

MicroRNA-126 affects ovarian cancer cell differentiation and invasion by modulating expression of vascular endothelial growth factor

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Abstract. Primary ovarian cancer is the main cause of gynecological cancer-associated mortality. However, the mechanism behind the spread of ovarian cancer requires elucidation. The present study aimed to investigate the effects of microRNA-126 (miR-126) on differentiation and invasion, and its mechanism in primary ovarian cancer. Ovarian cancer SKOV3 cells transfected with LV3-has-miR-126 mimics and LV3-has-miR-126 inhibitor were produced; it was revealed that LV-miR-126 mimics could induce cell cycle arrest at G₁ phase, suppress cell invasion through Matrigel-coated membranes and downregulate the expression of vascular endothelial growth factor (VEGF). Furthermore, LV-has-miR-126 inhibitor-transfected cells could increase the number of cells in S phase, induce cell invasion and upregulate the expression of VEGF. The present study, to the best of our knowledge, is the first to report that miR-126 may serve tumor suppressor roles by inducing G₁ cell cycle arrest and suppressing invasion in ovarian cancer cells, at least in part by targeting VEGF expression.

Introduction

Primary ovarian cancer is the leading cause of gynecological cancer-associated mortality cause of death in patients with and most cases are diagnosed at an advanced stage with lymph node metastasis (1-3). Overall 5-year survival rates of patients with advanced ovarian cancer undergoing comprehensive surgical procedures remain low, owing to the spread of ovarian cancer cells throughout the peritoneal cavity (4). The majority of women are diagnosed with stage III or IV cancer (5,6).

Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis that induces the formation of novel blood vessels, and the growth and metastasis of cancers (7,8). The growth and spread of tumors depends on the formation of an adequate vascular support and amoeboid mode of tumor cell invasion (9). Furthermore, several studies have indicated that VEGF serves a role in promoting endothelial cell proliferation and migration, and induces a direct effect on cell proliferation and invasiveness (3,10,11). Previous studies have indicated that suppressing the expression or activity VEGF in ovarian cancer cells may represent a potential anticancer therapy (12-14).

MicroRNAs (miRNAs/miRs) are 22-nucleotide-long non-coding RNAs that regulate the expression of target mRNAs and are involved in cellular processes, including proliferation, differentiation, apoptosis and development (15-17). miR-126 is upregulated in the endothelium and originates from a common precursor structure located within the epidermal growth factor-like protein 7 gene (18,19). Several online databases are available to identify potential miRNA targets. Analysis performed using the PicTar algorithm (<http://www.pictar.org/>) (20), miRBase (www.mirbase.org) (21) and TargetScan target prediction (version 6.2; www.targetscan.org) (22) tools revealed that VEGF was the putative target of miR-126 that may serve a role in angiogenesis owing to regulation of VEGF signaling (23,24). Alteration of expression of miR-126 was also reported in various breast, lung and prostate cancer cells (24-26). However, the cellular mechanisms underlying the effect of miR-126 on ovarian cancer cells remains to be elucidated.

The present study aimed to characterize the role of miR-126, and the signaling pathway that it may be involved in, in the pathogenesis of ovarian cancer cells. Using SKOV3 ovarian cancer cells with up or downregulated miR-126, the present study attempted to identify the role of miR-126 in ovarian cancer cells.

Materials and methods

Cell culture. The ovarian cancer SKOV3 cell line (American Type Culture Collection, Manassas, VA, USA) was used in the present study. Cells were maintained and propagated *in vitro* by

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serial passage in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified environment in 5% CO₂ at 37°C.

