MiR-15a-3p suppresses the growth and metastasis of ovarian cancer cell by targeting Twist1

B. FAN[1], L.-P. CHEN[1], Y.-H. YUAN[1], H.-N. XIAO[1], X.-S. LV[2], Z.-Y. XIA[1]

[1]Obstetrics and Gynecology, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan, China
[2]Gynecology Department, Maternal and Child Health Care Hospital, Laiwu, Shandong, China

Bo Fan and *Li Ping Chen* contribute equally to this article

Abstract. – **OBJECTIVE:** To investigate the roles of miR-15a-3p in ovarian cancer cell growth and metastasis.

PATIENTS AND METHODS: A key role of miR-15a-3p was identified via gene profiling and bioinformatics analysis. The impact of miR-15a-3p on ovarian cancer cell growth, migration and invasion was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), wound-healing and transwell invasion assays. Bioinformatics and luciferase reporter assays were applied to identify that twist family BHLH transcription factor 1 (Twist1) was the target gene of miR-15a-3p. The miR-15a-3p level and the expression of Twist1 were detected using quantitative Real-time polymerase chain reaction (qRT-PCR) assay. The expressions of N-cadherin and E-cadherin were measured by immunofluorescence staining. Small interfering RNA targeting Twist1 and pCDNA3.1 containing Twist1 were applied to decrease and increase the expression of Twist1, respectively.

RESULTS: miR-15a-3p was markedly down-regulated in ovarian cancer. Exogenous up-regulation of miR-15a-3p inhibited the growth, colony formation, migration and invasion of ovarian cancer cell in vitro. Furthermore, a xenograft model indicated that miR-15a-3p inhibited tumour growth and the metastatic potential of ovarian cancer cell in vivo. We found that Twist1 was the direct target of miR-15a-3p in ovarian cancer and that its expression was negatively correlated with the level of miR-15a-3p in ovarian cancer tissues. Up-regulation of miR-15a-3p rescued the inhibitory impact of miR-15a-3p on ovarian cancer cell growth, migration and invasion. Finally, down-regulation of Twist1 mimicked the suppressive effects of miR-15a-3p on ovarian cancer cell.

CONCLUSIONS: We demonstrated that miR-15a-3p is down-regulated in ovarian cancer. Up-regulation of miR-15a-3p restrains the growth and metastasis of ovarian cancer cell by regulating Twist1.

Introduction

Ovarian cancer is one of the most common female malignant tumours and the leading cause of cancer mortality among women[1-3]. Despite the improvements in the treatment of this disease, the local infiltration and metastases of ovarian cancer remain major challenges. Previous investigations indicate that cancer cell metastasis is a complicated process that enables tumour cells to escape from primary tumour sites and form distant metastases[4,5]. Accumulating evidence suggests that epithelial-mesenchymal transition (EMT) is a crucial process that exerts vital roles in the metastasis of various cancer types, including breast cancer, colorectal carcinoma and ovarian cancer[6,7]. During the EMT process, cancer cells acquire mesenchymal cell characteristics and lose epithelial cell markers, which increase migration and invasion capabilities. The EMT process is regulated by many transcription factors, including Snail Family Transcriptional Repressor 1/2 (Snail1/2) and Twist1[8]. Many studies have demonstrated the core role of Twist1 in the cancer cell EMT process, and the up-regulation of Twist1 remarkably induces morphological changes and increases the metastasis of cancer cells by regulating the expression of c-Fos proto-oncogene (c-fos), metalloproteinase-9 (MMP-9), N-cadherin and E-cadherin.

MicroRNAs (miRNAs), which are small non-coding RNAs, post-transcriptionally regulate the expression of downstream target genes through binding to the 3'-untranslated region (3'-

Corresponding Author: Zhi Yong Xia, MD; email: xiazhiyongxzy@foxmail.com

UTR) of target mRNAs[9,10]. In particular, miRNAs act as either tumour suppressors or tumour promoters by targeting anti-oncogenes or oncogenes, respectively[11]. A large number of studies [12,13] have demonstrated that dysregulation of miRNAs is closely associated with human cancer progression. In hepatocellular carcinoma (HCC), miR-382 is significantly down-regulated, and miR-382 inhibits HCC metastasis by targeting Golgi Membrane Protein 1 (GOLM1). In oesophageal carcinoma, miR-1 promotes the proliferation and suppresses the apoptosis of oesophageal cancer cells by negatively regulating the expression of the SRC proto-oncogene (Src) gene.

MiR-15a is a part of the miR-15a/16 cluster, which is encoded[14,15] by its host gene deleted in leukaemia 2. The members of the miR-15a/16 cluster have been identified as tumour promoters or tumour suppressors in multiple tumour types, including prostate carcinoma, pituitary adenomas and lymphocytic lymphoma[16]. In cervical cancer, up-regulation of miR-15a-3p promotes the radio-sensitivity of cancer cells by regulating tumour protein D52[17]. In addition, down-regulation of miRNA-15a-3p is associated with the prognosis of patient and negatively regulates the proliferation and migration of osteosarcoma. MiR-15a-3p is also down-regulated in gastric carcinoma and inhibits gastric cancer cell metastasis by negatively regulating the expression of the Twist1 gene. Since miR-15a-3p has been reported to be very important in several cancers, the precise mechanism by which miR-15a-3p regulates ovarian cancer cells has not been investigated, and further investigation of the roles of miR-15a-3p in the progression of ovarian cancer is required[18].

Currently, we demonstrate that the level of miR-15a-3p is markedly decreased in ovarian cancer. Up-regulation of miR-15a-3 significantly inhibits the growth and metastatic potential of ovarian cancer cells both *in vitro* and *in vivo*. In addition, we demonstrate that Twist1 is the target gene of miR-15a-3p and plays a crucial role in the inhibitory effect of miR-15a-3p on ovarian cancer. Our study elucidates a molecular link between Twist1 and miR-15a-3p that determines the growth and metastasis of ovarian cancer.

Patients and Methods

Ovarian Cancer Tissues and Cell Lines

Forty-five pairs of ovarian cancer and paracancerous tissues were obtained from the Maternal and Child Health Care Hospital (Laiwu, Shandong, China). This research was approved by the Institutional Research Committee of the Maternal and Child Health Care Hospital, and informed consent was obtained from all patients. Written informed consent for participation in the study was obtained from all patients before participation in this study. Ovarian cancer cell lines (OVCR3, SKOV3 and A2780) and the human ovarian surface epithelial cell line HOSEpiC were obtained from Guangzhou Jennio Biotech Co., Ltd. (Guangzhou, Guangdong, China). 293T cells were purchased from Nanjing Cobioer Biotech Co., Ltd. (Nanjing, Jiangsu, China). 293T, A2780 and OVCR3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 1% pen-strep (Beyotime, Nanjing, Jiangsu, China). SKOV3 cells were maintained in McCoy's 5a modified medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% pen-strep (Beyotime, Nanjing, Jiangsu, China). All cells were cultured in an incubator containing 5% CO_2 at 37°C.

Cell Transfections

Cells (1×10^5) were seeded in 6-well plates and transfected with 25 nM miR-15a-3p mimic or the negative control (miR-NC) (GenePharma, Shanghai, China). The Twist1 overexpression vector pCDNA3.1-Twist1 was synthesized by GenePharma (Shanghai, China). Small interfering RNA (siRNA) was used for the knockdown of Twist1. Non-targeting control siRNA (siCon) was used as an internal control. Pooled siRNAs against Twist1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl- 2-H-Tetrazolium Bromide (MTT) Assay

Cells were cultured in 96-well culture plates for 1, 2, 3 or 4 days. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the cells were incubated for 4 h. Next, the supernatant was removed, and 200 µl of dimethyl sulfoxide (DMSO) were added to 96-well plates. The optical density (OD) value was detected at 490 nm [19].

