miR-NC (Figure 2B). After miR-181 transfection, SKOV3 and HO8910 cell proliferation was significantly suppressed compared with that of cells transfected with miR-NC (Figure 2C). The colony formation ability also revealed a significant decrease in miR-181-transfected SKOV3 and HO8910 ovarian cancer cells compared with those miR-NC-transfected cells (Figure 2D). In addition, overexpression of miR-181 in SKOV3 and HO8910 cells repressed cell invasiveness dramatically in comparison to the control cells (Figure 2E). Moreover, flow cytometric analysis indicated that the apoptosis was significantly promoted in SKOV3 and HO8910 cells overexpressing miR-181 compared to cells transfected with miR-NC or control (Figure 1F), strongly suggesting that miR-181 plays a critical role in regulating ovarian cancer cell apoptosis in vitro. Taken together, these results indicated that miR-181 functions as a tumor suppressor in SKOV3 and HO8910 ovarian cancer cells.

Altered miR-181 Expression Affects Cell Proliferation, Apoptosis, and Invasion by Mediating NF-κB Pathway in Ovarian Cancer Cells In Vitro

To explore the underlying mechanism by which miR-181 functions as a tumor suppressor, we measured the expression of signaling molecules involved in the NF-κB and cellular apoptosis pathways. We found a dramatic decrease in NF-κB expression in SKOV3 and HO8910 cells transfected with miR-181 mimics (Figure 2G). Meanwhile, miR-181 transfection significantly enhanced IκBα protein, an inhibitor of NF-κB, expression in ovarian cancer cells (Figure 2G). In addition, the level of the antiapoptotic protein Bcl-2 was decreased