Plasmid construction, lentivirus package and cell infection. The lentiviral vector pGLV3/H1/GFP+Puro (pGLV3; Shanghai GenePharma Co., Ltd., Shanghai, China) was used to construct the pGLV3-miR-126 plasmid. miR-126 mimics, miR-126 inhibitor and negative control (NC) oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd. The following sequences were used: miR-126, 5'-TCGTACCGTGAGTAATAATGCG-3'; hsa-miR-126 inhibitor, 5'-CGCATTATTACTCACGGTACGA-3'; and miR-NC, 5'-TTCTCCGAACGTGTCACGT-3'. miR-126 shDNA double chain template sequence was synthesized artificially and cloned into pGLV3-miRNA lentivirus plasmid. The miR-126 mimic sequence was chemosynthesized by Shanghai GenePharma Co., Ltd. using the following primers: hsa-miR-126-*Bam*HI forward, 5'-GATCCGTCGTACCGTGAGTAATAATGCGTTCAAGAGACGCATTATTACTCACGGTACGACTTTTTTG-3' and hsa-miR-126-*Eco*RI reverse, 5'-AATCAAAAAAGTCGTACCGTGAGTAATAATGCGTCTCTTGAACGCATTATTACTCACGGTACGACG-3'. The miRNA-126 inhibitor sequence was synthesized using the following primers: hsa-miR-126-*Bam*HI forward, 5'-GATCCGAGCATGGCACTCATTATTACGCTTCAAGAGAGCGTAATAATGAGTGCCATGCTCTTTTTTG-3' and hsa-miR-126-*Eco*RI reverse, 5'-AATTCAAAAAAGAGCATGGCATGCTCG-3'. The NC was pGLV3-shDNA-NC and the following sequence was used: NC-*Bam*HI forward, 5'-GATCCGTCGTACCGTGAGTAATAATGCGTTCAAGAGACGCATTATTACTCACGGTACGACTTTTTTG-3' and shNC-*Eco*RI reverse, 5'-AATTCAAAAAAGTCGTACCGTGAGTAATAATGCGTCTCTTGAACGCATTATTACTCACGGTACGACG-3'. All the sequences of resulting vectors were verified with sequence analysis by Shanghai GenePharma Co., Ltd.

The 293T cell line (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) was maintained in DMEM, 10% FBS, 4.0 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C and 95% CO₂ in order to produce lentivirus packing plasmids. At 1 day prior to transfection, 5x10⁶ cells were seeded into a 15-cm dish. pGLV3-miR-126 or pGLV3 vector and packing plasmids, including pGag/Pol, pRev and pVSV-G were co-transfected using RNAi-mate transfection reagent (both from Shanghai GenePharma Co., Ltd.), according to the manufacturer's protocol. A total of 72 h following transfection, the supernatant was collected and centrifuged at 8,500 x g at 4°C for 4 min, and passed through a 0.45- μ m syringe filter. Subsequently, the sample was centrifuged again at 48,400 x g at 4°C for 2 h. The viral titer of miR-126 mimic, miR-126 inhibitor and the negative control was measured according to the expression level of green fluorescent protein (GFP) according to the manufacturer's protocol (Shanghai GenePharma Co. Ltd.). Packaged lentiviruses were named LV3-has-miR-126, LV3-has-miR-126 inhibitor and LV3-NC. All sequences of resulting vectors were verified with sequence analysis by Shanghai GenePharma Co., Ltd.

SKOV3 cells were infected with LV3-has-miR-126, LV3-has-miR-126 inhibitor and LV3-NC as LV3-has-miR-126 mimic group, LV3-has-miR-126 inhibitor group and NC group respectively, while uninfected SKOV3 cells were the untreated SKOV3 cells group. The SKOV3 cells of the first three groups were infected at a multiplicity of infection (MOI) of 15 in the presence of 5 μ g/ml polybrene (Shanghai GenePharma Co., Ltd.). Efficiency of infection was ~90% as assessed by GFP and fluorescent microscopy. Cells were used for subsequent experimentation 72 h after transfection.

Cell cycle assay. Cells from all treatment groups were harvested at 48 h following transfection, fixed in 70% ice-cold ethanol overnight at 0°C and washed with 1X Buffer A (centrifugation at 4,200 x g for 5 min at 0°C). Subsequently, 5 μ l propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was added into 0.5 ml of cell suspension (5x10⁵ cells) for 15 min at room temperature. Experiments were performed in triplicate for each sample and analyses were performed using a flow cytometer (FACSCanto II; BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's protocol. Multicycle software for Windows (Phoenix Flow Systems, San Diego, CA, USA) was used to determine the percentage of cells in G₁, G₂ and S stages.

