

MicroRNA-195 suppresses cell proliferation, migration and invasion in epithelial ovarian carcinoma via inhibition of the CDC42/CCND1 pathway

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Abstract. Epithelial ovarian carcinoma (EOC) is the most common cause of gynecological cancer mortality, and poses a threat to women. MicroRNA-195 (miR-195) has been reported to induce apoptosis of human OVCAR-3 cells by inhibiting the VEGFR2/AKT pathway. However, the role of miR-195 in EOC remains unknown. A previous study reported that cell division cycle 42 (CDC42) can serve as a target gene of miR-195 and mediate malignant progression of esophageal squamous cell carcinoma (ESCC). The aim of the present study was to investigate the role of miR-195 in EOC and the regulation in CDC42/CCND1 pathway. Tissues samples and clinical materials were collected from 78 enrolled patients with EOC to analyze the expression and clinical significance of miR-195, CDC42 and cyclin D1 (CCND1). Human EOC cell lines OVCA420, OVCAR-3, A2780 and SKOV3 cell lines were used to assess the expression and function of miR-195, CDC42 and CCND1 *in vitro*. Cell proliferation, the cell cycle and apoptosis, as well as the cell migratory and invasive abilities were detected *in vitro* using BrdU incorporation, colony formation, wound healing and Transwell invasion assays, along with flow cytometry. miR-195 was downregulated, while CDC42 and CCND1 were upregulated in human EOC tissues and cells, and the aberrant expression of both was associated with increased EOC malignancy. Moreover, miR-195 expression was negatively correlated with CDC42 and CCND1 expression levels, and negatively regulated these expression levels. Thus, it was suggested that miR-195 functions as a tumor suppressor, but CDC42 and CCND1 act as tumor promoters based their abilities to enhance cell proliferation, cell cycle

entry, migration and invasion, as well as decrease apoptosis in OVCAR-3 cells. the present results demonstrated that miR-195 inhibited human EOC progression by downregulating CDC42 and CCND1 expression. Furthermore, it was identified that miR-195, CDC42 and CCND1 may be effective biomarkers for EOC diagnosis and treatment.

Introduction

Ovarian cancer (OC) is one of the most common malignancies of the female reproductive organs. For instance, its morbidity is second only to cervical cancer and corpus carcinoma; however, the mortality of OC ranks first (1). The 5-year survival rates of OC are ~90 and ~15% in the early and advanced phases, respectively (2). OC can be divided into epithelial, germ and stromal cell neoplasms (3). Epithelial ovarian carcinoma (EOC), which accounts for >70% of the cases of OC, has a 30% 5-year survival rate and the highest mortality rate among all gynecological tumors worldwide, and therefore, poses a serious threat to women (4,5). As ovary tumor tissues are small and reside deeply in the pelvic cavity, and patients lack typical symptoms, the diagnosis of EOC during the early stage is very difficult. Moreover, <30% of patients with EOC are reported to have tumors confined to the ovary during surgery; the majority of tumors spread to the pelvic and abdominal organs, and thus, early diagnosis is important (6). However, EOC typically presents at an advanced stage, leading to an elevated mortality rate (7). Considering the difficulty in early diagnosis and the distinctive metastasis of EOC, investigating the biological and molecular mechanisms of advance metastasis is crucial for the development of effective therapeutic approaches against EOC (8).

MicroRNAs (miRNAs/miRs) are short, single-stranded, non-coding RNAs present in different tissues and bodily fluids that can regulate target gene expression post-transcriptionally. Previous studies have reported that miRNAs are abnormally expressed in various tumors and can act as tumor suppressors or oncogenes, with roles in tumor development via negative regulation of specific genes (9-11). For example, miR-195 has been observed to be downregulated in EOC, and was revealed to decrease cell proliferation and induce apoptosis

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of human OVCAR-3 cells by inhibiting VEGFR2 and AKT signaling (12). In addition, miR-195 is downregulated in human laryngeal squamous cell carcinoma, while the overexpression of miR-195 inhibits cell viability, migration and invasion, as well as promotes apoptosis of AMC-HN-8 cells (13). However, the roles of miR-195 in the cell cycle, migration and invasion of EOC cells are yet to be fully elucidated.

Cell division cycle 42 (CDC42) has been identified as a target gene of miR-195 (14). miR-195 expression is downregulated after overexpression of CDC42 in esophageal squamous cell carcinoma (ESCC), and the combined aberrant expression of miR-195 and CDC42 could predict malignant progression and unfavorable prognosis of ESCC (15). Furthermore, down-regulated expression of miR-195, but upregulated cyclin D1 (CCND1) expression has been observed in papillary thyroid carcinoma (PTC) cells, and miR-195 inhibits tumor growth and metastasis in PTC by targeting CCND1 and fibroblast growth factor 2 (16). CDC42/CCND1 signaling pathways are also involved in endothelial cell migration and proliferation (17). However, it remains to be determined whether the CDC42/CCND1 pathway serves a role in EOC, and whether miR-195 acts as a tumor inhibitor in EOC by targeting the CDC42/CCND1 signaling pathway.

The present study hypothesized that miR-195 may suppress cell proliferation, migration and invasion in EOC via inhibition of the CDC42/CCND1 pathway.

Materials and methods

Patients and tumor tissues. Human EOC tumor tissues and adjacent non-tumor tissues were obtained from 78 patients (mean age, 52.33±9.87 years; age range, 42–60 years) diagnosed with EOC via pathology, and who had undergone surgical excision with no prior anticancer treatments at The Second Hospital of Shanxi Medical University between January 2018 and December 2018. After resection, tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C for the following experiments. The clinical cases of the patients were acquired to analyze the association between miR-195, CDC42 and CCND1 expression levels and age, differentiation degree, TNM staging, serum CA12-5 content and International Federation of Gynecology and Obstetrics (FIGO) staging. The present work was approved by the Clinical Ethical Committee of The Second Hospital of Shanxi Medical University and written informed consent was obtained from all patients before the start of the study.