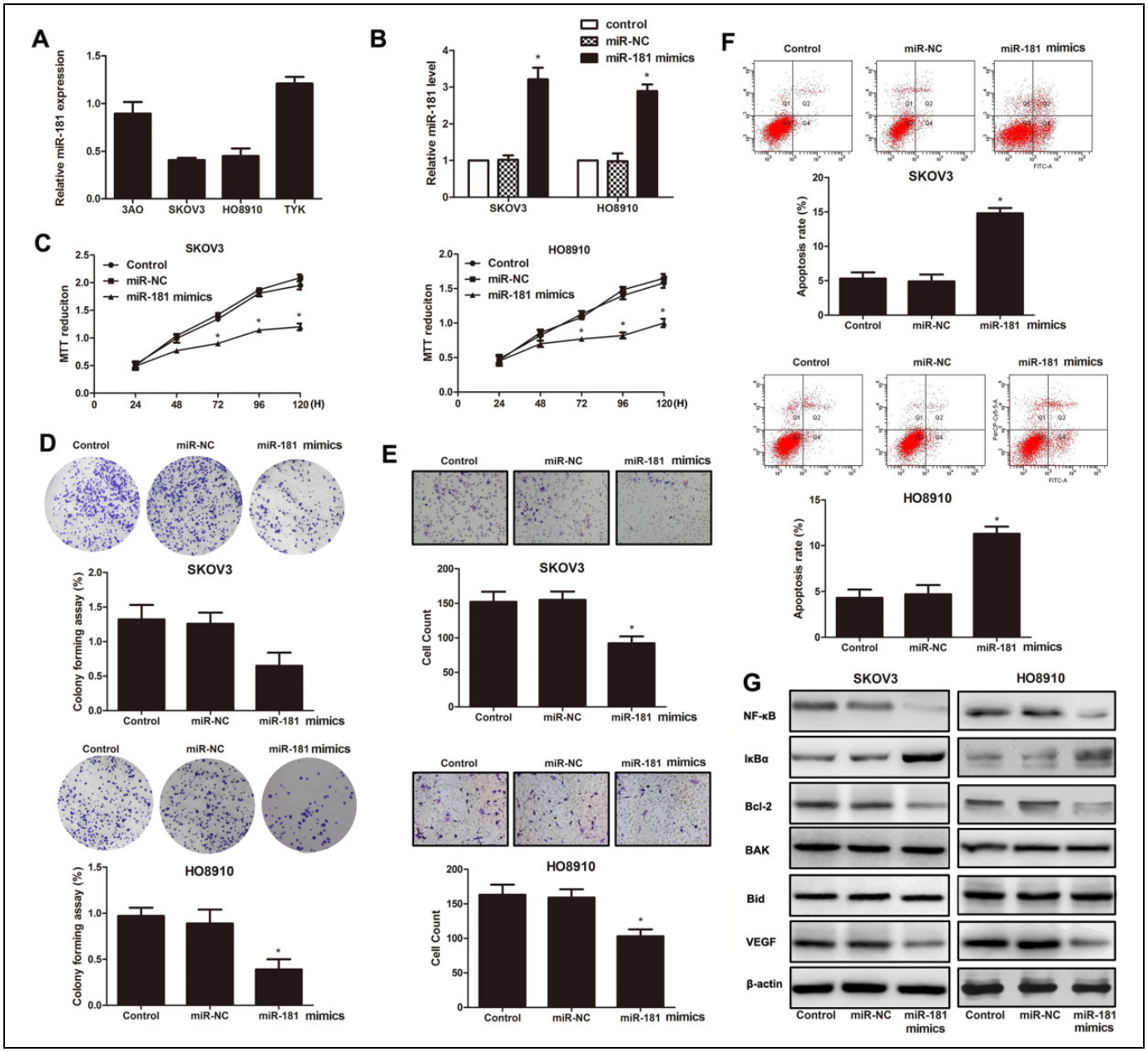

Figure 2. A, The miR-181 expression was measured by qRT-PCR in 3AO, SKOV3, HO8910, and TYK ovarian cancer cell lines. All expression was normalized to the level of U6 small nuclear RNA expression. *P < .05. B, The qRT-PCR analysis showed increased levels of miR-181 expression in both the SKOV3 and HO8910 miR-181 mimics-transfected cell lines, whereas a line stably expressing NC did not significantly increase miR-181 expression. C, The MTT assays show that the growth inhibition ratio decreased in SKOV3 and HO8910 cells overexpressing miR-181. *P < .05. D, The miR-181 significantly suppressed colony formation ability in cells overexpressing miR-181 compared to controls. *P < .05. E, Apoptosis rate was significantly higher in SKOV3 and HO8910 cells transfected with miR-181 mimics as examined by PI staining and flow cytometry. *P < .05. F, The miR-181 overexpression in SKOV3 and HO8910 cells by miR-181 mimics transfection decreased invasion compared with control cells in a Matrigel assay. *P < .05. G, Altered NF-κB signaling and apoptosis are associated with overexpression of miR-181. Detection of NF-κB and apoptosis-associated proteins in SKOV3 and HO8910 ovarian cancer cells by Western blotting. MTT indicates 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; miR-181, microRNA-181.

in miR-181-transfected SKOV3 and HO8910 cells, whereas the levels of the apoptotic proteins BAK and Bid were not changed. Furthermore, a significant decrease in the level of vascular endothelial growth factor (VEGF) after miR-181 transfection was visualized (Figure 2G). These data suggest that activating miR-181 function in ovarian cancer cells may suppress the NF-κB pathway, which not only enhances the apoptosis of the cells by decreasing the levels of cellular antiapoptosis proteins but also suppresses the invasiveness by downregulating VEGF expression in ovarian cancer cells in vitro.