Colony Formation Assay

Cells were cultured in 96-well plates for 4 weeks. Then, colonies were stained with 1% crystal violet, and the number of colonies for each group was analyzed.

Wound-Healing Assay

Cells were cultured in 6-well plates for 24 h until confluent. Then, a wound was made using a 200 μl pipette tip. Non-adherent cells were removed, and cells were cultured in medium without fetal bovine serum (FBS). The wound areas were photographed at 0 h and 48 h[20].

Transwell Invasion Assay

Cells that were transfected with miR-15-3p or miR-NC were cultured in the upper chamber with an 8 μm pore size that was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and the chamber was placed into 24-well plates. The complete medium containing 20% fetal bovine serum (FBS) was added to the lower chamber. After 36 h, the invaded cells were stained using 1% crystal violet. The number of invaded cells was counted in 5 fields per filter[21].

Luciferase Reporter Assay

The wild-type (wt) or mutant (mut) 3'-untranslated regions (3'-UTRs) of the Twist1 gene were amplified from the reverse-transcribed cDNA of SKOV3 cells and cloned into the pGL3 luciferase reporter plasmid. The wt or mut pGL3-Twist1-3'-UTR and miR-15a-3p were co-transfected into 293T cells using Lipo3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the luciferase activity was detected using a luciferase assay system (Promega, Madison, WI, USA).

Immunoblotting Assay

Total protein was extracted using RIPA buffer (Pierce Biotechnology, Waltham, MA, USA). Proteins (30 μg) were separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDA-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked using 5% non-fat milk and then incubated with Twist1 antibody (Cell Signaling Technology, Danvers, MA, USA) or anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA). Next, the membrane was washed three times using Tris-buffered saline Tween (TBST) solution and incubated with secondary antibody. Target bands were detected by an enhanced chemiluminescence (ECL) detection system (Amersham Bioscience, Piscataway, NJ, USA).

Immunofluorescence Staining

First, cells were fixed using methanol, and the cell membrane was permeabilized using 0.1% Triton X-100 (Sangon Biotech, Shanghai, China). Next, the cells were incubated with primary antibodies (N-cadherin or E-cadherin) overnight at 4°C. After 24 h, the cells were incubated with FITC-conjugated goat anti-rabbit antibody for 2 h. Cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) (Beyotime Biotech, Haimen, Jiangsu, China). Images were photographed using an inverted microscope (Carl Zeiss, Hallbergmoos, Germany).

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

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed to single-stranded cDNA using reverse transcription reagent (TaKaRa, Dalian, Liaoning, China). The qRT-PCR assay was conducted using SYBR Mix (TaKaRa, Dalian, Liaoning, China) on a Bio-Rad Multicolor RT-PCR System (Bio-Rad, Hercules, CA, USA). The level of miR-15a-3p was measured using a microRNA first-strand synthesis and miRNA quantitation kit (TaKaRa, Dalian, Liaoning, China). The threshold cycle (Ct) values of GAPDH and U6 were used as internal controls to normalize the levels of Twist1 and miR-15a-3p. The threshold cycle value was applied to analyze the fold change in the transcript level using the $2^{-\Delta\Delta CT}$ method. The primers used for PCR were as follows (Forward Primer and Reverse Primer, respectively): GAPDH: 5′-TGTGGGCATCAATGGATTTGG-3′ and 5′-ACACCATGTATTCCGGGTCAAT-3′; Twist1: 5′-GGACAAGCTGAGCAAGATTCA-3′ and 5′-CGGAGAAGGCGTAGCTGAG-3′; E-cadherin: 5′-CGAGAGCTACACGTTCACGG-3′ and 5′-GGGTGTCGAGGGAAAAATAGG-3′; N-cadherin: 5′-TTTGATGGAGGTCTCCTAACACC-3′ and 5′-ACGTTTAACACGTTGGAAATGTG-3′; U6: 5′-AAAGCAAATCATCGGACGACC-3′ and 5′-GTACAACACATTGTTTCCTCGGA-3′; miR-15a-3p: 5′-GGGGCAGGCCATATTGTG-3′ and 5′-TGCGTGTCGTGGAGTC-3′.

Xenografted Tumour

Experimental animal procedures were approved by the Institutional Animal Care and Use

Committee (IACUC) of the Maternal and Child Health Care Hospital (Laiwu, Shandong, China). MiR-NC- or miR-15a-3p-transfected SKOV3 cells (1×10^6) were subcutaneously inoculated into female nude mice. The tumour size was measured using a calliper once a week and calculated as the volume (0.5 x length x width)[22].

Experimental Metastasis Assay

MiR-15a-3p- or miR-NC-transfected SKOV3 cells (5×10^5) were injected into nude mice via the lateral tail vein. Mice were sacrificed four weeks after inoculation[23]. The number of metastatic lung nodules was determined using an inverted microscope (Carl Zeiss, Hallbergnoos, Germany). Animal experiments were approved by the IACUC of the Maternal and Child Health Care Hospital (Laiwu, Shandong, China).

Results

MiR-15a-3p is Down-regulated in Ovarian Cancer

To analyze the dysregulation of miRNAs in ovarian cancer, microRNA array analysis was conducted to compare the miRNA in ovarian carcinoma tissues with corresponding normal tissues using GSE83693, and we found that miR-15a-3p was remarkably down-regulated in ovarian cancer tissues (Figure 1A). Then, we detected the levels of miR-15a-3p in 45 pairs of ovarian cancer tissues and adjacent normal tissues using qRT-PCR to investigate the levels of miR-15a-3p in ovarian cancer and paracancerous tissues. As shown in Figure 1B, the level of miR-15a-3p was significantly lower in ovarian cancer tissues than in normal tissues. Finally, the levels of miR-15a-
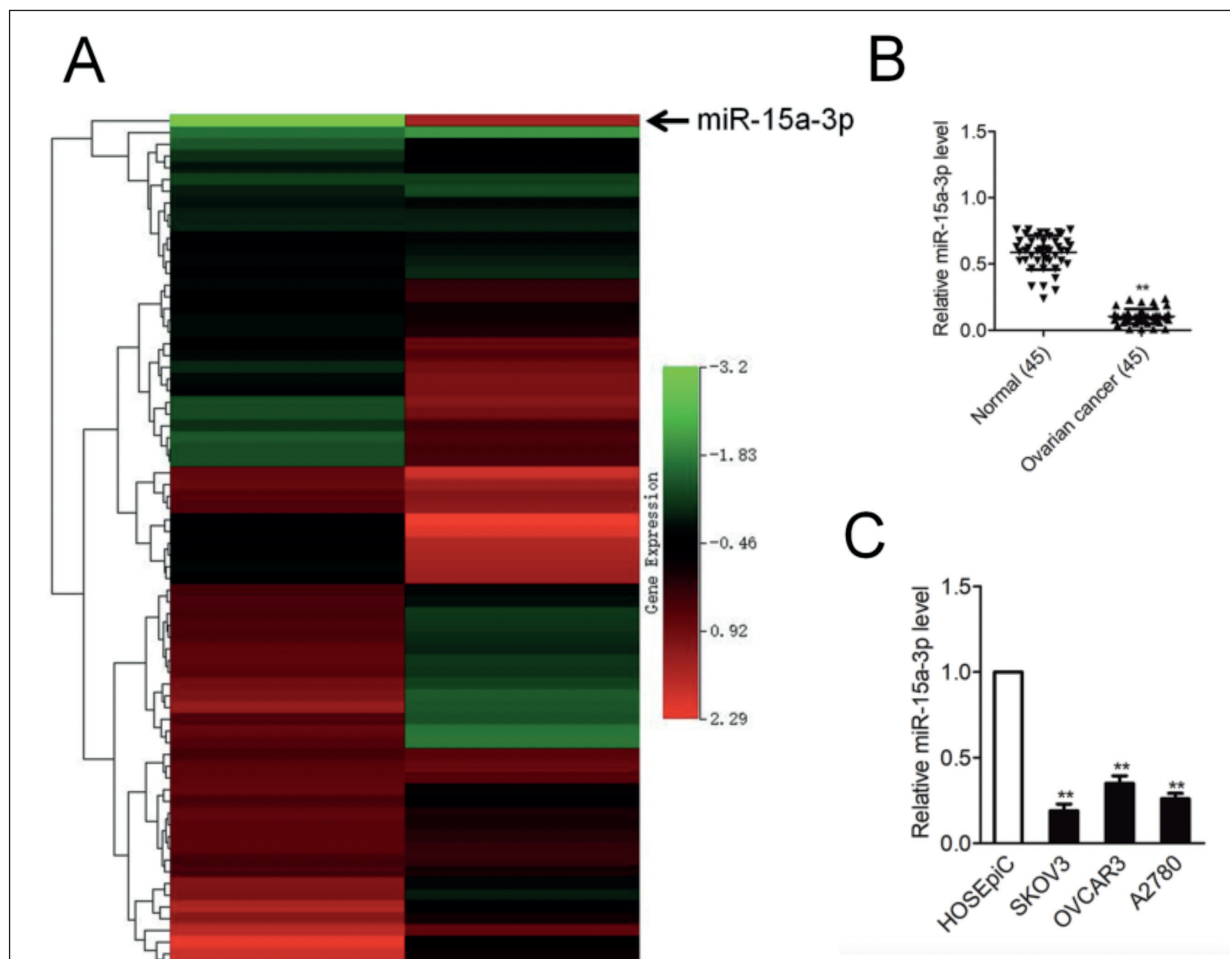