Cell culture. The wild-type human ovarian epithelial cell line HS832.Tc, and human EOC cell lines OVCA420, OVCAR-3, A2780 and SKOV3, were purchased from the Cell Bank of Chinese Academy of Sciences. All cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) or RPMI 1640 (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS (Sigma-Aldrich; Merck KGaA) and 0.5% penicillin or streptomycin and maintained in a 5% CO₂ humidified chamber at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was conducted to determine miR-195 expression in tissues and cells, as well as to determine CDC42 and CCND1

mRNA expression levels in OVCAR-3 cells, as previously described (12). Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature, according to the manufacturer's instructions. Following cDNA synthesis using the TaqMan™ miRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 65°C for 1 h. PCR was performed using TaqMan™ miRNA assay (Invitrogen; Thermo Fisher Scientific, Inc.) under the following thermocycling conditions for 42 cycles: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec, and a final extension step at 72°C for 5 min. Relevant primer sequences: hsa-miR-195 forward, 5'-ACACTCCAGCTGGGTAGCAGC ACAGAAAT-3' and reverse, 5'-TGGTGTCTGTTGAGATCG-3'; human U6 (used as an internal control of miR-195) forward, 5'-CTCGCTTCGGCAGCACACA-3' and reverse, 5'-AACGCT TCACGAATTTGCGT-3'; human CDC42 forward, 5'-GCC CGTGACCTGAAGGCTGTCA-3' and reverse, 5'-TGCTTT TAGTATGATGCCGACACCA-3'; human CCND1 forward, 5'-TCCTACTACCGCCTCACACA-3' and reverse, 5'-ACC TCCTCCTCCTCCTCT-3'; and human GAPDH (used as an internal control of CDC42 and CCND1) forward, 5'-AAGGTG AAGGTCCGAGTCA-3' and reverse, 5'-GGAAGATGGTGA TGGGATTT-3'. Relative quantification was performed using the 2^{-ΔΔC_q} method (18).

Western blotting. Western blotting was performed to detect the protein expression levels of CDC42 and CCND1 from tissues and cells, as previously described (13). Total proteins were extracted with RIPA buffer (Beyotime Institute of Biotechnology). The BCA kit (Beyotime Institute of Biotechnology) was used to determine the concentration. In total, 80 μg of each sample were resolved using 10% SDS-PAGE at 75 V for 2 h. After transfer to PVDF membranes at 350 mA for 2 h, membranes were blocked with 5% skim milk diluted in TBS for 1 h at room temperature. The membranes were probed overnight at 4°C with the following primary antibodies: Monoclonal anti-CDC42 (rabbit; 1:1,000; cat. no. 2466S; Cell Signaling Technology, Inc.), monoclonal anti-CCND1 (rabbit; 1:1,000; cat. no. 3300S; Cell Signaling Technology, Inc.) and monoclonal anti-GAPDH (rabbit; 1:1,000; cat. no. 5174S; Cell Signaling Technology, Inc.; used as an internal control). After probing with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG; 1:5,000; cat. no. TA130059; OriGene Technologies, Inc.) at room temperature for 1 h, proteins were detected using an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.), and an Alpha Innotech instrument (Bio-Rad Laboratories, Inc.) was used to scan and quantify the images. GAPDH was used to normalize the signals.

Immunohistochemistry. The human EOC tumor tissues and adjacent non-tumor tissues were collected, fixed with 4% paraformaldehyde at 25°C for 24 h and dehydrated with a sucrose solution (gradient, 10, 20 and 30%) at room temperature. After the tissues were harvested and dehydrated with 30% sucrose overnight, these were sectioned at 15-μm thickness using a freezing microtome (Leica CM1900; Leica Microsystems GmbH). The sections were washed three times for 5 min each time in 0.01 M PBS. The endogenous oxidase

activity was blocked with 3% hydrogen peroxide at 25°C in a closed environment for 15 min. Then, sections were washed three times (5 min/time) with PBS and incubated with 2% goat serum (Sigma-Aldrich; Merck KGaA) at 37°C in a wet box for 20 min. The antigen retrieval protocol was performed at 95°C in a microwave for 15 min with 0.01 M citrate buffer (pH 6.0). The tissue sections were then incubated for 30 min at 37°C in a water bath, followed by overnight incubation at 4°C with a polyclonal CDC42 antibody (rabbit; 1:300; cat. no. 2462S, Santa Cruz Biotechnology, Inc.) or a polyclonal CCND1 antibody (rabbit; 1:200; cat. no. 12531S, Santa Cruz Biotechnology, Inc.). After washing with 0.1 mol/l PBS three times for 5 min, the sections were incubated for 30 min at 37°C with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. W10814; Thermo Fisher Scientific, Inc.), followed by three washes for 5 min with 0.1 mol/l PBS and added liquid detergent. The 3,3'-diaminobenzidine staining reaction was performed at room temperature for 30 sec, followed by the addition of hematoxylin at room temperature for 15 min for counterstaining and differentiation for 1 sec. The negative control was PBS. After the slides were naturally dried, sealed the slides with neutral resin (Beyotime Institute of Biotechnology). Observation was performed under light microscope at x40 magnification and analysis was performed using ImageJ software (v1.8.0, National Institutes of Health).

RNA interference and transfection assays. For overexpression or knockdown of miR-195, miR-195 mimic (5'-UAGCAG CACAGAAUAUUGGC-3'), miR-195 mimic NC (5'-UUC UCCGAACGUGUCACGUTT-3'), miR-195 inhibitor (5'-GCC AAUAUUUCUGUGCUGCUA-3') and inhibitor-NC (5'-CAA UAUAUUUCUGUGCUGCUA-3') all were from Shanghai GenePharma Co., Ltd. Transfection into OVCAR-3 cells were performed using Lipofectamine® 2000 Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

To generate OVCAR-3 cells overexpressing CDC42 or CCND1, pcDNA 3.1-CDC42 and pcDNA 3.1-CCND1 (Generay Biotech Co., Ltd.) were cloned into a pcDNA 3.1 vector (Promega Corporation) and transfected into OVCAR-3 cells at 10 nM concentration. Lipofectamine® 2000 Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to mediate the transfection. The empty vector was used as the control. To knockdown CDC42 or CCND1 expression in OVCAR-3 cells, HS_CDC42 small interfering (si)RNA (5'-CATCAGATTTTGAAAATATTTAA-3'), HS_CCND1 siRNA (5'-GCCACAGATGTGAAGTTCA-3') and NC siRNA (5'-AATTCTCCGAACGTGTCACGT-3'), provided by GeneCopoeia, Inc., were transfected into OVCAR-3 cells using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) at 50 nM, according to the manufacturer's instructions.

Cell proliferation evaluation. Cell proliferation was evaluated using a Bromodeoxyuridine (BrdU) incorporation assay and a colony formation assay. The BrdU incorporation assay was performed at 24 h following transfection of OVCAR-3 cells, as previous reported (19). Colony formation was performed at 48 h after transfection, as previous reported (20).

Cell cycle and cell apoptosis assay. After transfection for 48 h, cell cycle and apoptosis were detected using flow cytometry (21). The cell cycle was detected using 20 µg/ml PI (Becton, Dickinson and Company) at 37°C for 30 min in dark. Cell apoptosis was detected using a BD apoptosis assay kit (Becton, Dickinson and Company) according to the manufacturer's instruction. A flow cytometer (Flow Sight; Merck KGaA) was used to assess cell cycle distribution and cell apoptosis. Cell cycle was analyzed using a FACS Calibur system (Cytek Development Inc.). FlowJo software (v10.0.7; Becton, Dickinson and Company) was used to analyze the percentage of apoptotic cells.