Cell invasion assay. A total of 48 h following transfection, the invasive ability of cells was assayed using a Transwell invasion assay (8-mm pore size). Transwell inserts were put into the 24-well plates. A total of 0.1 ml Matrigel (50 mg/ml; BD Biosciences) was added onto the plate surface and incubated for 4-5 h. SKOV3 cells were freshly trypsinized (0.25% trypsin) at 37°C for 3 min and washed with PBS, then suspended in DMEM with 0.1% BSA (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, 0.1 ml cell suspension (1x10⁵ cells) was added to the upper chamber of each insert coated with Matrigel. A total of 0.5 ml DMEM containing 20% FBS was added into the lower chamber and cells were allowed to invade for 24 h at 37°C in a 5% CO₂ humidified incubator. Following the incubation, cells on the membrane were removed using a cotton swab and 0.05% crystal violet stain was used to identify migratory cells at 20°C for 30 min. The number of migratory cells on the lower surface of the membrane was counted under Nikon Labophot 2 light microscope (Nikon Corp., Tokyo, Japan; 10 fields of vision) at a magnification of x200.

Immunofluorescence staining and western blot analysis. Analysis preformed using the PicTar algorithm (<http://www.pictar.org/>) (20), miRBase (www.mirbase.org) (21) and TargetScan target prediction (version 6.2; www.targetscan.org) (22) tools revealed that VEGF was the putative target of miR-126. Therefore, the expression of VEGF was explored in all groups. A total of 48 h after transfection, cells were fixed in 4% paraformaldehyde for 30 min at 20°C, washed three times with PBS, and incubated for 5 min at -20°C in 95% ethanol (v/v in PBS). Cells were subsequently washed three times with PBS, blocked for 1 h at 20°C in 5% normal goat serum (Wuhan Boster Biological Technology, Ltd., Wuhan, China) in PBS with 0.1% Triton X-100 and incubated overnight with anti-VEGF antibodies (cat no. BA0407; 1:200; Wuhan Boster Biological Technology, Ltd.) at 4°C. Cells were subsequently

washed three times with PBS and incubated for 40 min at 37°C with the corresponding secondary antibody (goat anti-rabbit immunoglobulin (Ig)G (H+L)-tetramethylrhodamine (TRITC; cat no. BA1090; 1:100; Wuhan Boster Biological Technology, Ltd.). Cells were washed with PBS and mounted onto the glass slides. Immunostained SKOV3 cultures were examined under a Zeiss laser scanning confocal microscope at a magnification of $\times 200$ (LSM 510 META; Carl Zeiss AG, Oberkochen, Germany) for detection of the TRITC fluorophore by semi-quantitative confocal laser scanning analysis-Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD USA).

For western blot analysis, proteins were extracted from SKOV3 cells. Proteins were solubilized in Radio-immunoprecipitation assay buffer (Sangon Biotech Co., Ltd., Shanghai, China) and proteins quantified using the BCA Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China) were separated at 30 μ g per lane on 10% SDS-PAGE and electro-transferred onto polyvinylidene difluoride membrane. The membrane was blocked in 5% skimmed milk powder prepared in Tris-buffered saline with 0.1% Tween-20 (TBS-T; Sangon Biotech Co., Ltd.) for 30 min at 20°C. For VEGF detection, membranes were incubated at 4°C overnight with anti-VEGF antibodies (cat no. BA0407; 1:500; Wuhan Boster Biological Technology, Ltd.) and anti- β -actin antibodies (cat no. BM0627; 1:200; Wuhan Boster Biological Technology, Ltd.). Membranes were washed 3 times for 10 min in TBS-T and incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (cat no. BA1054; Wuhan Boster Biological Technology, Ltd.) for 2 h. Membranes were washed 6 times for 20 min with TBS-T prior to development with a standard enhanced chemiluminescence kit (ECL kit; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Densitometry analysis of the bands was performed using Quantity One software (version 4.6; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation ($n=5$). Statistical analysis was performed using SPSS software (version 14.0; IBM Corp., Armonk, NY, USA). The significance of any differences was evaluated using one-way analysis of variance followed by a Tukey post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell cycle assay. Cell cycle analysis was used to determine whether the effect of miR-126 on proliferation of SKOV3 cells was due to cell cycle alterations. The results demonstrated that the percentage of cells in G₁ phase increased to 54.65%, whereas the percentage of cells in S phase decreased to 34.44% in the miR-126 mimic group compared with the NC group (G₁ phase, 44.47% and S phase, 53.12% in the NC group). The percentage of cells in G₂ phase was 2.41% in the NC group and 10.90% in the miR-126 mimic group ($P<0.05$). These results indicate that the overexpression of miR-126 in SKOV3 cells induced G₁ cell cycle arrest. Cell cycle analysis was also conducted in the miR-126 inhibitor group. Following transduction with the LV3-miR-126 inhibitor for 48 h, flow cytometry analysis revealed that SKOV3 cells were promoted from the G₁ phase into the S phase. The percentage of cells