Figure 1. MiR-15a-3p is down-regulated in ovarian cancer. *(A)* The heatmap of miRNA profiling in GEO; *(B)* miR-15a-3p expression level was measured by qRT-PCR. There were eight cases of ovarian cancer tissues that had no corresponding paratumor tissues. **$p < 0.01$ as compared to normal; *(C)* The level of miR-15a-3p was measured in four different ovarian cancer cell lines by qRT-PCR assay. **$p < 0.01$ as compared to HOSEpiC cell.

3p in ovarian cancer cell lines, including SKOV3, OVCAR3, and A2780, as well as in the human ovarian surface epithelial cell line HOSEpiC were detected using qRT-PCR. As shown in Figure 1C, the level of miR-15a-3p was also markedly down-regulated in ovarian cancer cells. These findings indicate that miR-15a-3p might act as a suppressor in ovarian carcinoma.

Up-regulation of miR-15a-3p Suppresses the Proliferation and Colony Formation of Ovarian Cancer Cells

To explore the effect of miR-15a-3p on the proliferation of ovarian cancer cells *in vitro*, OVCR3 and SKOV3 cells were transfected with miR-15a-3p mimics to increase the level of miR-15a-3p. As shown in Figure 2A, qRT-PCR indicated that the level of miR-15a-3p was significantly increased in ovarian cancer cells after miR-15a-3p transfection. Then, we investigated the impact of miR-15a-3p on the proliferation of SKOV3 and OVCR3 cells using MTT assays. As shown in Figure 2B, induction of miR-15a-3p markedly

suppressed the growth of SKOV3 and OVCR3 cells *in vitro*. Consistently, the colony formation assay indicated that up-regulation of miR-15a-3p suppressed the colony formation of ovarian carcinoma cells *in vitro* (Figure 2C). Altogether, these findings suggest that miR-15a-3p serves as a tumour suppressor in ovarian cancer.

Up-regulation of miR-15a-3p Inhibits the Aggressiveness of Ovarian Cancer Cells In Vitro

Next, we found changes in ovarian cancer cell morphology after miR-15a-3p transfection. As shown in Figure 3A, ovarian carcinoma cells changed from a mesenchymal morphology to an epithelial morphology. To analyze whether upregulation of miR-15a-3p induced mesenchymal-epithelial transition (MET) of ovarian carcinoma cells, we detected the expression of a mesenchymal marker (E-cadherin) and an epithelial marker (E-cadherin) using qRT-PCR and immunofluorescence staining assays. As shown in Figure 3B, up-regulation of miR-15a-3p increased
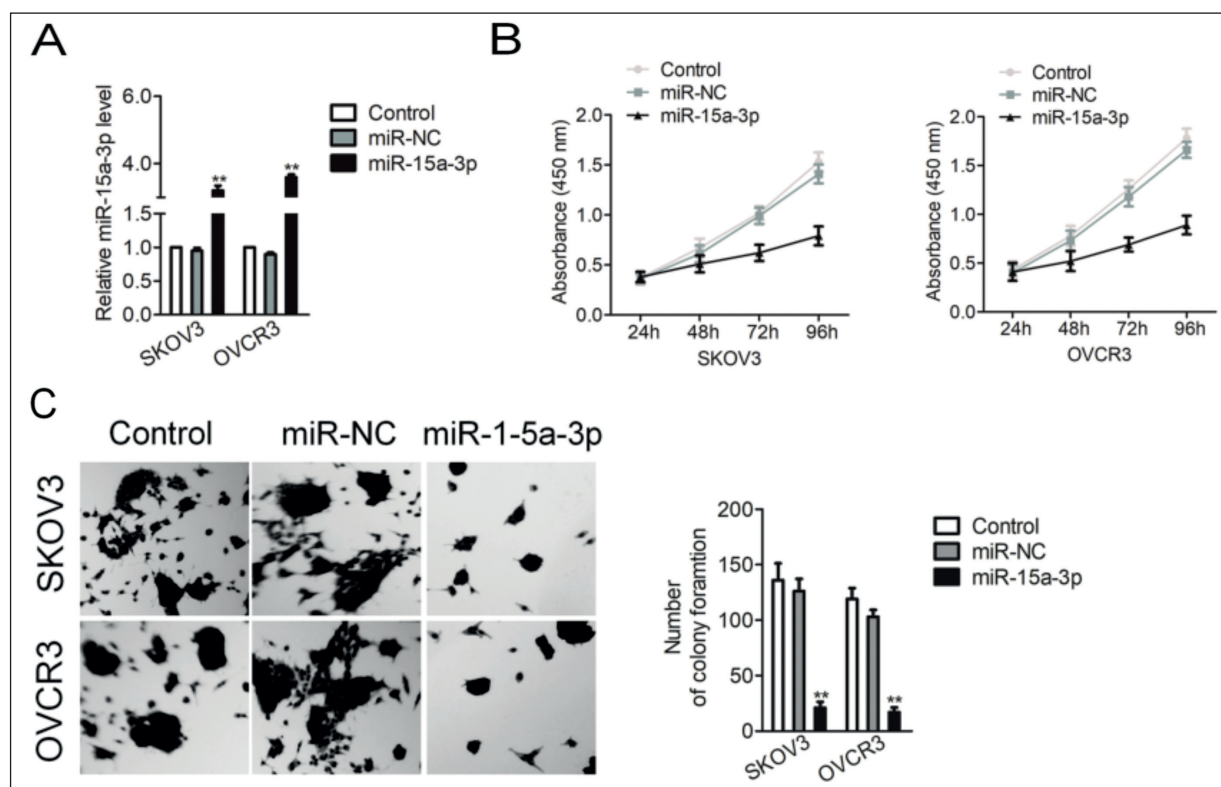