Cell migration and invasion assay. A total of 24 h after transfection, cell migration was assessed using the wound healing assay. Cells were scrapped vertically using pipette head tips. Then, cell were washed with PBS and cultured with serum-free medium at 37°C for 24 h.

A total of 48 h after transfection, cell invasion was measured using a Transwell invasion assay, as described in a previous study (22). Wound migration were observed under a light microscope at x40 magnification and cell invasion were observed under a light microscope at x200 magnification. Migrated cell and invaded cell were analyzed using ImageJ software (v1.8.0, National Institutes of Health).

Statistical analysis. SPSS 15.0 software (SPSS, Inc.) was used for statistical analyses. Each experiment was repeated three times. Data are presented as the mean ± standard deviation. One-way ANOVA followed by Tukey's multiple comparison test was used to compare the data between groups. Correlation between miR-195 and CDC42 or CCND1 was analyzed using Pearson's correlation coefficient. Expression levels of miR-195, CDC42 and CCND1 in healthy tissues and EOC tissues were compared using a paired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of miR-195, CDC42 and CCND1 in EOC tissues and cells. RT-qPCR results demonstrated that miR-195 was significantly downregulated in EOC tissues (Fig. 1A). Moreover, western blotting and immunohistochemistry results indicated that CDC42 and CCND1 were significantly upregulated in EOC tissues (Fig. 1B and C). It was identified that the downregulation of miR-195 or increased expression levels of CDC42 and CCND1 were associated with a reduced degree of differentiation, advanced clinical TNM stage, higher serum CA125 content and advanced FIGO staging (Table I).

RT-qPCR results suggested that miR-195 was downregulated in EOC tissues, while western blotting results identified that CDC42 and CCND1 were upregulated in various EOC cell lines, including OVCA420, OVCAR-3, A2780 and SKOV3 cells. Additionally, the lowest expression of miR-195 and the highest expression levels of CDC42 and CCND1 occurred in OVCAR-3 cells (Fig. 1D and E). Thus, subsequent experiments were performed in OVCAR-3 cells.

miR-195 could regulate the expression levels of CDC42 and CCND1. It was found that the miR-195 mRNA expression was

Table I. Associations of miR-195, CDC42 and CCND1 expression levels with the clinicopathological features in 78 patients with epithelial ovarian cancer.

Clinicopathologic parameters	Number	miR-195 expression	P-value	CDC42 expression	P-value	CCND1 expression	P-value
Age, years							
40-49	38	0.46±0.09	0.925	1.78±0.18	0.738	1.62±0.17	0.622
50-60	40	0.44±0.07		1.81±0.24		1.58±0.14	
Differentiation degree							
Low	26	0.34±0.06	<0.01	1.88±0.26	<0.01	1.74±0.16	<0.01
Middle	23	0.41±0.08		1.79±0.18		1.63±0.15	
High	29	0.55±0.10		1.62±0.13		1.52±0.13	
TNM stage							
I	23	0.61±0.12	<0.01	1.63±0.14	<0.01	1.53±0.13	<0.01
II	36	0.54±0.10		1.76±0.16		1.66±0.16	
III-IV	19	0.38±0.07		1.92±0.28		1.78±0.17	
Serum CA125 content (U/ml)							
>83	53	0.37±0.07	0.007	1.86±0.25	0.003	1.77±0.15	0.004
<83	25	0.63±0.13		1.58±0.11		1.52±0.12	
FIGO staging							
I	23	0.61±0.12	<0.01	1.63±0.14	<0.01	1.53±0.13	<0.01
II	35	0.53±0.09		1.77±0.15		1.64±0.15	
III	15	0.42±0.08		1.85±0.15		1.76±0.16	
IV	5	0.37±0.08		2.02±0.20		1.95±0.18	

Data are presented as the mean ± SD. FIGO, International Federation of Gynecology and Obstetrics.

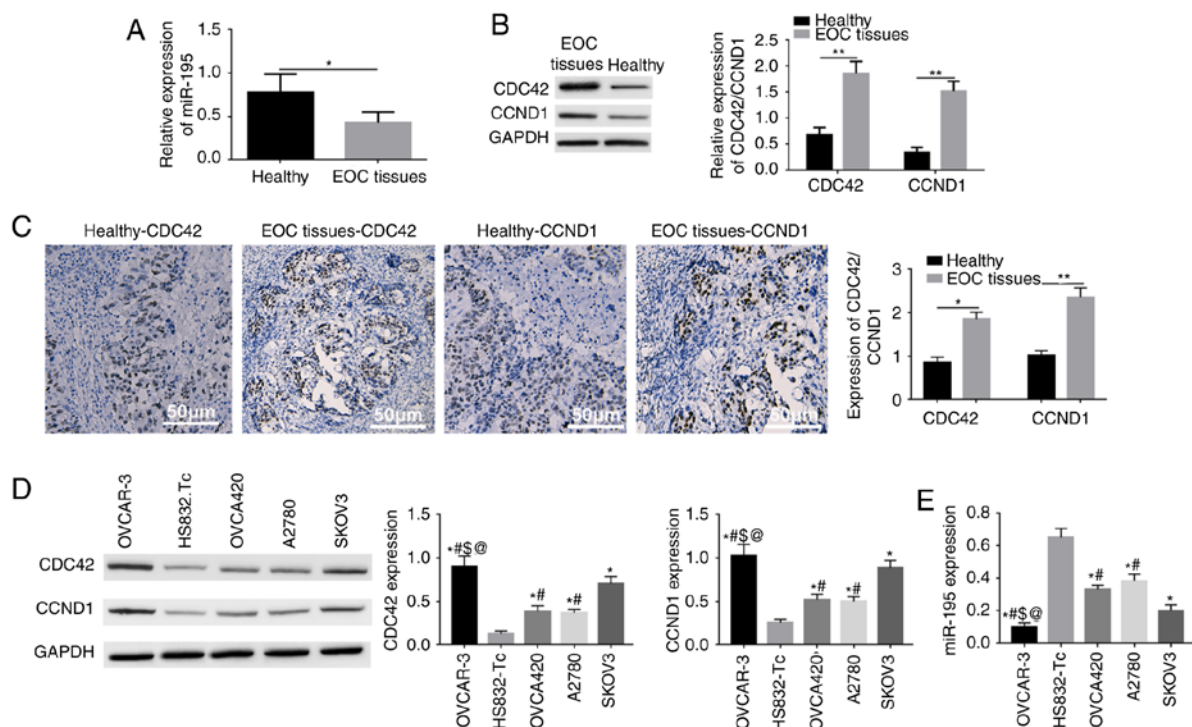


Figure 1. miR-195 is downregulated, while CDC42 and CCND1 are upregulated in human EOC tissues and cells. (A) mRNA expression of miR-195 in human EOC tissues. (B) Protein expression levels of CDC42 and CCND1 in human EOC tissues. (C) Immunohistochemical staining of CDC42 and CCND1 in human EOC tissues. Scale bar, 100 μ m. (D) Protein expression levels of CDC42 and CCND1 in human EOC cells. (E) mRNA expression of miR-195 in diverse human EOC cells. Data in Fig. 1A-C were analyzed using a paired t-test. * P <0.05, ** P <0.01 vs. healthy tissues. Data in the Fig. 1D were analyzed using a one-way ANOVA. * P <0.05 vs. HS832-Tc cells; # P <0.05 vs. SKOV3 cells; § P <0.05 vs. OVCA420 cells; @ P <0.05 vs. A2780 cells. EOC, epithelial ovarian carcinoma; miR, microRNA; CDC42, Cell division cycle 42; CCND1, cyclin D1.