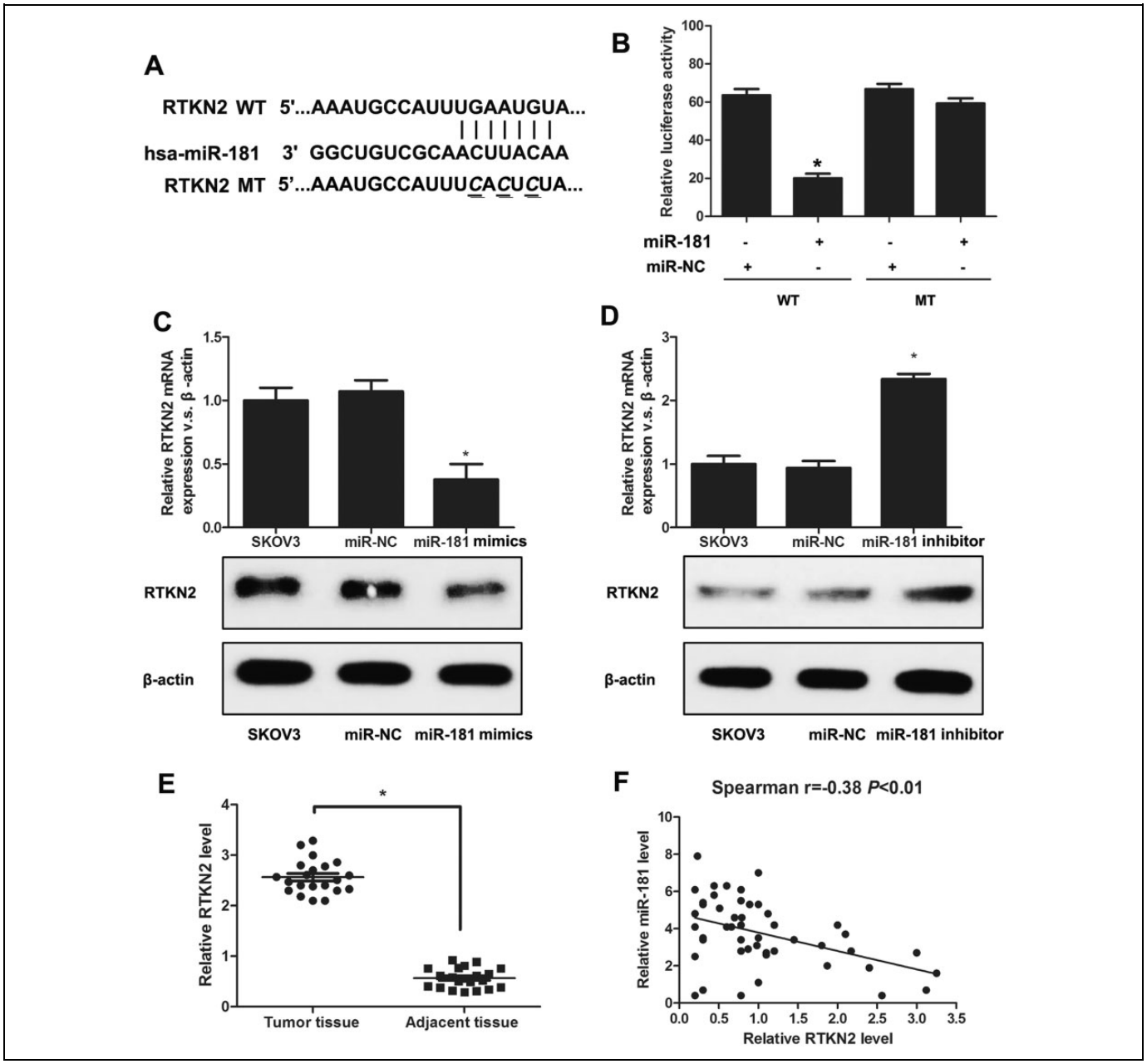

Figure 3. A, Sequence of wild-type and mutant miR-181 target sites in the RTKN2. B, Luciferase reporter assay in HEK293T Cells. *$P < .05$. C, The expression of RTKN2 was suppressed by miR-181 mimics in SKOV3 cells on protein and mRNA Levels. *$P < .05$. D, The protein expression of RTKN2 in SKOV3 cells transfected with miR-181 inhibitor or NC was analyzed by Western blotting. The mRNA levels of RTKN2 measured in SKOV3 cells transfected with miR-181 inhibitor by qRT-PCR. *$P < .05$. E, qRT-PCR analysis of RTKN2 mRNA expression in ovarian cancers. Results showed that RTKN2 mRNA expression was significantly increased in ovarian cancers compared to that in adjacent tissues. *$P < .05$. F, Correlation between RTKN2 mRNA expression and miR-181 expression. $P < .01$. qRT-PCR indicates quantitative reverse transcription polymerase chain reaction; miR-181, microRNA-181; mRNA, messenger RNA; RTKN2, Rhotekin2.