Figure 2. Up-regulation of miR-15a-3p inhibits cell proliferation *in vitro*. *(A)* OVCR3 and SKOV3 cells were transfected with miR-15a-3p mimics or miR-NC. The level of miR-15a-3p was measured by qRT-PCR; *(B)* Cells were seeded in 96-well culture plates and cultured for 24 h, 48 h, 72 h or 96 h. MTT assay was conducted; *(C)* The colony formation was carried out with cells that were transfected with miR-15a-3p or miR-NC. **$p < 0.01$ as compared to control.
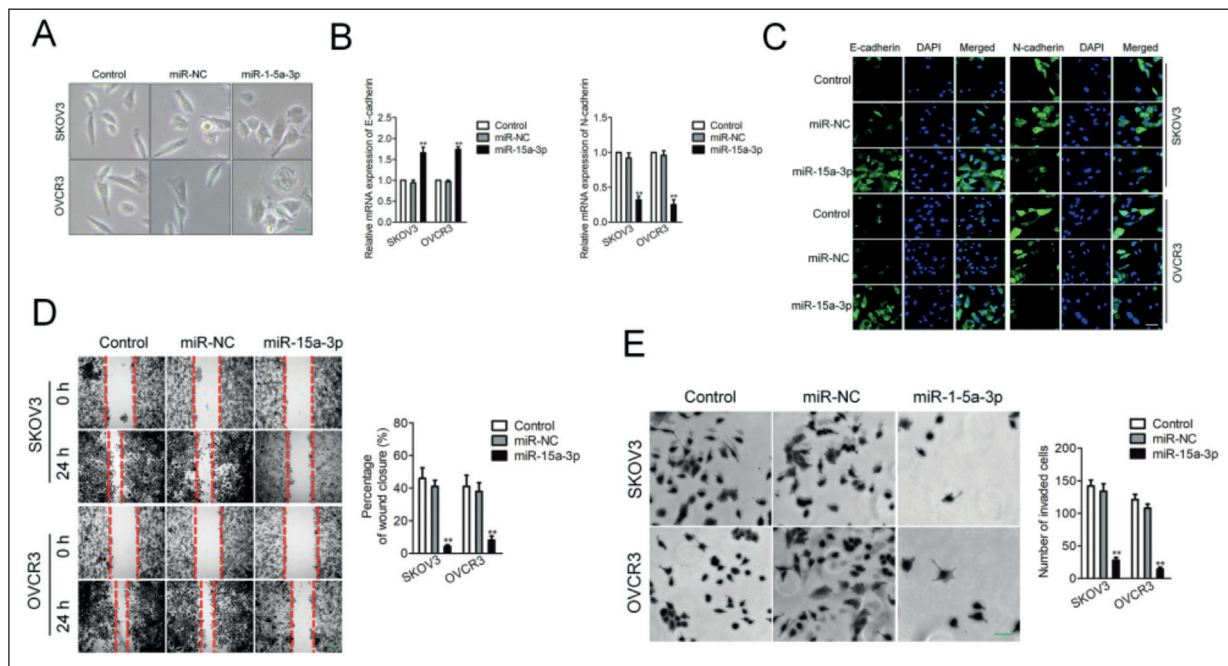
Figure 3. Up-regulation of miR-15a-3p inhibits migration and invasion of ovarian cancer cells *in vitro*. *(A)* Cell morphology was captured with optical microscope after transfected with miR-15a-3p; *(B)* Cells were transfected with miR-15a-3p or miR-NC. The mRNA levels of E-cadherin and N-cadherin were assessed by qRT-PCR assays; *(C)* Representative confocal images of immunofluorescence staining; *(D)* Wound healing assay; *(E)* Transwell invasion assay. **$p < 0.01$ as compared to control.

the level of E-cadherin but inhibited the level of N-cadherin in both SKOV3 and OVCR3 cells. Consistently, the immunofluorescence assay indicated that up-regulation of miR-15a-3p improved the expression of E-cadherin and suppressed the expression of N-cadherin (Figure 3C). The MET process of cancer cells accompanies the alteration of migration and invasion capabilities. Therefore, we explored the impact of miR-15a-3p on the migration of ovarian carcinoma cells. As shown in Figure 3D, up-regulation of miR-15a-3p dramatically suppressed the migration of SKOV3 and OVCR3 cells. Meanwhile, the invasion of SKOV3 and OVCR3 cells was also significantly inhibited by miR-15a-3p transfection (Figure 3E).

Up-regulation of miR-15a-3p Suppresses the Growth and Lung Metastasis of Ovarian Cancer Cells In Vivo

Then, we investigated whether miR-15a-3p affected the growth and metastasis of ovarian carcinoma cells *in vivo*. SKOV3 cells that were transfected with miR-NC or miR-15a-3p were subcutaneously implanted into nude mice. The tumour was monitored and measured every week. As shown in Figure 4A, up-regulation of miR-15a-3p markedly inhibited the tumour growth

of SKOV3 cells *in vivo*. Consistently, the weight of tumours derived from miR-15a-3p-transfected cells was significantly lower than that of the miR-NC group (Figure 4B). The level of miR-15a-3p in tumours was determined using qRT-PCR, and we found that the level of miR-15a-3p was increased in tumours formed by miR-15a-3p-transfected cells (Figure 4C). To reveal the role of miR-15a-3p in the metastasis of ovarian cancer cells *in vivo*, miR-NC- or miR-15a-3p-transfected cells were injected into mice via the lateral tail vein. Four weeks post-inoculation, metastasis was mainly observed in the lung tissue. As shown in Figure 4D, injection of miR-NC-transfected cells resulted in the formation of numerous lung metastases, whereas miR-15a-3p significantly inhibited pulmonary metastasis. All these findings demonstrate that up-regulation of miR-15a-3p suppresses the growth and lung metastasis of ovarian cancer cells *in vivo*.