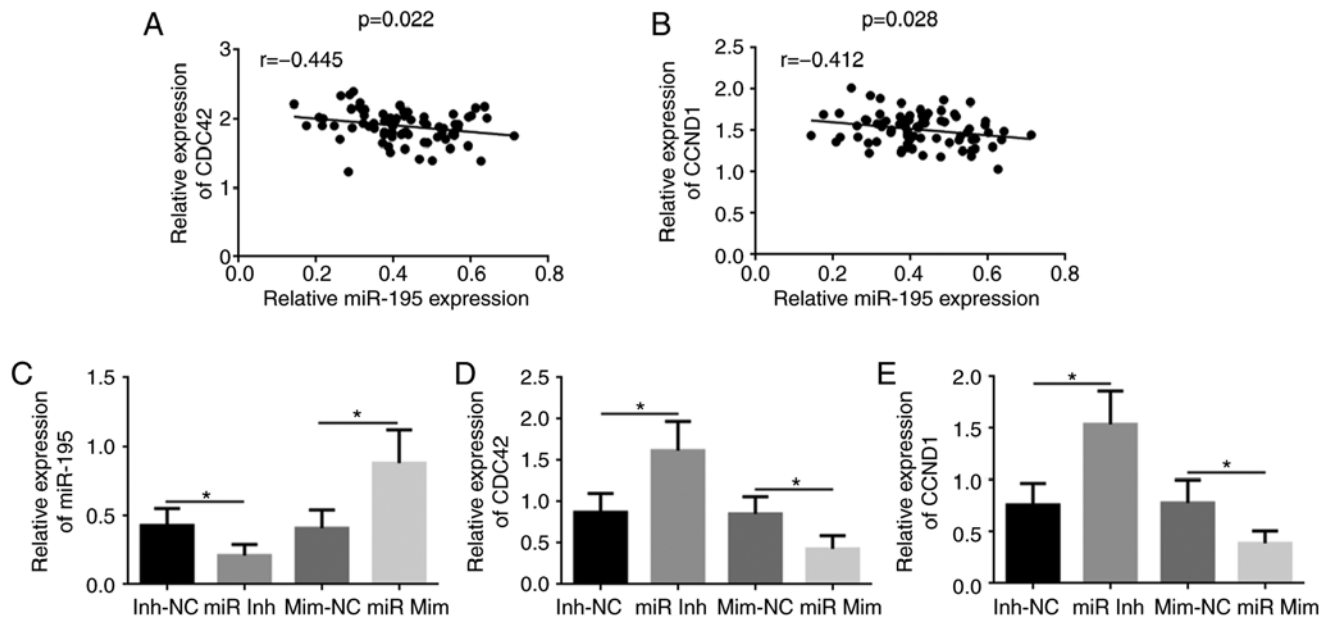


Figure 2. miR-195 can regulate CDC42 and CCND1 expression levels. Correlation between miR-195 and (A) CDC42 and (B) CCND1 expression levels in human epithelial ovarian carcinoma tissues were analyzed using Pearson's correlation coefficient. (C) mRNA expression of miR-195 in OVCAR-3 cells transfected with miR-195 mimic, mimic-NC, miR-195 inhibitor and inhibitor-NC. mRNA expression levels of (D) CDC42 and (E) CCND1 with overexpression or knockdown of miR-195 in OVCAR-3 cells. Data were analyzed using one-way ANOVA. * $P < 0.05$. miR, miR-195; Mim, mimic; NC, negative control; Inh, inhibitor; CDC42, Cell division cycle 42; CCND1, cyclin D1.

negatively correlated with CDC42 and CCND1 protein expression levels in human EOC tissues (Fig. 2A and B). Transfection of the miR-195 mimic led to significantly increased expression of miR-195 in OVCAR-3 cells compared with the mimic-NC group, while the miR-195 inhibitor significantly decreased miR-195 expression compared with the miR-195 inhibitor-NC group (Fig. 2C). miR-195 knockdown resulted in the upregulation of CDC42 and CCND1, while miR-195 overexpression caused downregulation of CDC42 and CCND1 in OVCAR-3 cells (Fig. 2D and E).

miR-195 represses cell proliferation by inhibiting CDC42 and CCND1 expression levels in OVCAR-3 cells *in vitro*. In order to investigate whether the effect of miR-195 in cell proliferation and apoptosis was CDC42/CCND1 dependent, OVCAR-3 cells overexpressing CDC42 or CCND1 and knocking down CDC42 or CCND1 in OVCAR-3 cells were generated (Fig. S1). RT-qPCR results indicated that knockdown of miR-195 significantly promoted CDC42 and CCND1 expression levels, but transfection with CDC42 or CCND1 siRNA reversed this trend, and induced downregulation of CDC42 or CCND1 in OVCAR-3 cells (Fig. 3A and B). By contrast, overexpression of miR-195 significantly reduced CDC42 and CCND1 expression levels, but transfection with a CDC42 or CCND1 overexpression vector resulted in upregulation of CDC42 and CCND1 (Fig. 3C and D).

Knockdown of miR-195 resulted in enhanced cell proliferation, with significantly increased percentages of BrdU-positive cells and clone formation; however, this enhanced cell proliferative ability was significantly reversed after CDC42 or CCND1 siRNA co-transfection (Fig. 3E and F). Overexpression of miR-195 and co-transfection with miR-195 mimic and the CDC42 or CCND1 overexpression vector demonstrated opposite outcomes to treatment with miR-195 inhibitor and CDC42

or CCND1 siRNAs (Fig. 3E and F). These findings indicated that miR-195 repressed cell proliferation by inhibiting CDC42 and CCND1 expression levels in OVCAR-3 cells *in vitro*.

miR-195 inhibits cell cycle entry and promotes apoptosis in OVCAR-3 cells by downregulating CDC42 and CCND1 expression levels *in vitro*. Flow cytometry with PI staining identified that the miR-195 inhibitor increased cell cycle entry, which arrested OVCAR-3 cells at S and G₂ phases, with significantly fewer EOC cells appearing in G₁ phase (Fig. 4). However, following co-transfection with CDC42 or CCND1 siRNA, cell cycle entry was inhibited, with reduced S and G₂ phase cells and increased G₁ phase OVCAR-3 cells compared with cells transfected with NC siRNA. Additionally, overexpression of miR-195 restrained OVCAR-3 cell cycle entry, while overexpression of CDC42 or CCND1 reversed this pattern and facilitated cell cycle entry (Fig. 4).