Rhotekin2 Is a Novel Target of miR-181

We speculate that RTKN2 is a potential target of miR-181 because it contains a putative miR-181 target sites in its 3′Untranslated Region (UTR) by using target scan. We cloned the target site or its mutant into an identical luciferase reporter vector (Figure 3A). We found that the reporter vectors with the putative target sequence resulted in an approximately 36.3% (1.9%) decrease in relative luciferase activity compared to the mutant introduced with miR-181 in HEK293T cells (Figure 3B). In addition, Western blot and qRT-PCR analysis showed that the protein and mRNA levels of endogenous RTKN2 were reduced by transfection of a miR-181 mimic in SKOV3 cells (Figure 3C). In contrast, when the expression of miR-181 was effectively downregulated by the transfection of a miR-181 inhibitor, the level of RTKN2 protein and mRNA was

upregulated compared to the control (Figure 3D). Moreover, we measured the expression of RTKN2 and miR-181 in ovarian cancer specimens to investigate its clinical relevance in vivo. We found the mRNA expression of RTKN2 was significantly increased in ovarian cancer tissues compared to adjacent tissues by using qRT-PCR analysis (Figure 3E). Moreover, statistical analyses of mRNA expression indicated that RTKN2 expression was associated inversely with miR-181 expression ($r = -0.38$, $P < .01$; Figure 3F). These data strongly suggest RTKN2 is a direct target of miR-181 in ovarian cancer cells.

Effects of RTKN2 Downregulation on Proliferation, Colony Formation, Apoptosis, and Invasiveness Ability in Ovarian Cancer Cells In Vitro

Considering that RTKN2 plays a critical role in the pathological process of cell proliferation, apoptosis, and invasiveness in various malignant tumor cells, we then investigated its biological functions in ovarian cancer cells. The mRNA and protein expression levels of RTKN2 in SKOV3 and HO8910 cells were significantly higher than those in 3AO and TYK cells (Figure 4A and B). Next, SKOV3 and HO8910 cells with relatively high RTKN2 expression were chosen for RTKN2 siRNA treatment, and the Western blot results revealed that RTKN2 protein was significantly inhibited by RTKN2 siRNA but not by GFP siRNA (Figure 4C). We then measured the contribution of RTKN2 expression to the SKOV3 and HO8910 cell growth by using MTT assay. Downregulation of RTKN2 significantly suppressed cell growth in SKOV3 and HO8910 cells underexpressing RTKN2 compared to control cells (Figure 4D). In addition, we found that knockdown of RTKN2 suppressed the colony-forming capacity of SKOV3 and HO8910 compared to cells transfected with GFP-siRNA controls (Figure 4E). Furthermore, RTKN2-siRNA transfection was positively correlated with enhanced apoptosis of ovarian cancer cells (Figure 4F). Meantime, the invasiveness ability revealed a significant decrease in siRNA-RTKN2-transfected SKOV3 and HO8910 ovarian cancer cells compared with that control cells (Figure 4G). These results indicated that RTKN2 can function as a tumor oncogene in ovarian cancer progressions.

Rhotekin 2 Regulates miR-181-Mediated Effects Through NF-κB Pathway in Ovarian Cancer Cells

To investigate whether RTKN2 is a regulator in miR-181-induced cell growth, apoptosis, and invasiveness, full-length RTKN2 was transfected into SKOV3 and HO8910 cells treated with miR-181 mimics. The MTT assay and colony-forming assay revealed that the increase of RTKN2 in ovarian cancer cells overexpressing miR-181 reversed lower growth and colony-forming ability observed in cells only overexpressing miR-181 (Figure 5A and B). In addition, apoptosis assays indicated that miR-181-induced apoptosis in SKOV3 and HO8910 cells was prevented by increasing RTKN2 (Figure 5C). Moreover, invasive assay showed that upregulation of RTKN2 in SKOV3 and HO8910 cells expressing miR-181 mimics reversed the decreased invasiveness observed in cells expressing only the miR-181 mimics (Figure 5D). These results imply that miR-181 induced antitumor effects through a RTKN2-dependent mechanism.