MiR-15a-3p Directly Binds to the 3'-UTR of Twist1

miRBase and TargetScan were selected to predict the potential target of miR-15a-3p, and we found that Twist1 is the target gene of miR-15a-3p [24]. Sequence alignment showed the
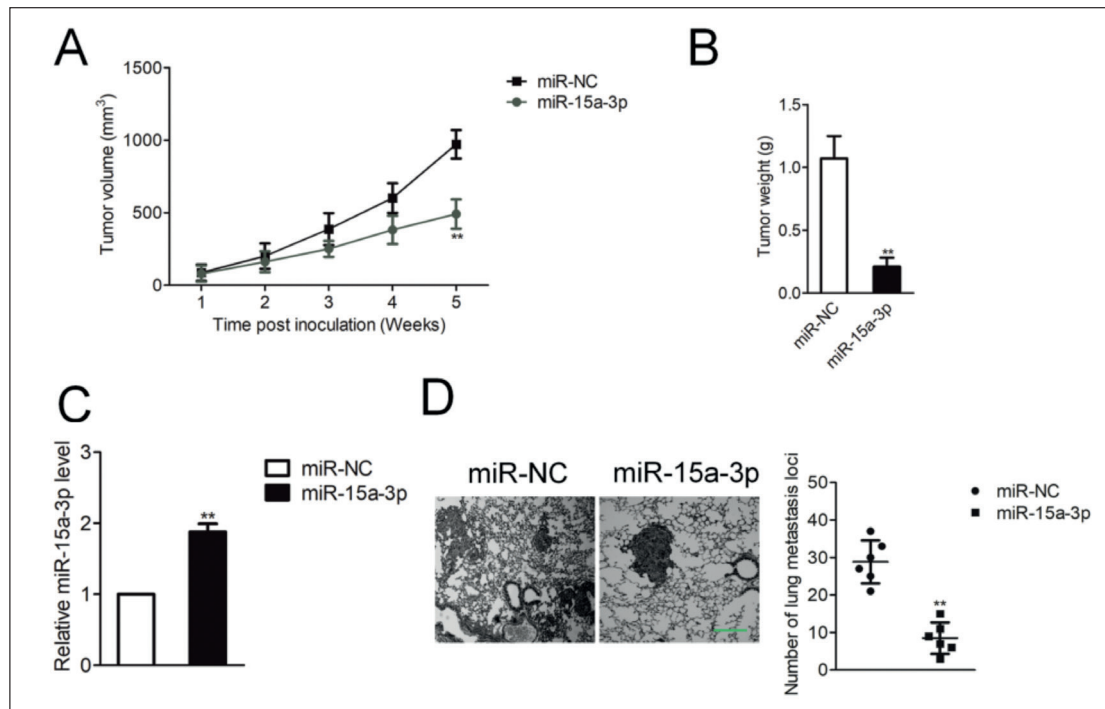
Figure 4. Up-regulation of miR-15a-3p inhibits the tumor growth and metastasis of SKOV3 cells *in vivo*. *(A)* miR-NC or miR-15a-3p transfected Cskov3 cells were implanted into nude mice. The volume of tumor was measured once a week; *(B)* The tumors of two group were taken out at the end of experiment, and the tumor images (*left*) and tumor weight (*right*) are shown; *(C)* The levels of miR-15a-3pin tumors were analyzed using qRT-PCR assays; *(D)* Representative pictures of hematoxylin-eosin (H&E) staining using lungs from mice that were injected with miR-NC or miR-15a-3p transfected SKOV3 cells. Numbers of lung metastasis were quantified and showed by each data point. **$p < 0.01$ as compared to miR-NC.

binding sites between the 3'-UTR of Twist1 and miR-15a-3p (Figure 5A). To investigate whether miR-15a-3p was bound to the 3'-UTR of Twist1, the wild-type (wt) or mutant (mut) 3'-UTR of Twist1 was inserted downstream of the luciferase reporter. 293T cells were cotransfected with the 3'-UTR of Twist1 and miR-15a-3p. The luciferase activity assay indicated that miR-15a-3p markedly reduced the luciferase activity of cells that were transfected with the wt 3'-UTR of Twist1, whereas miR-15a-3p transfection had no significant inhibitory effect on the luciferase activity of cells that were transfected with the mut 3'-UTR of Twist1 (Figure 5B). Then, both qRT-PCR and immunoblotting indicated that the level of Twist1 was decreased in ovarian cancer cells compared to human primary cervical epithelial cells, HCerEpiC (Figure 5C-D). We also analyzed the relationship between miR-15a-3p and Twist1 in ovarian cancer tissues. As shown in Figure 5E-F, the mRNA level of Twist was significantly higher in ovarian cancer tissues and was negatively associated with the level

of miR-15a-3p. Finally, the level of Twist1 was remarkably down-regulated when SKOV3 cells were transfected with miR-15a-3p (Figure 5G). These findings indicate that Twist1 is the target of miR-15a-3p.

The Inhibitory Impact of miR-15a-3p is Rescued by Twist1 Overexpression

To analyze whether miR-15a-3p suppressed the growth and aggressiveness of ovarian cancer cells by inhibiting Twist1, SKOV3 cells were cotransfected with Twist1 and miR-15a-3p. As shown in Figure 6A, qRT-PCR showed that the level of Twist1 was up-regulated and was not suppressed by miR-15a-3p. After that, the MTT and colony formation assays indicated that overexpression of Twist1 increased the growth and colony formation of ovarian cancer cells (Figure 6B-6C). In addition, wound-healing and transwell invasion assays revealed that the inhibitory effects of miR-15a-3p on the migration and invasion of ovarian cancer cells were neutralized by the overexpression of Twist1 (Figure 6D-6E). These results
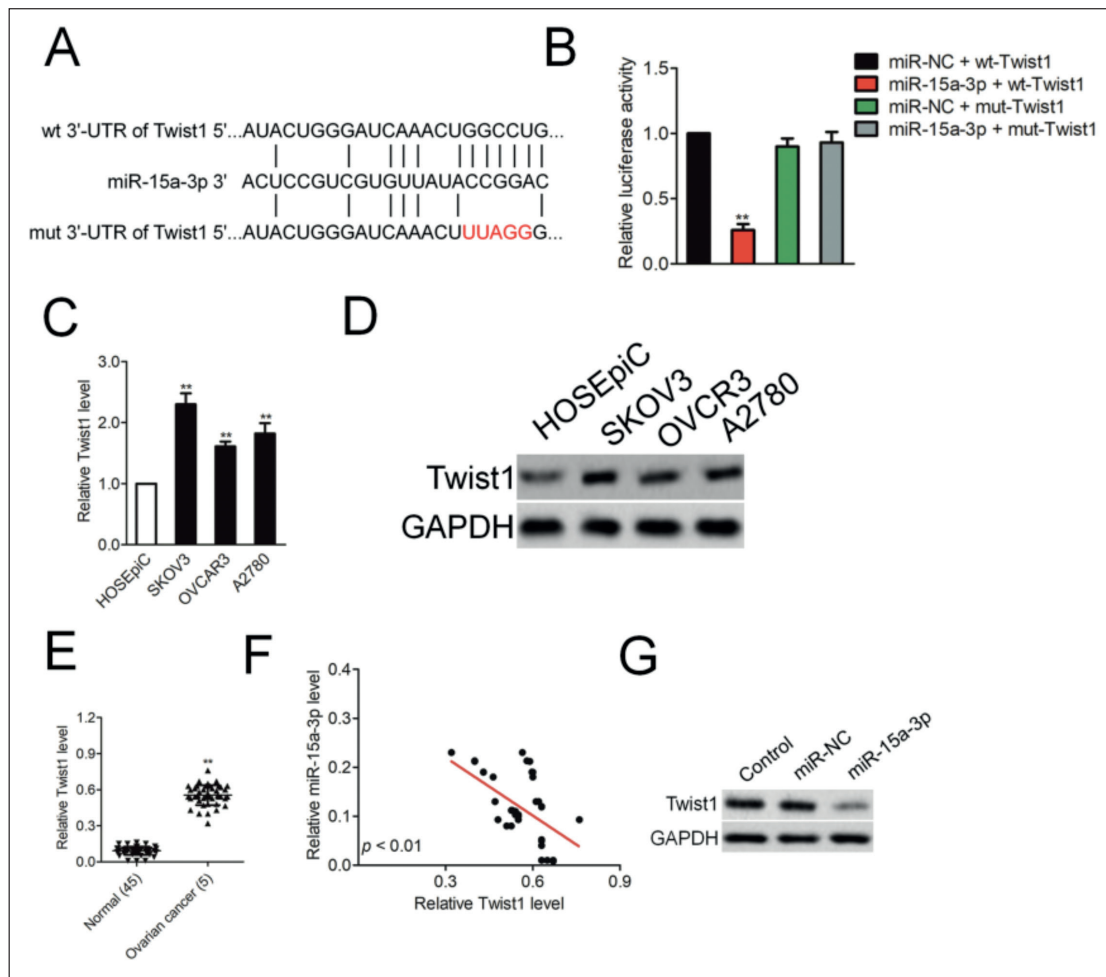
Figure 5. Twist1 is a direct and functional target of miR-15a-3p. *(A)* Predicted miR-15a-3p target sequences in 3'-UTR of Twist1; *(B)* Luciferase reporter assay. **$p < 0.01$ as compared to miR-NC + wt-Twist1; *(C)* The level of Twist1 in ovarian cancer cell lines was detected by qRT-PCR assay. **$p < 0.01$ as compared to HOSEpiC cell; *(D)* The expression of Twist1 in ovarian cancer cell lines was detected by western blot assay; *(E)* RNA was extracted from patients' ovarian cancer tissues, and the mRNA level of Twist1 was measured by qRT-PCR. **$p < 0.01$ as compared to normal; *(F)* The correlation analysis of miR-15a-3p and Twist1 expressions. G. The level of Twist1 in SKOV3 cells that were transfected with miR-15a-3p was analyzed by western blotting assay.