Flow cytometry with Annexin V-FITC/PI staining suggested that knockdown of miR-195 reduced apoptosis of OVCAR-3 cells *in vitro* compared with the miR-195 inhibitor-NC group. Conversely, following co-transfection with CDC42 or CCND1 siRNA, the apoptotic rate was significantly increased compared with the NC siRNA (Fig. 5). Moreover, transfection with miR-195 mimic and overexpression vectors of CDC42 or CCND1 exerted the opposite effects to the miR-195 inhibitor and CDC42 or CCND1 siRNAs (Fig. 5).

miR-195 suppresses migration and invasion in OVCAR-3 cells by inhibiting CDC42 and CCND1 expression levels *in vitro*. The wound healing assay results demonstrated that knockdown of miR-195 caused increased OVCAR-3 cell migration, compared with the miR-195 inhibitor-NC group. Furthermore, OVCAR-3 cells migrated toward the wound more slowly following co-transfection with CDC42 siRNA compared

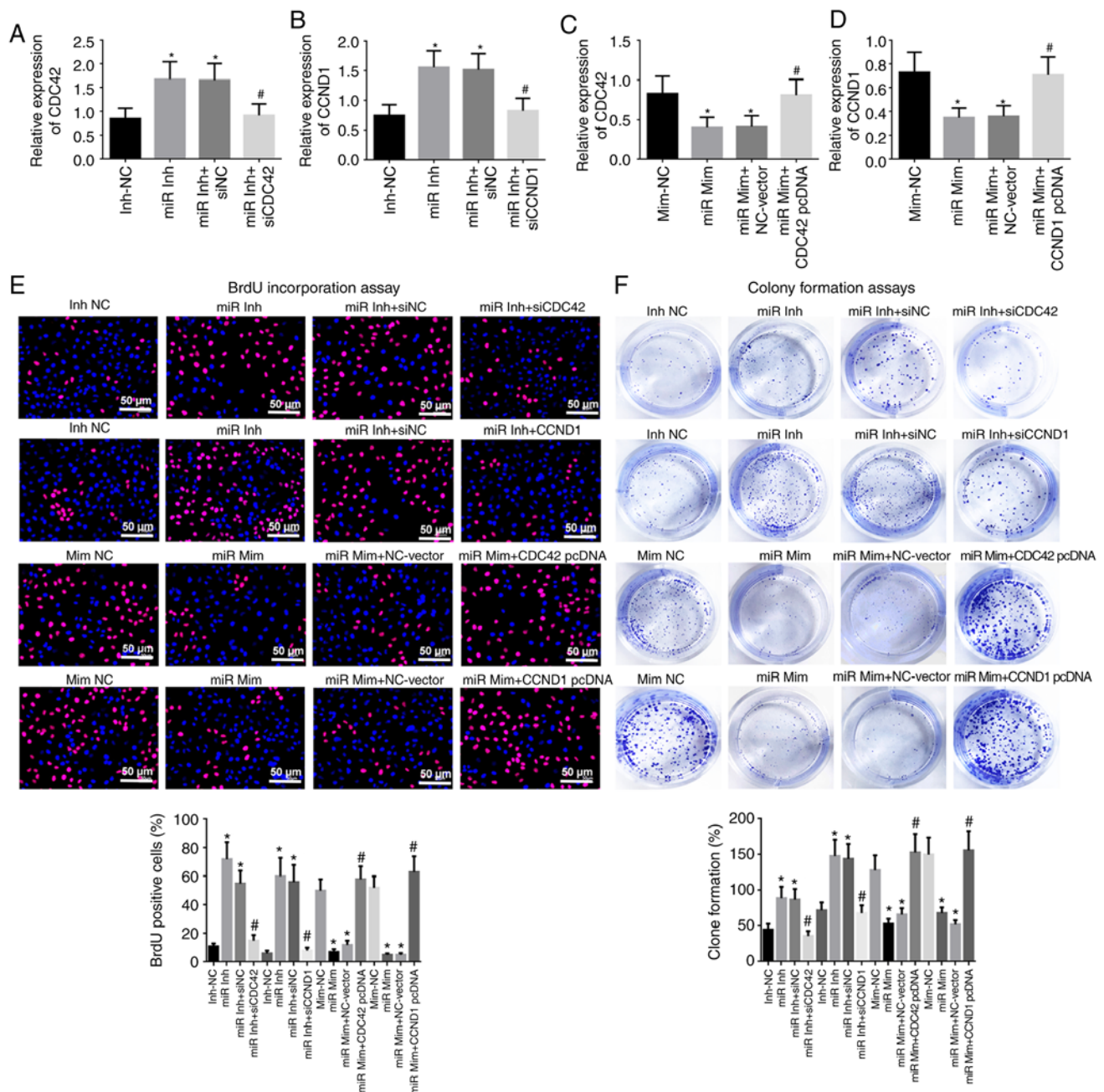


Figure 3. miR-195 inhibits cell proliferation by reducing CDC42 and CCND1 expression levels in OVCAR-3 cells *in vitro*. mRNA expression levels of (A) CDC42 and (B) CCND1 in OVCAR-3 cells transfected with miR-195 inhibitor, inhibitor-NC, miR-195 inhibitor + NC and miR-195 inhibitor + CDC42 or CCND1 siRNAs. mRNA expression levels of (C) CDC42 and (D) CCND1 in OVCAR-3 cells transfected with miR-195 mimic, mimic-NC, miR-195 mimic + NC-vector and miR-195 mimic + CDC42 or CCND1 pcDNA. (E) Cell proliferation was assessed using BrdU immunostaining at 24 h after transfection. Red, OVCAR-3 cells labeled with BrdU; blue, nuclei counterstained by BrdU. Scale bar, 50 μ m. (F) Evaluation of cell proliferation via colony formation assays at 48 h after transfection. Data were analyzed using one-way ANOVA. * P <0.05 vs. Inh-NC or Mim-NC groups; # P <0.05 vs. miR Inh-siNC or miR Mim + NC-Vector groups. BrdU, 5-bromo-2-deoxyuridine; miR, miR-195; Inh, inhibitor; NC, negative control; siRNA, short interfering RNA; Mim, mimic; CDC42, Cell division cycle 42; CCND1, cyclin D1.

with cells transfected with the NC siRNA. The migration of OVCAR-3 cells was significantly weakened after overexpression of miR-195 and reversed following transfection with the CDC42 or CCND1 overexpression vector (Fig. 6A and B).