We next explored whether the NF-κB pathway was directly regulated by RTKN2 in ovarian cancer cells by using Lenti-RTKN2 to enhance RTKN2 expression. Increasing RTKN2 expression dramatically increased the levels of NF-κB and decreased the levels of IκBα, leading to increased Bcl-2 and VEGF expressions (Figure 5E). These data indicate that RTKN2 may be involved in the regulation of NF-κB pathway in these ovarian cancer cell lines. Notably, the effect of miR-181 on NF-κB and IκBα was abrogated by RTKN2 transfection (Figure 5E). Furthermore, when RTKN2 was transfected into both cell lines to increase RTKN2 expression, suppression of Bcl-2 and VEGF expressions by miR-181 was blocked (Figure 5E). These data indicate that miR-181-mediated apoptosis and invasiveness in ovarian cancers are likely associated with NF-κB pathway, which is regulated by RTKN2.

Discussion

Recently, miRNAs has been regarded as a critical regulator of carcinogenesis and tumor progression. MicroRNAs reveal differential expression modes in tumors and function as cancer suppressor genes or oncogenes by targeting special genes. The miR-181 family, which is considered as tumor suppressor gene or oncogene, has been indicated to play a critical role in occurrence and progression of malignant cancers such as leukemia, glioma, breast cancer, and lung cancer.[6,8,9,13] Mounting evidence have shown miR-181 that acts as tumor suppressors is expressed at relatively low levels, whereas oncogenic miR-181 shows relatively high expression levels in cancer.[10] Notably, the miR-181 family has been recognized as an important suppressor of AML by targeting Protein kinase C delta type (PRKCD).[10] In addition, Ouyang et al demonstrate that decreased miR-181a levels reduced glucose deprivation-induced apoptosis and mitochondrial dysfunction in astrocytes.[14] Furthermore, miR-181 upregulation could enhance TNF-induced apoptosis in L929 murine fibroblasts.[15] The miR-181 has also been implicated in prevention of proliferation and significantly enhanced the chemosensitivity to temozolomide in glioma stem cells.[13] These findings indicated that miR-181 might function as suppressor miRNA in various diseases. In agreement with these results, we found miR-181 was expressed at low levels in human ovarian cancer specimens and at relatively high levels in human adjacent tissues. Furthermore, overexpression of miR-181, resulting in decreased level of RTKN2 and low level of NF-κB activity, was related to the dramatic decrease in proliferation and invasiveness in SKOV3 and HO8910 cells. Therefore, we strongly suggested that miR-181 may functions as an anti-oncogene in ovarian cancers.

The RTKN2, a Rho effector protein, has an N-terminal Rho-GTPase binding domain (designated HR-1) and a mid-sequence Pleckstrin homology domain. It produces oncogenic