in G₁ phase was from 44.47% in the NC group and 9.42% in the miR-126 inhibitor group, while the percentage of cells in S phase was 53.12% in the NC group and 90.58% in the miR-126 inhibitor group ($P<0.05$). There were no significant alterations in cell cycle ratios between the untreated group and the NC group ($P>0.05$; Fig. 1). The results demonstrated that miR-126 mimic arrested SKOV3 cells at the G₁ phase while the miR-126 inhibitor induced cell cycle progression.

Cell invasion assay. The number of SKOV3 cells in the miR-126 mimic group that invaded through the Matrigel-coated membranes was significantly lower than that in the untreated group (164.6 ± 25.8 vs. 253.1 ± 14.9 cells, respectively; $P<0.05$; Fig. 2). Furthermore, the depletion of miR-126 significantly promoted SKOV3 cell invasion through Matrigel-coated membranes, compared with the untreated group (290.9 ± 13.2 cells vs. 253.1 ± 14.9 cells, respectively; $P<0.05$; Fig. 2). There were no significant differences between the number of invaded NC cells and that of the untreated cells (256.5 ± 15.2 vs. 253.1 ± 14.9 cells respectively; $P>0.05$; Fig. 2). These results indicated that downregulation of miR-126 may promote cell invasion, whereas upregulation of miR-126 inhibits the invasion of cells through Matrigel-coated membranes.

Immunofluorescence staining and western blot analysis. Immunofluorescence double staining and semi-quantitative confocal laser scanning analysis revealed that miRNA vectors and VEGF were expressed in the four treatment groups. Green fluorescence, indicating vector-transfected cells, was detected in all cell nuclei and only certain cytoplasmic areas. The red fluorescence detected in the cytoplasm of SKOV3 cells indicated VEGF expression. The results of immunofluorescence staining demonstrated that transfection with miR-126 mimics mediated the inhibition of VEGF expression. The mean immunofluorescence intensity of VEGF in the miR-126 inhibitor group was significantly increased compared with that in the control group ($P<0.05$; Fig. 3). Furthermore, the expression level of VEGF decreased as a result of overexpression of miR-126 in LV3-has-miR-126-transfected SKOV3 cells, compared with untreated and negative control cells (Fig. 3). These results further indicated that miR-126 has an inhibitory role on VEGF expression in SKOV3 cells.

VEGF protein was detected in all treatment groups as a single band at ~ 28 kDa. Densitometry analysis of the VEGF bands revealed a significant increase in expression levels ($110.4\pm 6.8\%$) in the LV3-has-miR-126 inhibitor group and a significant decrease ($31.2\pm 2.2\%$) in the LV3-has-miR-126 mimic group, compared with the untreated group ($71.4\pm 2.8\%$; both $P<0.05$; Fig. 4). There was no significant difference between the NC control group and the untreated group (70.9 ± 3.8 vs. $71.4\pm 2.8\%$, respectively; $P>0.05$; Fig. 4). The above results indicate that VEGF was upregulated in SKOV3 cells transfected with LV3-has-miR-126 inhibitor, whereas VEGF expression was inhibited following LV3-has-miR-126 mimic transfection.