demonstrate that miR-15a-3p inhibits the growth, migration and invasion of ovarian cancer cells by down-regulating Twist1 and that the suppressive effect of miR-15a-3p could be rescued by Twist1 overexpression.

Knockdown of Twist1 Mimics the Inhibitory Impacts of miR-15a-3p on the Growth and Metastasis of Ovarian Cancer Cells

Up-regulation of miR-15a-3p suppresses the growth and aggressiveness of ovarian cancer cells by regulating Twist1, which suggests that Twist1 might also regulate ovarian cancer cell progression. Then, we explored whether Twist1 knockdown mimicked the suppressive effects of miR-15a-3p on the growth and metastasis of ovarian cancer cells. siRNA targeting Twist1 was applied to decrease the expression of Twist1 in SKOV3 cells (Figure 7A). To investigate the role of Twist1 in ovarian cancer cells, the impact of Twist1 knockdown on the growth and colony formation of ovarian cancer cells was examined. As shown in Figure 7B-7C, down-regulation of Twist1 in SKOV3 cells restrained the growth and colony formation of SKOV3 cells, suggesting an oncogenic effect of Twist1 on ovarian cancer growth. Consis-
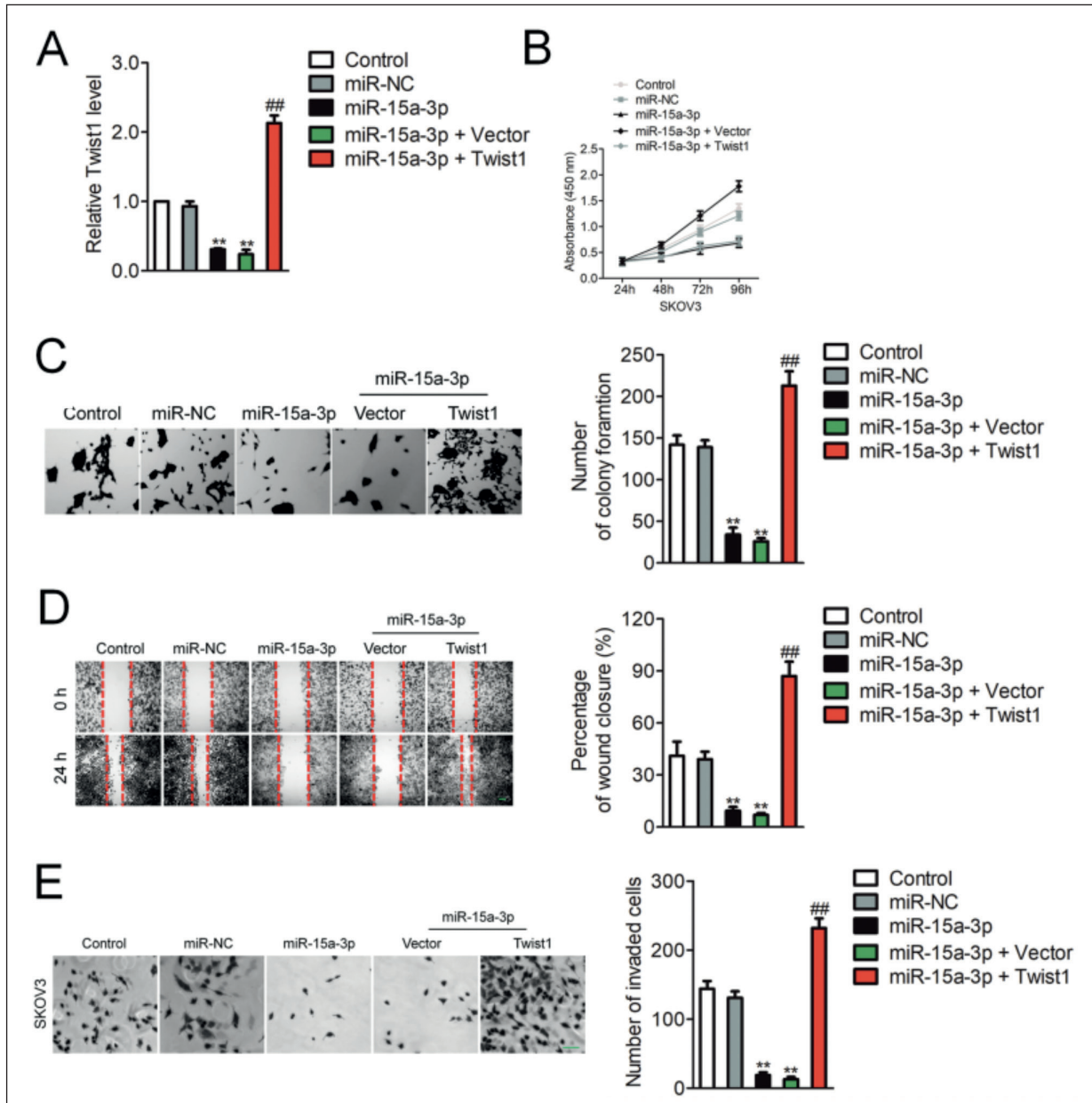
Figure 6. The effect of miR-15a-3p on ovarian cancer can be rescued by Twist1. *(A)* SKOV3 cells were transfected with miR-15q-3p alone or cotransfected with miR-15a-3p and Twist1. The level of Twist1 was detected using qRT-PCR; *(B)* Cell proliferation activity was measured by MTT; *(C)* Colony formation assay; *(D)* SKOV3 cells were transfected with miR-15q-3p alone or cotransfected with miR-15a-3p and Twist1. Wound healing assay was conducted; *(E)* Cell invasion ability was measured by the Transwell invasion assay. **$p < 0.01$ as compared to control, ## $p < 0.01$ as compared to miR-NC + Vector.

tently, down-regulation of Twist1 in SKOV3 cells inhibited the migration and invasion of SKOV3 cells (Figure 7D-7E). Altogether, these results demonstrate that Twist1 knockdown has similar effects on ovarian cancer cells as the up-regulation of miR-15a-3p.

