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

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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 luciferas[illegible]porter assays were applied to identify th[illegible] family BHLH transcription factor 1 (Twist[illegible]as the target gene of miR-15a-3p. The miR-1[illegible]p level and the expression of Twist1 were dete[illegible] using quantitative Real-time polymerase ch[illegible] reaction (qRT-PCR) assay. The[illegible]ssions [illegible] N-cadherin and E-cadherin [illegible]ured by immunofluorescence stain[illegible]Small[illegible]erfering RNA targeting Twist1 an[illegible]DNA3.1[illegible]taining Twist1 were applied to [illegible]se the expression of Twi[illegible] re[illegible]y.

RESULTS: miR-15[illegible] was m[illegible]y down-regulated in ovarian[illegible]er. Exogen[illegible]p-regulation of miR-15[illegible]ibited the g[illegible], colony formation[illegible]gra[illegible]nd invasion of ovarian cancer[illegible]ce[illegible] in vitro[illegible]thermore, a xenograft mo[illegible]dicated that[illegible]5a-3p inhibited tumour[illegible]wth and the meta[illegible]tic potential of ovari[illegible]ancer [illegible] in vivo. We found that Twist1 was[illegible] direc[illegible]get of miR-15a-3p in ovarian can[illegible]d[illegible] its ex[illegible]ssion was negatively correla[illegible]n the l[illegible]of miR-15a-3p in ovar-[illegible]canc[illegible]ues[illegible]p-regulation of miR-15a-[illegible]cued [illegible]tory impact of miR-15a-3p on [illegible]ian can[illegible]cell growth, migration and invas[illegible]Finally, down-regulation of Twist1 mim-ic[illegible]ressive effects of miR-15a-3p on [illegible]ian cancer cell.

CONCLUSIONS: We demonstrated that [illegible]5a-3p is down-regulated in ovarian cancer[illegible]-regulation of miR-15a-3p restrains the growth and metastasis of ovarian cancer cell by regulating Twist1.

Key Words:
Ovarian[illegible]ancer,[illegible]5a-3p, Migration, Invasion, Twist1.

[illegible]roduction

[illegible]ian can[illegible]s one of the most common fem[illegible]tumours and the leading cause of cancer[illegible]tality among women[1-3]. Despite the improvements in the treatment of this disease, the [illegible]ltration and metastases of ovarian cancer [illegible]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'-

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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 pr[illegible]sis of patient and negatively regulates the [illegible]ation and migration of osteosarcoma. MiR-[illegible]3p is also down-regulated in gastric carcinoma[illegible] inhibits gastric cancer cell metastasis by n[illegible]atively regulating the expressio[illegible] the Twis[illegible] gene. Since miR-15a-3p has b[illegible]ed to be very important in several [illegible]cers, [illegible] precise mechanism by which mi[illegible]-3p re[illegible]ver-ian cancer cells has n[illegible] further investigation[illegible] miR-15a-3p in the progression [illegible]varian can[illegible] required[18].

Currently, w[illegible]nstrate that [illegible]evel of miR-15a-3p i[illegible] decreased [illegible] ovarian cancer. Up[illegible]ulation o[illegible]15a-3 significantly inhibits t[illegible]growth and i[illegible]tic potential of ovarian[illegible]cer cells both *in v[illegible]* and *in vivo*. In addit[illegible] we de[illegible]strate that Twist1 is the target ge[illegible] miR[illegible]3p and plays a crucial role in the i[illegible] effect [illegible]iR-15a-3p on ovarian [illegible]er. C[illegible]dy el[illegible]ates a molecular link be-[illegible]Twist[illegible]-15a-3p that determines the gro[illegible] and me[illegible]sis 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 th[illegible] nal and Child Health Care Hospital, a[illegible] consent was obtained from all pa[illegible]s. Written informed consent for participat[illegible] the study was obtained from all patients [illegible] partici-pation in this study. Ovari[illegible] cance[illegible] lines (OVCR3, SKOV3 and A[illegible]0) and the ovarian surface epitheli[illegible]l line [illegible]OSEpiC obtained from Guan[illegible]ou Jen[illegible] Biotech Co., Ltd. (Guangzhou, Gu[illegible]ng[illegible]na). 29[illegible] cells were purchased [illegible]n [illegible] Cobio[illegible]otech Co., Ltd. (Nan[illegible], Jiangsu, [illegible]), [illegible], A2780 and OVCR3 [illegible]ere maintai[illegible] Dulbecco's Modified [illegible]e [illegible]dium (DMEM; Invitrogen, Carlsbad, [illegible]A, US[illegible] supplemented with 10% fetal b[illegible]ne serum (F[illegible] Invitrogen, Carlsbad, CA[illegible] and 1% pen-st[illegible] (Beyotime, Nanjing, J[illegible]gsu, China). SKOV3 cells were maintained [illegible]McCoy's 5a[illegible]odified medium (Invitrogen, [illegible]sbad, CA, [illegible]A) supplemented with 10% fe[illegible]ovine se[illegible] (FBS; Invitrogen, Carlsbad, CA, [illegible] pen-strep (Beyotime, Nanjing, Jiangsu, [illegible]a). All cells were cultured in an in-[illegible]bator 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 T gene were amplified from the reverse-transcribed DNA of SKOV3 cells and cloned into the pGL luciferase reporter plasmid. The wt and mut pGL 3'-UTR and miR-15a-3p were co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the luciferase activity was detected using a dual-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 (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using 5% non-fat milk and then incubated with Twist1 antibody (Cell Signaling Technology, Danvers, MA, USA) and 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 and E-cadherin) overnight at 4°C. After 24 h, cells were incubated with FITC-conjugated secondary 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 ($1x10^6$) 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, we found that miR-15a-3p was remarkably down-regulated in ovarian cancer tissues (Fig. 1A). Then, we detected the levels of miR-15a-3p in pairs of ovarian cancer tissues and adjacent normal tissues using qRT-PCR to investigate the expression 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-