Discussion

miR-126 has been reported to be downregulated in human breast, lung, stomach, cervix and pancreas cancer (24,27-30). Furthermore, patients with non-small cell lung cancer and

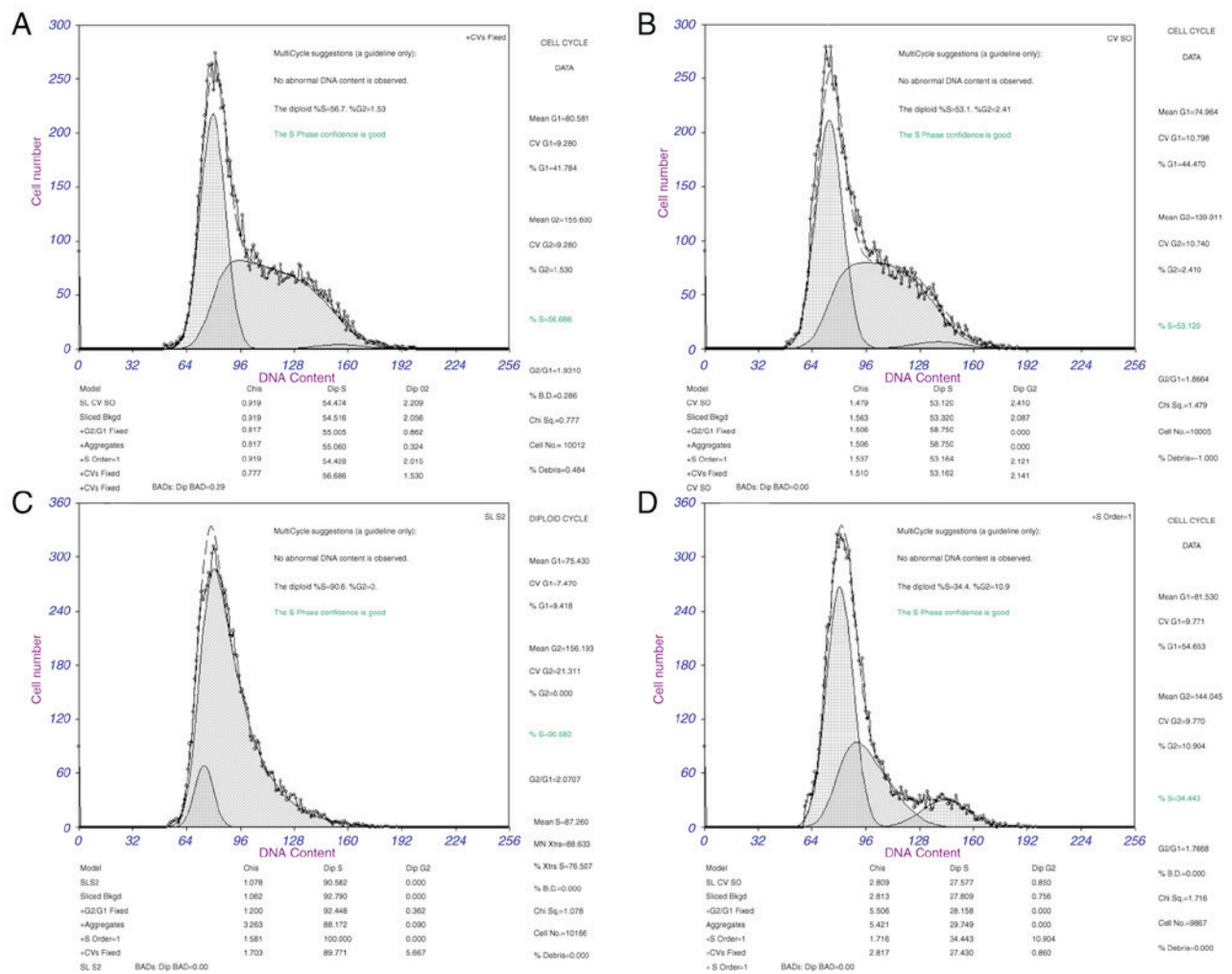


Figure 1. SKOV3 cell cycle analysis detected by flow cytometry. (A) Untreated SKOV3 cells. (B) SKOV3 cells transfected with LV3 negative control. (C) SKOV3 cells transfected by LV3-has-miR-126 inhibitor. (D) SKOV3 cells transfected by LV3-has-miR-126.