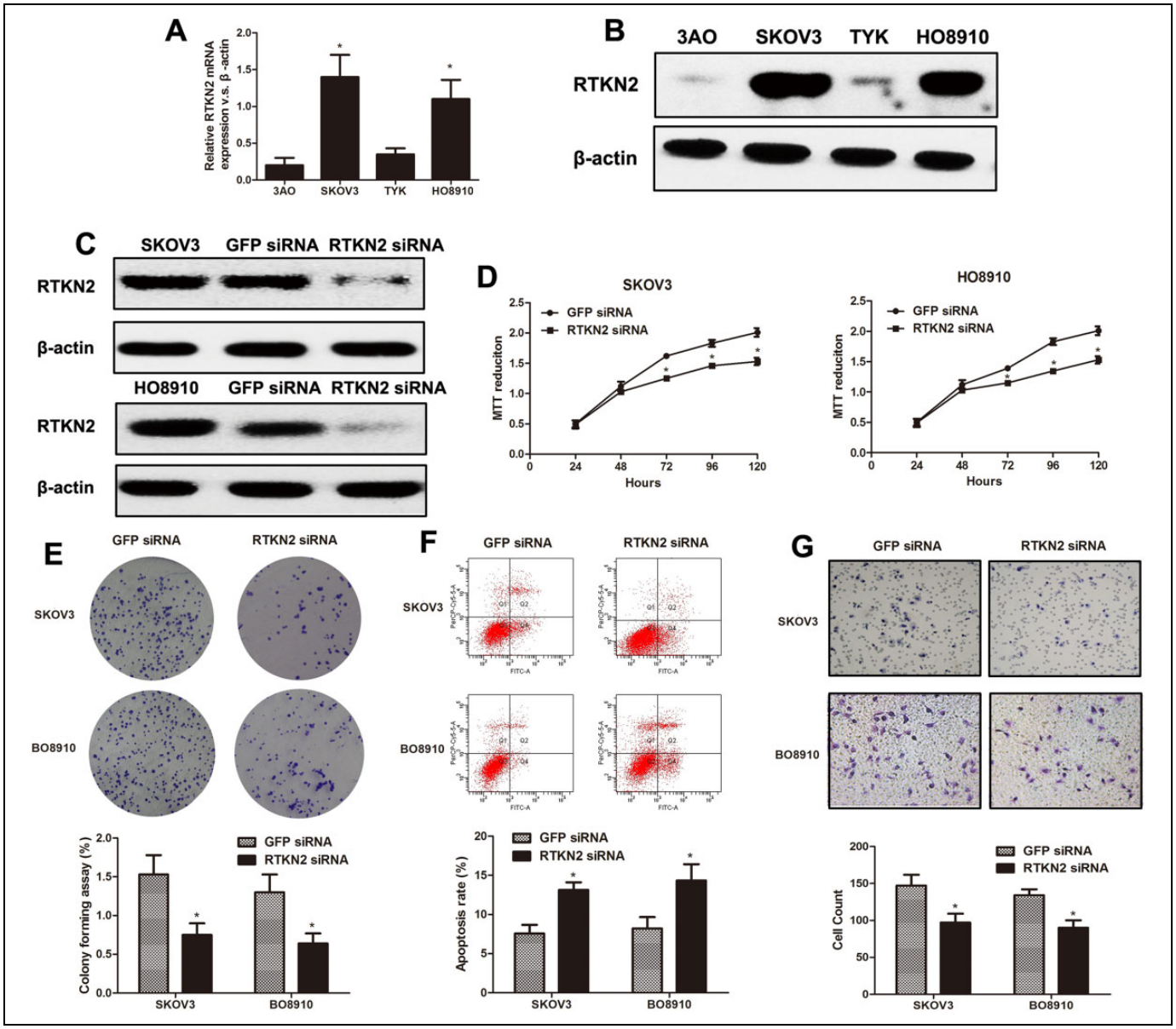

Figure 4. A, Expression of RTKN2 mRNA in 4 ovarian cancer cell lines measured by qRT-PCR. *$P < .05$. B, Protein levels of RTKN2 measured in 4 ovarian cancer cell lines by Western blot. C, Western blots analysis demonstrates that RTKN2 mRNA was significantly lower suppressed in SKOV3 and HO8910 cells treated with RTKN2-siRNA compared to cells treated with GFP-siRNA. *$P < .05$. D, Effect of RTKN2 knockdown on SKOV3 and HO8910 cell proliferation as measured by MTT assay. *$P < .05$. E, Effect of RTKN2 knockdown on SKOV3 and HO8910 cell colony formation ability as detected by colony formation assay. *$P < .05$. F, The number of apoptotic cells was significantly higher in SKOV3 and HO8910 cells transfected with RTKN2-siRNA as measured by flow cytometry. *$P < .05$. G, The RTKN2 inhibition in SKOV3 and HO8910 cells by RTKN2-siRNA transfection decreased invasion compared with GFP-siRNA and control cells in Matrigel assays. *$P < .05$. MTT indicates 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; mRNA, messenger RNA; RTKN2, Rhotekin2.