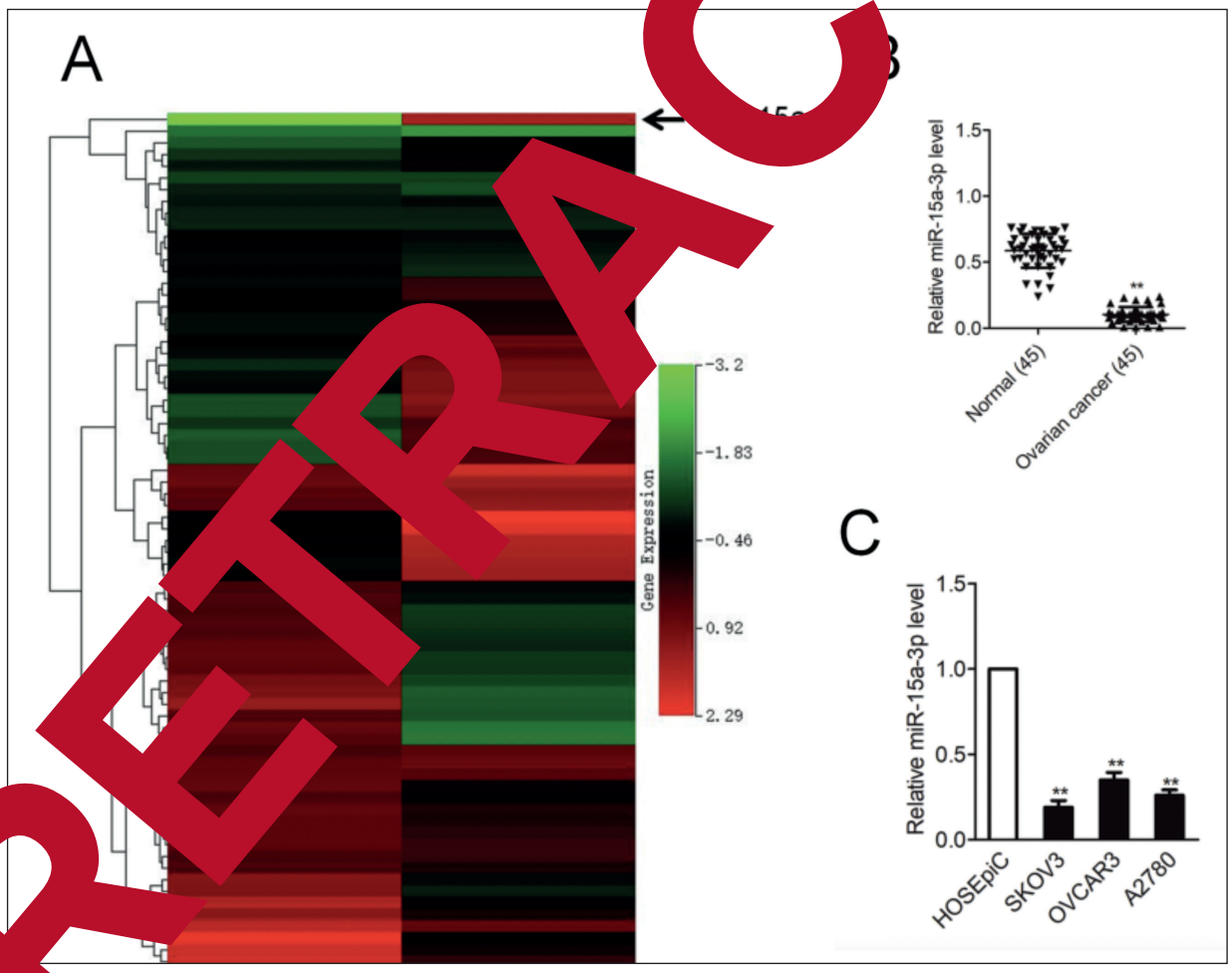

Figure 1. MiR-15a-3p is down-regulated in ovarian cancer. ***(A)*** The heatmap of miRNA profiling in GEO; ***(B)*** miR-15a-3p expression level was measured by qRT-PCR. There were eight cases of ovarian cancer tissues that had no corresponding paratumor tissues. $^{**}p < 0.01$ as compared to normal; ***(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 [illegible] 3p suppressed the colony formation [illegible] carcinoma cells *in vitro* (Figure 2[illegible]). Altogether, these findings suggest that miR[illegible]p serves as a tumour suppressor in ovarian ca[illegible].

Up-regulation of miR[illegible]-3p Inhibits Aggressiveness of O[illegible]arian Cancer Cells In Vitro