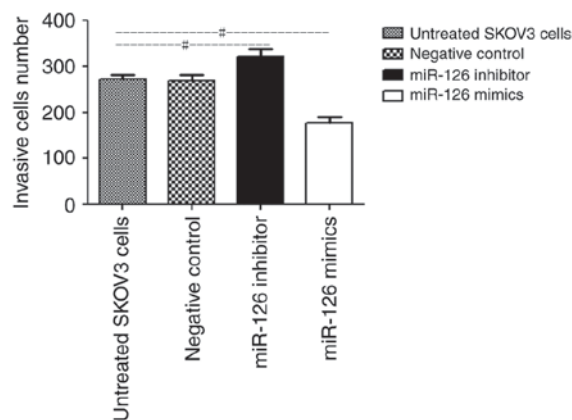


Figure 2. Number of invasive cells in the untreated SKOV3 group, SKOV3 cells transfected with LV3 negative control, SKOV3 cells transfected with LV3-has-miR-126 inhibitor and SKOV3 cells transfected with LV3-has-miR-126. Data are presented as mean \pm standard deviation. * $P < 0.05$ with comparisons shown by lines. miR, microRNA.

primary breast tumors exhibiting low miR-126 expression displayed poor survival compared with patients with high miR-126 levels (25). These studies indicated the presence of an association between the expression of miR-126 and

metastasis-free survival. The present study demonstrated that ovarian cancer cells transfected with miR-126 exhibited a significantly increased frequency of cells in G₁ phase and a lower frequency of cells in S phase. The percentage of cells in G₁ phase in the miR-126 mimic group was markedly higher than that in the NC group. These results demonstrated that miR-126 could induce ovarian cancer cell cycle arrest, whereas inhibition miR-126 promoted cell cycle progression and induced ovarian tumor cell proliferation.

Previous studies demonstrated that VEGF serves a role in the modulation of cellular functions, including angiogenesis, tumor cell proliferation and invasiveness (31-33). VEGF increases vascular permeability and leads to malignant ascites in later stages of ovarian cancer, indicating disease progression and treatment failure (31,34). Therefore, anti-VEGF treatment could aid in improving patient survival (35). Clinical trials have demonstrated that certain anti-VEGF agents, including bevacizumab, can increase the risk of treatment-associated death caused by pulmonary hemorrhage, hypertension, proteinuria or bleeding, owing to disruption of normal vasculature (36,37). These side effects limit the therapeutic usage of anti-VEGF agents. The targeting of endogenous miRNAs provides an alternative approach to anti-VEGF treatment (38). Online accessible algorithms, including PicTar, miRBase and TargetScan,