The Transwell invasion assay identified a significant increase in the number of invading OVCAR-3 cells in the miR-195 inhibitor group compared with the miR-195 inhibitor-NC group; however, the number of invading OVCAR-3 cells was reduced following co-transfection with CDC42 or CCND1 siRNA compared with NC siRNA (Fig. 6C). In

addition, the miR-195 mimic induced a significant decrease in invading cells compared with the NC mimic, while a higher number of invading OVCAR-3 cells was observed in the group co-transfected with the CDC42 or CCND1 overexpression vector compared with the NC-vector group (Fig. 6B).

Discussion

EOC is the most frequent histopathological type of OC, with a 5-year survival rate of ~30% and the highest mortality rate

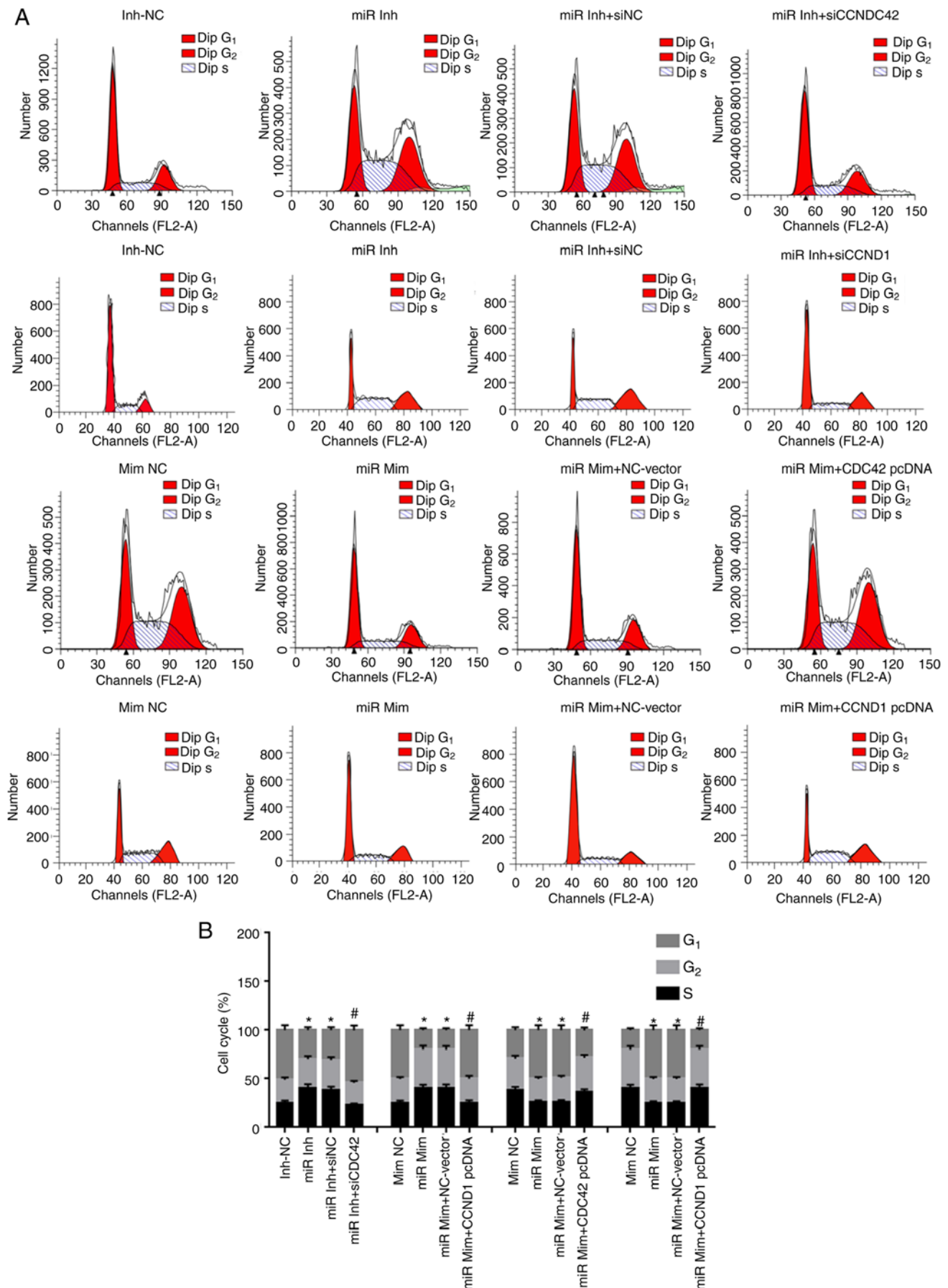


Figure 4. miR-195 inhibits cell cycle entry in OVCAR-3 cells by downregulating CDC42 and CCND1 expression levels *in vitro*. (A) Effects of miR-195, CDC42 and CCND1 on the percentage of PI-stained OVCAR-3 cells at G₁, S and G₂ phases. (B) Cell cycle of each group. Data were analyzed via one-way ANOVA. *P<0.05 vs. Inh-NC or Mim-NC groups; #P<0.05 vs. miR Inh-siNC or miR Mim + NC-Vector groups. miR, miR-195; Inh, inhibitor; NC, negative control, siRNA, short interfering RNA; Mim, mimic; CDC42, Cell division cycle 42; CCND1, cyclin D1.