Discussion

Ovarian cancer has the highest mortality rate due to metastatic progression. Therefore, investigating the underlying mechanisms of cancer cell metastasis is important for the develop-
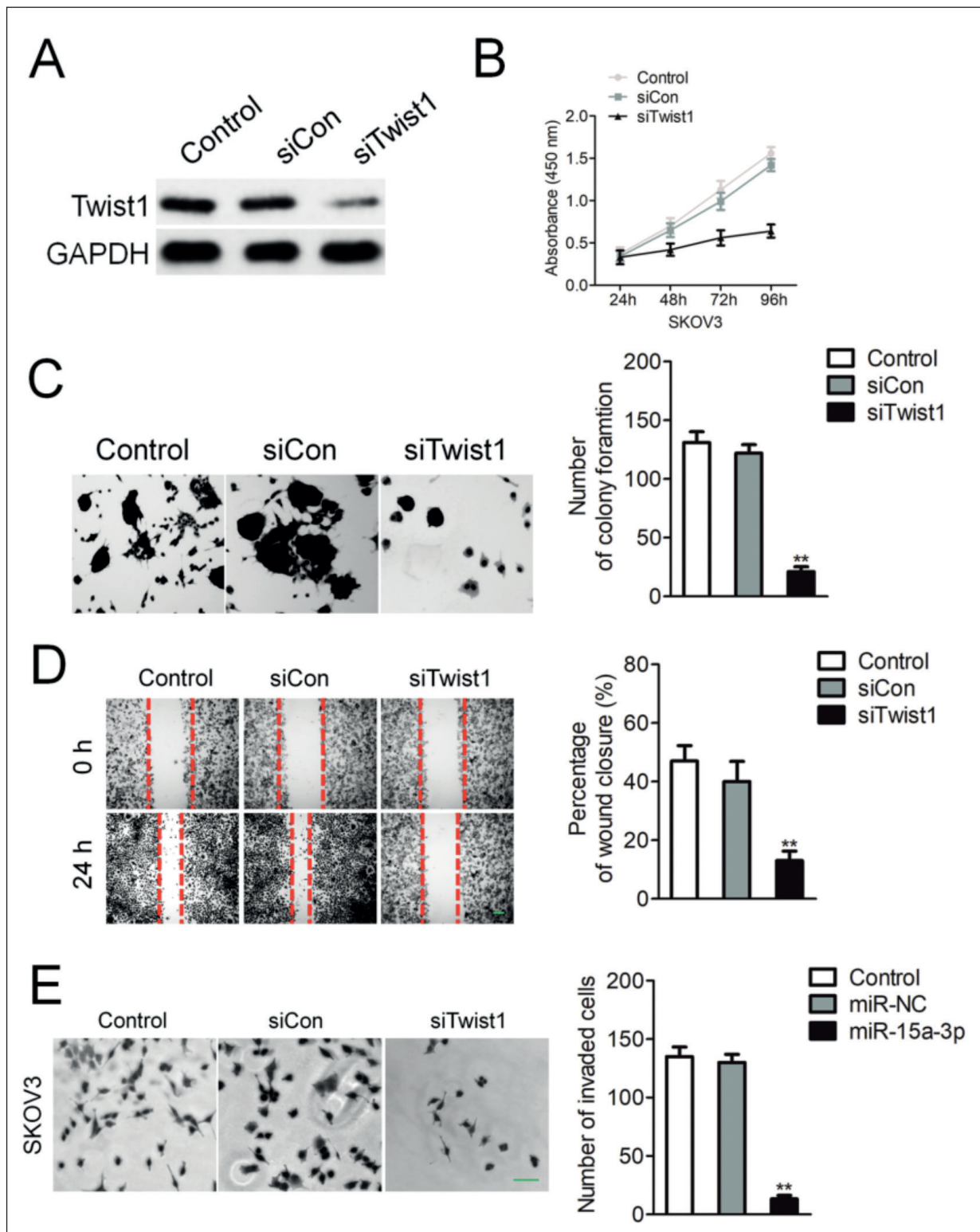
Figure 7. Twist1 knockdown mimics the effects of miR-15a-3p on ovarian cancer cells. *(A)* siTwist1 and siCon was transfected into SKOV3 cells. The level of Twist1 was detected by western blot; *(B)* Cell proliferation activity was measured by MTT; *(C)* Colony formation assay; *(D)* SKOV3 cells were transfected with siCon or siTwist1. Wound healing assay was conducted; *(E)* Cell invasion ability was measured by the Transwell invasion assay. **$p < 0.01$ as compared to control.

ment of targeted therapies[25]. Cancer metastasis is a complex process, including local invasion, intravasation, circulation, extravasation, proliferation and formation of distant metastases[26]. Emerging evidence suggests that dysregulation of miRNAs is involved in cancer metastasis by regulating tumor suppressors and oncogenes. Investigating the underlying mechanisms of ovarian cancer invasion and metastasis is crucial for developing effective therapies for ovarian cancer[27,28]. The current study demonstrates that miR-15a-3p is down-regulated in ovarian cancer. Furthermore, miR-15a-3p inhibits the growth and metastasis of ovarian cancer cells by regulating Twist1.

Previous investigations demonstrated that miR-15a targets oncogenes and that down-regulation of miR-15a-3p alters the expression of target genes in tumors[17]. MiR-15a suppresses the mobility and invasion of osteosarcoma cells by down-regulating the expression of B-cell lymphoma-2 (Bcl-2). Moreover, down-regulation of miR-15a promotes the occurrence and progression and induces the EMT process of non-small cell lung cancer (NSCLC) cells *in vitro*[29]. In chronic myeloid leukemia, miR-15a-5p negatively regulates the survival and metastasis of leukemia cells by targeting the C-X-C motif chemokine 10 (CXCL10)[30]. Recently, miR-15a-3p has been demonstrated to negatively regulate the expression of Twist1 and inhibit invasion and metastasis of gastric cancer cells[31]. Nevertheless, the effect of miR-15a-3p in ovarian carcinoma has not been well investigated. Our study showed that miR-15a-3p was down-regulated in ovarian cancer and that up-regulation of miR-15a-3p inhibited the growth and metastasis of ovarian carcinoma cells *in vitro* and *in vivo*.

In our research, the expression of Twist1 was remarkably decreased in SKOV3 and OVCR3 cells that were transfected with miR-15a-3p. It was reported that Twist1 is a regulator of EMT and has been proven to be targeted by miR-15a-3p in gastric cancer. In ovarian cancer cells, we revealed that the expression of Twist1 was markedly decreased after cells were transfected with miR-15a-3p. Combined with the luciferase reporter assay, all these findings indicate that Twist1 is the direct target gene of miR-15a-3p. Furthermore, the level of Twist was higher in ovarian cancer tissues and was negatively correlated with the level of miR-15a-3p. In addition, our results showed that knocking down Twist1 mimics the suppressive impacts of miR-15a-3p on

the growth and metastasis of ovarian cancer cells. Although some important findings were obtained in this study, there were some limitations. First, in a subcutaneous tumor model, miR-15a-3p-transfected cells were injected into nude mice. In fact, the cells that were stably transfected with miR-15a-3a were more convincing. Second, according to the *in vitro* results, the correlation between miR-15a-3p and distant metastasis and prognosis of patients with ovarian cancer needs to be investigated in the future. Finally, the downstream pathway that is regulated by Twist1 in ovarian cancer is not yet clear.

Conclusions

We demonstrate that miR-15a-3p is down-regulated in ovarian carcinoma. Up-regulation of miR-15a-3p inhibits the growth, migration, and invasion as well as induces the mesenchymal-epithelial transition (MET) process of ovarian cancer cell. In addition, miR-15a-3p suppresses the tumor growth and lung metastasis of ovarian cancer cells *in vivo*. Twist1, which we identified, is the direct target of miR-15a-3p, which exerts a crucial role in the inhibitory impact of miR-15a-3p on ovarian carcinoma cell. These results elucidate a molecular linkage between the Twist1 and miR-15a-3p pathways that determine growth and metastasis of ovarian cancer.