action by promoting NF-κB signaling in various human malignant cancer cells, such as bladder and hepatocellular carcinoma cells.[16,17] Recently, a study by Liu et al have found that RTKN2 overexpression confers cell resistance to apoptosis by induction of a number of NF-κB regulated antiapoptotic genes in gastric tumorigenesis.[18] A number of studies have shown that RTKN2 played a critical role in the process of intrinsic apoptosis and this was dependent on both NF-κB signaling and expression of downstream BCL-2 genes.[18-20] In addition to stimulating growth and invasion of ovarian cancer, overactivation of NF-κB pathway leads to resistance to standard chemotherapy agents including paclitaxel and cisplatin, highlighting its importance in ovarian cancer.[21,22] Here, we demonstrated that NF-κB pathway was directly regulated by RTKN2 in ovarian cancer cells, and the effect of miR-181 on NF-κB pathway activity was abrogated by RTKN2

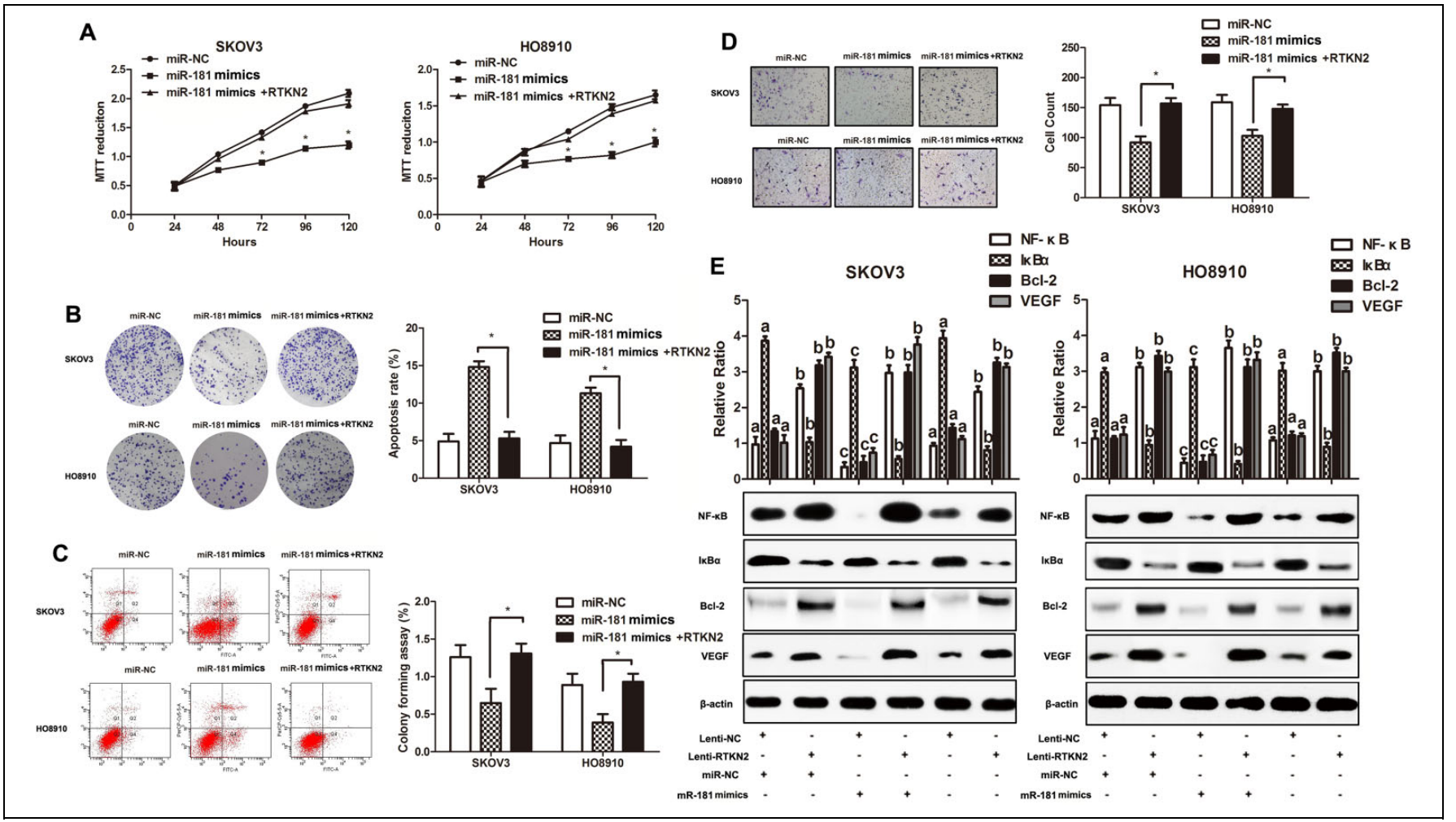

Figure 5. A, An MTT assay of SKOV3 and HO8910 cells cotransfected with miR-181 and RTKN2 or the control. *$P < .05$. B, Colony formation assay of SKOV3 and HO8910 cells cotransfected with miR-181 and RTKN2 or the control. *$P < .05$. C, Apoptosis assay of SKOV3 and HO8910 cells cotransfected with miR-181 and RTKN2 or the control. *$P < .05$. D, Apoptosis assay of SKOV3 and HO8910 cells cotransfected with miR-181 and RTKN2 or the control. *$P < .05$. E, Matrigel assay of SKOV3 and HO8910 cells cotransfected with miR-181 and RTKN2 or the control. *$P < .05$. F, The RTKN2 overexpression blocks the effect of miR-181 on NF-κB signaling. The miR-181 suppresses Bcl-2 and VEGF protein expression through RTKN2. Values representing the mean (SD; n = 3) with different letters are significantly different. *$P < .05$. MTT indicates 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; miR-181, microRNA-181; RTKN2, Rhotekin2; Bcl-2, B-cell lymphoma-2; VEGF, vascular endothelial growth factor.