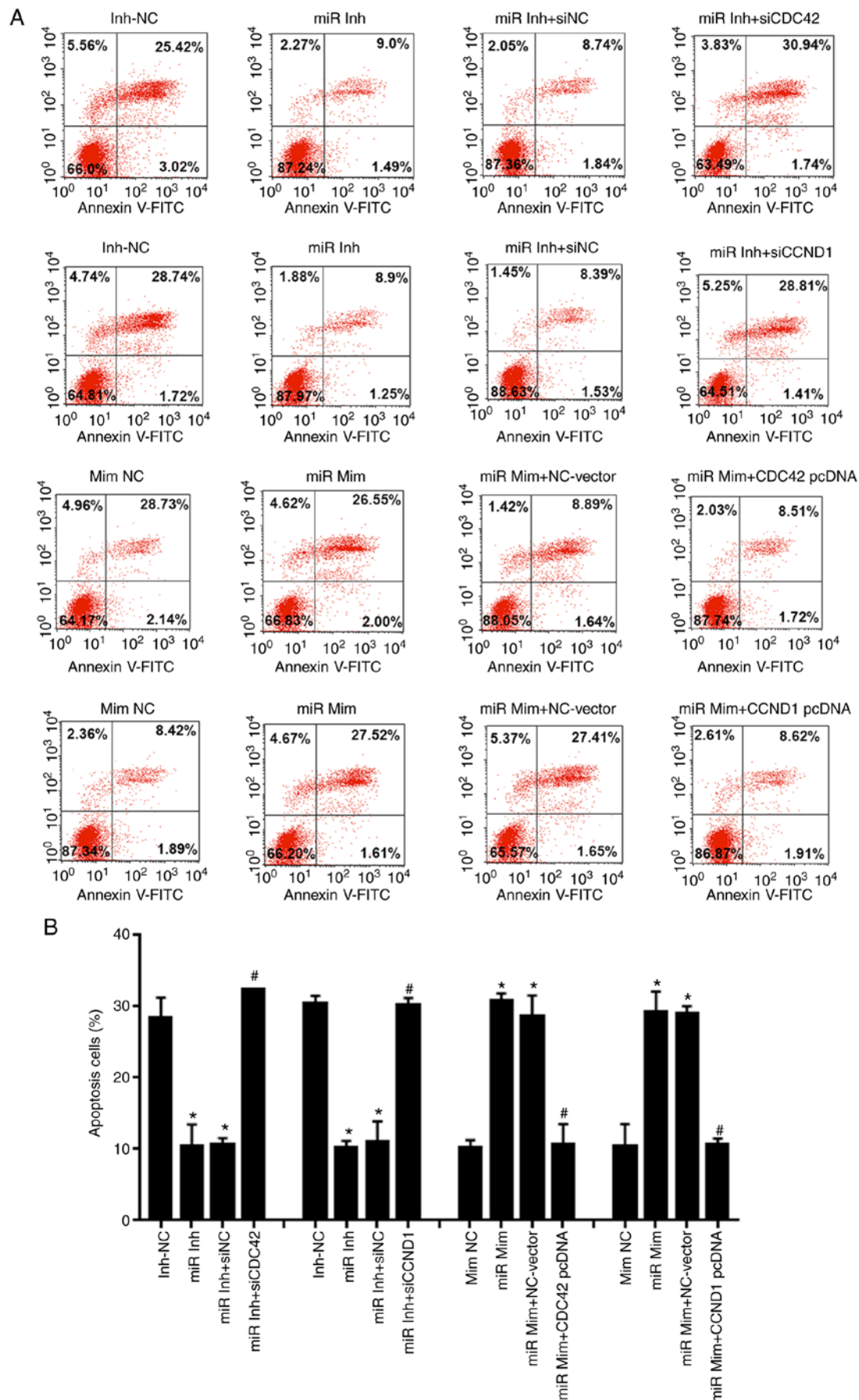


Figure 5. miR-195 promotes apoptosis in OVCAR-3 cells by downregulating CDC42 and CCND1 expression levels *in vitro*. (A) Effects of miR-195, CDC42 and CCND1 on cell apoptosis. (B) Apoptotic cells in each group. Data were analyzed using one-way ANOVA. * $P < 0.05$ vs. Inh-NC or Mim-NC groups; # $P < 0.05$ vs. miR Inh-siNC or miR Mim + NC-Vector groups. miR, miR-195; Inh, inhibitor; NC, negative control, siRNA, short interfering RNA; Mim, mimic; CDC42, Cell division cycle 42; CCND1, cyclin D1.

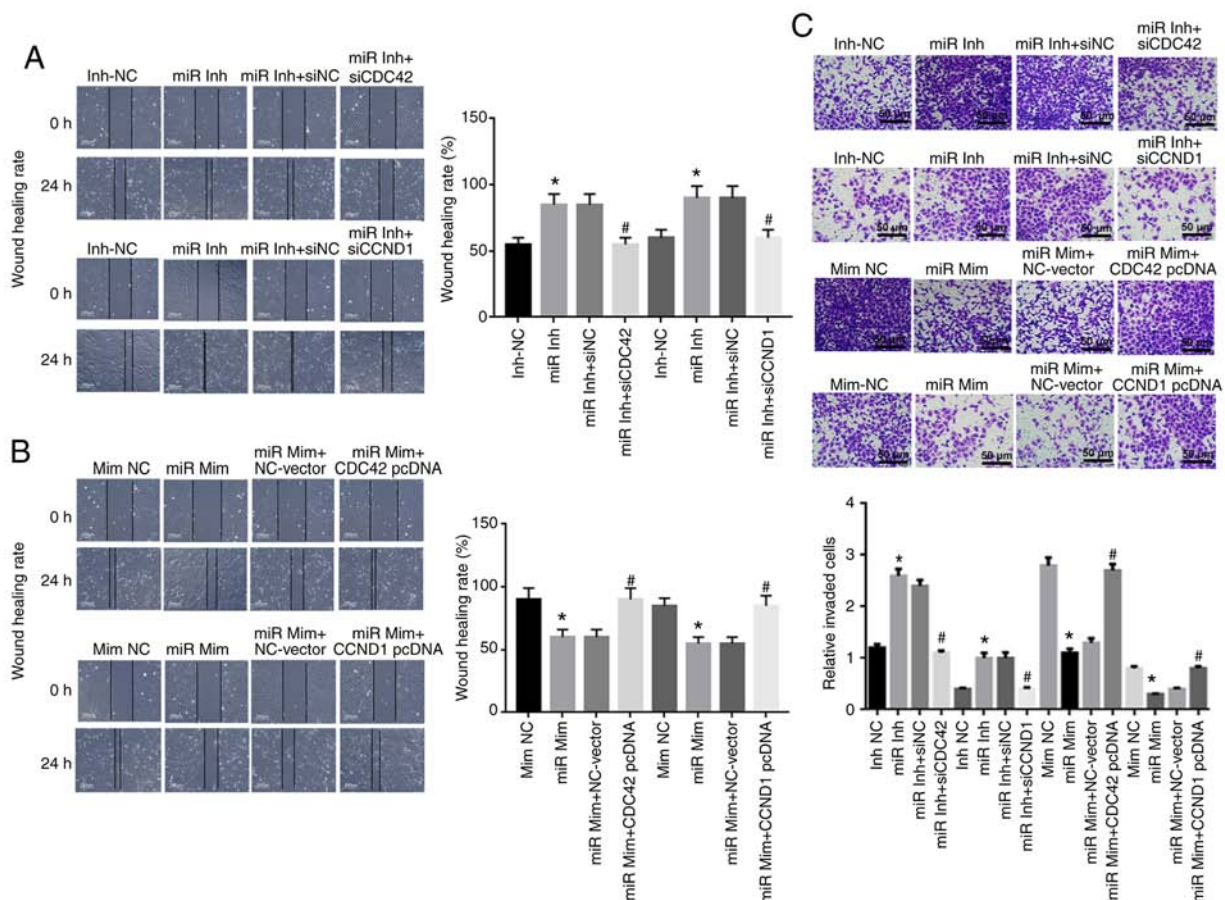


Figure 6. miR-195 suppresses cell migration and invasion in OVCAR-3 cells by inhibiting CDC42 and CCND1 expression levels *in vitro*. Effects of miR-195 (A) inhibitor or (B) mimic, CDC42 and CCND1 on OVCAR-3 cell migration using wound healing assay at 0 and 24 h after transfection. Scale bar, 200 μ m. (C) Effects of miR-195, CDC42 and CCND1 on OVCAR-3 cell invasion via Transwell invasion assay at 48 h after transfection. Scale bar, 50 μ m. Data were analyzed using one-way ANOVA. * P <0.05 vs. Inh-NC or Mim-NC groups; # P <0.05 vs. miR Inh-siNC or miR Mim + NC-Vector groups. miR, miR-195; Inh, inhibitor; NC, negative control, siRNA, short interfering RNA; Mim, mimic; CDC42, Cell division cycle 42; CCND1, cyclin D1.