References

1) Ye H, Liu XJ, Hui Y, Liang YH, Li CH, Wan Q. USF1 gene polymorphisms may associate with the efficacy and safety of chemotherapy based on paclitaxel and prognosis in the treatment of ovarian cancer. Neoplasma 2018; 65: 153-160.

2) Wu D, Lu P, Mi X, Miao J. Downregulation of miR-503 contributes to the development of drug resistance in ovarian cancer by targeting PI3K p85. Arch Gynecol Obstet 2018; 297: 699-707.

3) Xu QF, Tang YX, Wang X. LncRNA EBIC promoted proliferation, metastasis and cisplatin resistance of ovarian cancer cells and predicted poor survival in ovarian cancer patients. Eur Rev Med Pharmacol Sci 2018; 22: 4440-4447.

4) Mukhopadhyay A, Bizzarri N, Bradbury M, Sinha S, Bhaumik J, Helm CW. Metastatic involvement of lesser sac in advanced epithelial ovarian cancer. Int J Gynecol Cancer 2018; 28: 293-301.

5) Minlikeeva AN, Moysich KB, Mayor PC, Etter JL, Cannioto RA, Ness RB, Starbuck K, Edwards RP, Segal BH, Lele S, Odunsi K, Diergaarde B, Modugno F. Anthropometric characteristics and ovarian cancer risk and survival. Cancer Causes Control 2018; 29: 201-212.

6) Yang L, Hou J, Cui XH, Suo LN, Lv YW. MiR-133b regulates the expression of CTGF in epithelial-mesenchymal transition of ovarian cancer. Eur Rev Med Pharmacol Sci 2017; 21: 5602-5609.

7) Jin Y, Feng SJ, Qiu S, Shao N, Zheng JH. LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. Eur Rev Med Pharmacol Sci 2017; 21: 3176-3184.

8) Wei C, Zhang X, He S, Liu B, Han H, Sun X. MicroRNA-219-5p inhibits the proliferation, migration, and invasion of epithelial ovarian cancer cells by targeting the Twist/Wnt/beta-catenin signaling pathway. Gene 2017; 637: 25-32.

9) Yang J, Zhou Y, Ng SK, Huang KC, Ni X, Choi PW, Hasselblatt K, Muto MG, Welch WR, Berkowitz RS, Ng SW. Characterization of MicroRNA-200 pathway in ovarian cancer and serous intraepithelial carcinoma of fallopian tube. BMC Cancer 2017; 17: 422.

10) Yang A, Wang X, Yu C, Jin Z, Wei L, Cao J, Wang Q, Zhang M, Zhang L, Zhang L, Hao C. microRNA-494 is a potential prognostic marker and inhibits cellular proliferation, migration and invasion by targeting SIRT1 in epithelial ovarian cancer. Oncol Lett 2017; 14: 3177-3184.

11) Li LW, Xiao HQ, Ma R, Yang M, Li W, Lou G. miR-152 is involved in the proliferation and metastasis of ovarian cancer through repression of ERBB3. Int J Mol Med 2017; 41: 1529-1535.

12) Weidle UH, Birzele F, Kollmorgen G, Nopora A. Potential microRNA-related targets for therapeutic intervention with ovarian cancer metastasis. Cancer Genomics Proteomics 2018; 15: 1-15.

13) Dou YD, Huang T, Wang Q, Shu X, Zhao SG, Li L, Liu T, Lu G, Chan WY, Liu HB. Integrated microRNA and mRNA signatures in peripheral blood lymphocytes of familial epithelial ovarian cancer. Biochem Biophys Res Commun 2018; 496: 191-198.

14) Aqeilan RI, Calin GA, Croce CM. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell Death Differ 2010; 17: 215-220.

15) Bhattacharya R, Nicoloso M, Arvizo R, Wang E, Cortez A, Rossi S, Calin GA, Mukherjee P. MiR-15a and MiR-16 control Bmi-1 expression in ovarian cancer. Cancer Res 2009; 69: 9090-9095.

16) Bandi N, Zbinden S, Gugger M, Arnold M, Kocher V, Hasan L, Kappeler A, Brunner T, Vassella E. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. Cancer Res 2009; 69: 5553-5559.

17) Wu Y, Huang J, Xu H, Gong Z. Over-expression of miR-15a-3p enhances the radiosensitivity of cervical cancer by targeting tumor protein D52. Biomed Pharmacother 2018; 105: 1325-1334.

18) Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, Bartucci M, Muto G, Peschle C, De Maria R. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 2008; 14: 1271-1277.

19) Hu M, Fu X, Cui Y, Xu S, Xu Y, Dong Q, Sun L. Expression of KAP1 in epithelial ovarian cancer and its correlation with drug-resistance. Int J Clin Exp Med 2015; 8: 17308-17320.

20) Zhao W, Geng D, Li S, Chen Z, Sun M. LncRNA HOTAIR influences cell growth, migration, invasion, and apoptosis via the miR-20a-5p/HMGA2 axis in breast cancer. Cancer Med 2018; 7: 842-855.

21) Gomez Zubieta DM, Hamood MA, Beydoun R, Pall AE, Kondapalli KC. MicroRNA-135a regulates NHE9 to inhibit proliferation and migration of glioblastoma cells. Cell Commun Signal 2017; 15: 55.

22) Li H, Cai Q, Godwin AK, Zhang R. Enhancer of zeste homolog 2 promotes the proliferation and invasion of epithelial ovarian cancer cells. Mol Cancer Res 2010; 8: 1610-1618.

23) Wang J, Chen D, He X, Zhang Y, Shi F, Wu D, Chen J, Zhang Y, Zhao F, Dou J. Downregulated lincRNA HOTAIR expression in ovarian cancer stem cells decreases its tumorgeniesis and metastasis by inhibiting epithelial-mesenchymal transition. Cancer Cell Int 2015; 15: 24.

24) Qin Y, Huo Z, Song X, Chen X, Tian X, Wang X. mir-106a regulates cell proliferation and apoptosis of colon cancer cells through targeting the PTEN/PI3K/AKT signaling pathway. Oncol Lett 2018; 15: 3197-3201.

25) Xu CX, Xu M, Tan L, Yang H, Permuth-Wey J, Kruk PA, Wenham RM, Nicosia SV, Lancaster JM, Sellers TA, Cheng JQ. MicroRNA MiR-214 regulates ovarian cancer cell stemness by targeting p53/Nanog. J Biol Chem 2016; 291: 22851.

26) Zhou F, Chen J, Wang H. MicroRNA-298 inhibits malignant phenotypes of epithelial ovarian cancer by regulating the expression of EZH2. Oncol Lett 2016; 12: 3926-3932.

27) Iorio MV, Croce CM. MicroRNA profiling in ovarian cancer. Methods Mol Biol 2013; 1049: 187-197.

28) Langhe R. microRNA and ovarian cancer. Adv Exp Med Biol 2015; 889: 119-151.

29) HE J. Knocking down MiR-15a expression promotes the occurrence and development and induces the EMT of NSCLC cells in vitro. Saudi J Biol Sci 2017; 24: 1859-1865.

30) CHEN D, WU D, SHAO K, YE B, HUANG J, GAO Y. MiR-15a-5p negatively regulates cell survival and metastasis by targeting CXCL10 in chronic myeloid leukemia. Am J Transl Res 2017; 9: 4308-4316.

31) WANG T, HOU J, LI Z, ZHENG Z, WEI J, SONG D, HU T, WU Q, YANG JY, CAI JC. miR-15a-3p and miR-16-1-3p negatively regulate Twist1 to repress gastric cancer cell invasion and metastasis. Int J Biol Sci 2017; 13: 122-134.