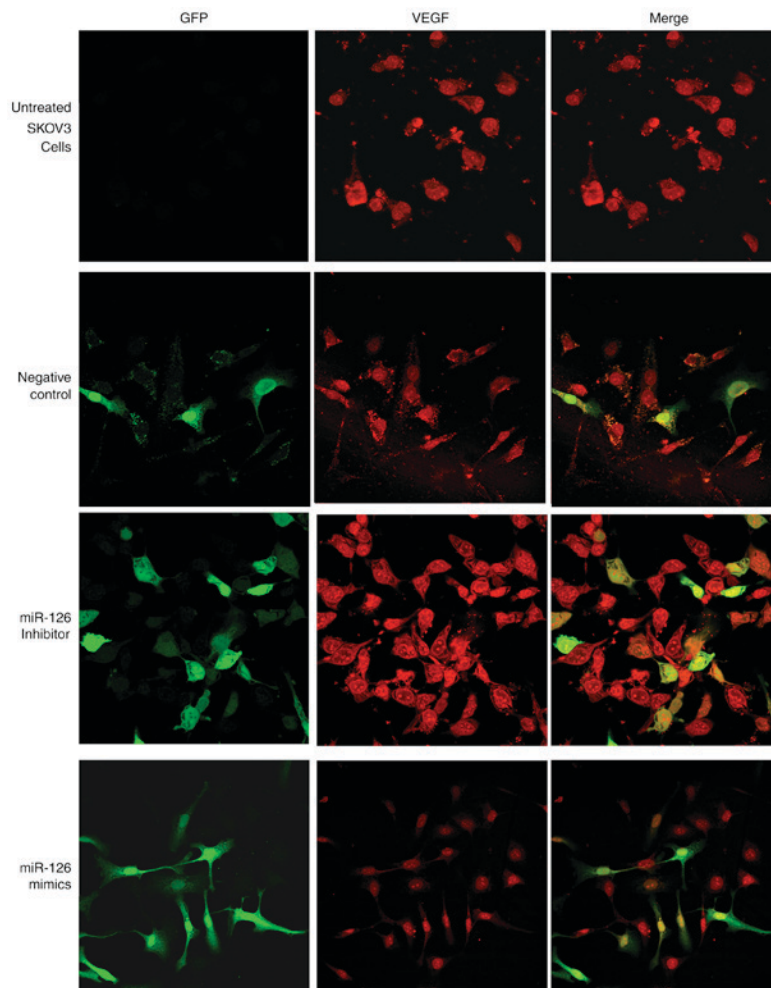


Figure 3. Immunofluorescence staining demonstrated suppression of VEGF by miR-126 in transfected cells. Cells were grown on coverslips overnight and subsequently subjected to immunostaining with anti-VEGF antibody. VEGF-positive cells were stained red, whereas GFP-positive cells were stained green. Merged images present VEGF and GFP. miR-126, microRNA-126; VEGF, vascular endothelial growth factor; GFP, green fluorescent protein.

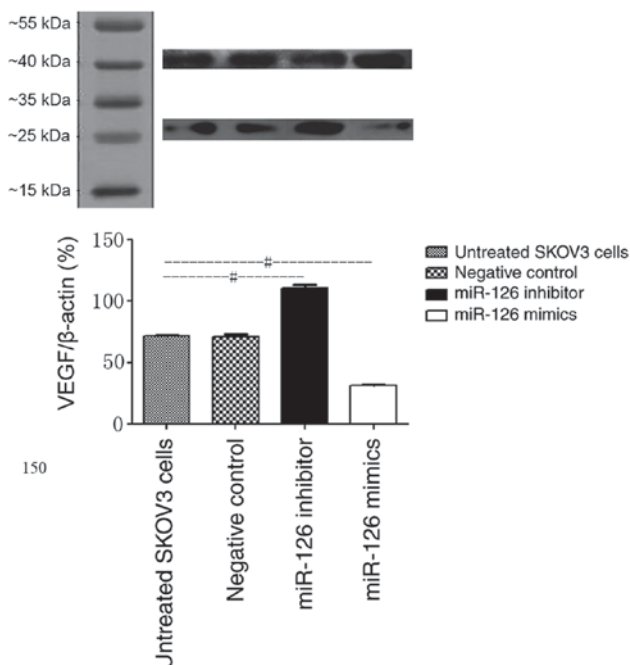


Figure 4. Western blot analysis of cells in all groups. Data are presented as the mean \pm standard deviation. * $P < 0.05$ with comparisons shown by lines. miR-126, microRNA-126; VEGF, vascular endothelial growth factor.

demonstrated that one of the potential targets of miR-126 is VEGF (18,19). The present study demonstrated that miR-126 was a target of VEGF and the upregulation of miR-126 markedly inhibited VEGF expression. The present study aimed to elucidate the interaction between miR-126 and VEGF in ovarian cancer cells. The results demonstrated that SKOV3 cells infected with LV-miR-126 can efficiently reduce the expression of VEGF. Furthermore, SKOV3 cells infected with LV-miR-126 inhibitor exhibited overexpression of VEGF and the cell proliferation as 90.58% cells of S phase vs. 53.12% in the NC group.

Invasion through the basement membrane is one of the features of aggressive tumors (39); therefore, the present study further investigated the association between the expression of miR-126 and ovarian cell invasion. Cells transfected with LV-has-miR-126 inhibitor exhibited increased invasiveness compared with the untreated group. The results also demonstrated that increasing the expression of miR-126 in ovarian cancer cells could reduce the invasiveness. We hypothesize that low expression of miR-126 promotes VEGF expression and cell invasion, leading to the development of ovarian cancer.

In conclusion, to the best of our knowledge, the present study is the first to present data indicating that miR-126 is a tumor suppressor that can induce ovarian cancer cell cycle arrest and suppress invasion, at least in part, by targeting VEGF

expression. miR-126 may be used as a potential marker for clinical prognosis and treatment of ovarian cancer in the future.

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Competing interests

The authors declare that they have no competing interests.

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