of all gynecological tumors worldwide (1). Thus, EOC is a serious threat to the lives of women. As EOC is difficult to diagnose at an early stage, there are limited treatment options and high rates of metastasis and recurrence following operative therapy (7,8). Therefore, the identification of biological targets for the diagnosis and treatment of EOC is an urgent issue. In the present study, miR-195 expression was found to be downregulated in human EOC tissues and multifold cells, particularly in OVCAR-3 cells, compared with the corresponding control groups. Moreover, this downregulation of miR-195 was closely associated with reduced differentiation, higher clinical TNM stage and increased serum EOC marker/CA125 content. Thus, miR-195 may be a potential target of EOC.

Previous studies have reported that aberrant expression of miRNAs may be a marker of the development and progression of various tumors or cancer types, as they are associated with abnormal cell viability, proliferation, migration, invasion, apoptosis and differentiation (23-25). The present results suggested that miR-195 was downregulated in human EOC tissues and cells, which led to an increase in cell proliferation, cell cycle entry, migration and invasion, as well as a decrease in apoptosis of OVCAR-3 cells *in vitro*. Another published report regarding the role of miR-195 in OC indicated that miR-195 was downregulated in human OC serum samples, which resulted in an increase in cell proliferation and decreased apoptosis of

human OVCAR-3 cells, which was in line with the current findings that knockdown of miR-195 induced an increase in cell proliferation and a decrease in cell apoptosis. Previous studies have also observed downregulation of miR-195 in other cancer types. For example, decreased miR-195 expression in hepatocellular carcinoma tissues significantly promotes proliferation, invasion and tumorigenesis (26). Moreover, miR-195 represses cell proliferation, migration and invasion, and accelerates apoptosis in glioma by decreasing spalt like transcription factor 4 protein expression (27). Downregulation of miR-195 in colon carcinoma tissues is also associated with increased cell proliferation, migration and invasion (28). Furthermore, miR-195 is downregulated in PTC tissues and cells, which facilitates cell cycle progression by inducing apoptosis (29). These previous findings were in accordance with the present results, and to the best of our knowledge, the current study was the first to demonstrate that miR-195 is downregulated in human EOC tissues and cells, and is associated with the CDC42/CCND1 pathway.

The present results suggested that the molecular mechanism of miR-195 in EOC was achieved via inhibition of the CDC42/CCND1 pathway. Previous studies have revealed that CDC42 and CCND1 are targets of miR-195 in ESCC and PTC, respectively (15,16). Thus, the present study did not perform a luciferase reporter assay. The current results

indicated that expression levels of CDC42 and CCND1 were negatively correlated with miR-195 in EOC tissues. In addition, CDC42 and CCND1 had a corresponding opposite effect following interference of miR-195 expression, which indirectly suggested that CDC42 and CCND1 are components of a downstream pathway of miR-195. In the present study, knock-down of miR-195 effectively increased cell proliferation and migration, decreased cell apoptosis and elevated CDC42 and CCND1 protein expression levels. A previous study identified that inhibition of CDC42 expression may be a key mechanism via which miR-18a impairs cancer cell proliferation (30). In the current study, overexpression of miR-195 significantly decreased cell proliferation and migration, promoted cell apoptosis and reduced CDC42 and CCND1 protein expression levels; these effects were reversed by CDC42 and CCND1 overexpression. The CDC42/CCND1 signaling pathway has been reported to promote cell migration and proliferation in endothelial cells. In addition, activation of the CDC42/CCND1 pathway could induce cell proliferation, migration and invasion to increase Ewing sarcoma metastasis (31). Overexpression of CDC42 and CCND1 has also been shown to cause increased cell migration, changes in cell morphology, G₁/S phase cell cycle progression and decreased apoptosis in colorectal cancer cells (30). Collectively, these previous findings are in line with the present results, which found that overexpression of CDC42 and CCND1 was associated with enhanced cell proliferation, cell cycle progression, migration and invasion, as well as attenuated apoptosis in EOC cells. Therefore, miR-195 may suppress cell proliferation, migration and invasion in EOC via inhibition of the CDC42/CCND1 pathway.

There are limitations to the present study. First, a luciferase assay was not performed to directly confirm the relationship between miR-195 and CDC42. At present, evidence that CCND1 can act as a target of miR-195 has been reported in ESCC (15). The present results can only indirectly demonstrate that miR-195 could target CDC42 via assessing the reverse effect of CDC42 and CCND1 siRNA or CDC42 and CCND1 overexpression. Secondly, western blotting and RT-qPCR were not conducted to measure markers of migration, invasion and apoptosis in cells. Finally, the present study did not investigate AKT and its inhibitors on the effect of miR-195. A previous study revealed that miR-195 inhibited VEGFR2 and AKT signaling (12); therefore, further evidence regarding the underlying molecular mechanism of miR-195 is required.

In conclusion, the present study demonstrated that miR-195 was downregulated, while CDC42 and CCND1 were upregulated, in EOC tissues and cells, which led to increased malignancy of EOC. miR-195 can repress human EOC cell cycle entry, migration and invasion, and CDC42 and CCND1 serve as tumor promoters in EOC progression. Furthermore, it was identified that inhibition of the CDC42/CCND1 pathway via overexpression of miR-195 may be a possible mechanism for suppression of EOC progression. Thus, miR-195 may be an effective agent for EOC therapy and diagnosis via anti-proliferative, migration-inhibiting and pro-apoptotic effects via inhibition of the CDC42/CCND1 signaling pathway.

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Availability of data and materials

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

Authors' contributions

XH made substantial contributions to the conception and design of the study. QJ and JY performed the experiments. XS and HG participated in this experiment, and analyzed the data. JG contributed to acquisition of data. YG organized the figure and interpreted the data. XH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Clinical Ethical Committee of The Second Hospital of Shanxi Medical University, and written informed consent was obtained from all patients before the start of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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