overexpression. Thus, it is reasonable to speculate that low expression of miR-181 could modulate several cellular signaling pathways, particularly RTKN2-NF-κB pathway, and by doing so, ultimately contributes to facilitate the malignant progression of ovarian cancer cells.

Emerging evidences show that VEGF, a highly specific mitogen for vascular endothelial cells, plays important roles in enhancing angiogenesis and promoting the vascular permeability. Overexpression of VEGF and its receptor has been found in various solid tumor types, including ovarian carcinoma.[23] Furthermore, Camerin et al demonstrate that high VEGF expression was associated with poorer outcomes in patients with ovarian cancer.[24] It is also noteworthy that VEGF receptor can stimulate endothelial division and proliferation, promote angiogenesis, and accelerate the exchange of nutrients tumor cells and blood vessels. It has been reported that inhibition of NF-κB activity decreases the VEGF mRNA expression in breast cancer cells.[25] In agreement with this notion, we found that upregulation of miR-181 significantly suppresses RTKN2 expression, which can directly regulate NF-κB pathway, and results in a decrease of VEGF-induced invasiveness. In light of above results, we strongly suggest that the significantly decreased cell invasiveness by miR-181 could be attributed to suppressed RTKN2-NF-κB-VEGF-induced invasiveness in ovarian cancers.

In conclusion, we demonstrated that RTKN2 is a direct target of miR-181. As a tumor suppressor in ovarian cancer, overexpression of miR-181 represses the proliferation, invasiveness, and colony formation ability and promoted the apoptosis of ovarian cancer cells in vitro. Our data showed that miR-181 regulates cell proliferation and invasiveness through the RTKN2-NF-κB pathway and indicates that miR-181 may be a promising therapeutic target for treating malignant ovarian cancers.

Authors' Note

Zilin Lin and Dehao Li contributed equally to this work.

Declaration of Conflicting Interests

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