Next, we found [illegible] ovarian cancer cell morphology [illegible] 3p transfection. As shown in [illegible]e 3A, ovarian carcinoma cells changed from mesenchymal morphology to an epithelial morphology. To analyze whether upregulation of miR[illegible]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

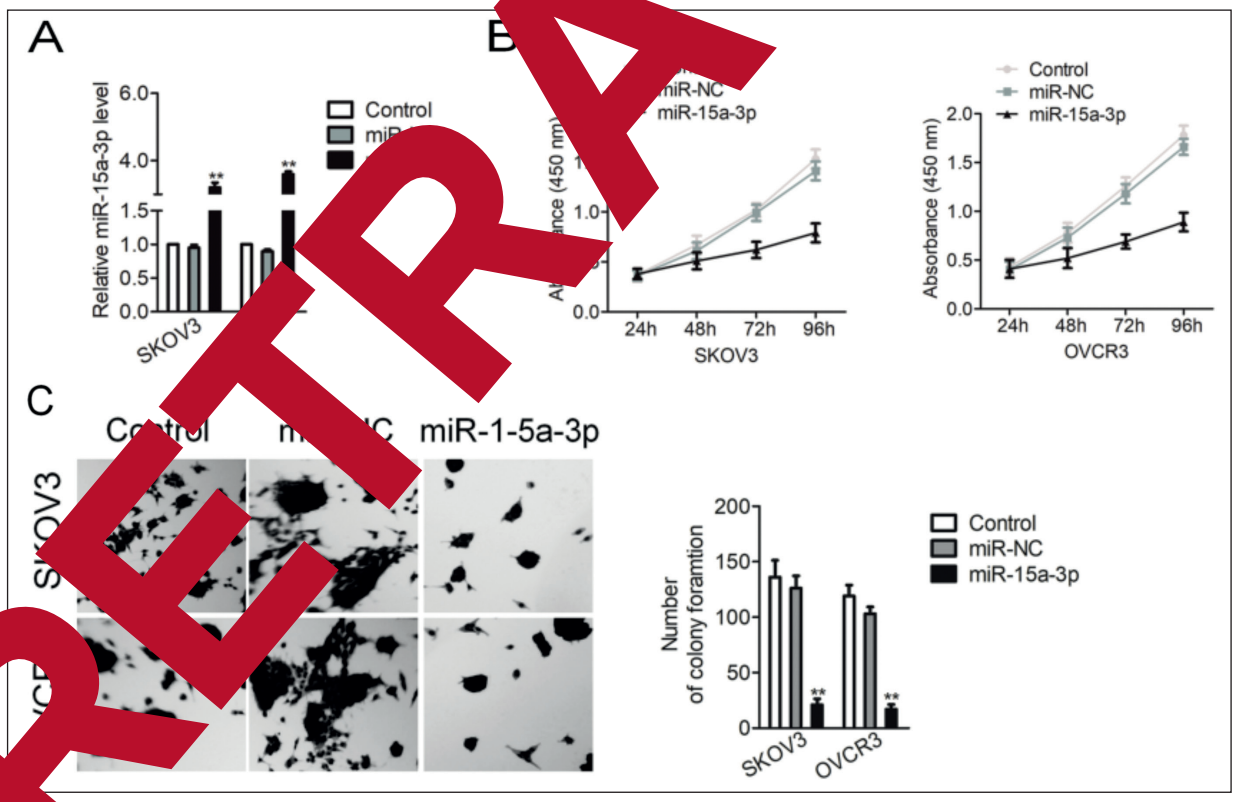

Figure 2. Up-regulation of miR-15a-3p inhibits cell proliferation *in vitro*. ***(A)*** OVCR3 and SKOV3 cells were transfected with mi[illegible]3p mimics or miR-NC. The level of miR-15a-3p was measured by qRT-PCR; ***(B)*** Cells were seeded in 96-well culture plates and cultured for 24 h, 48 h, 72 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.

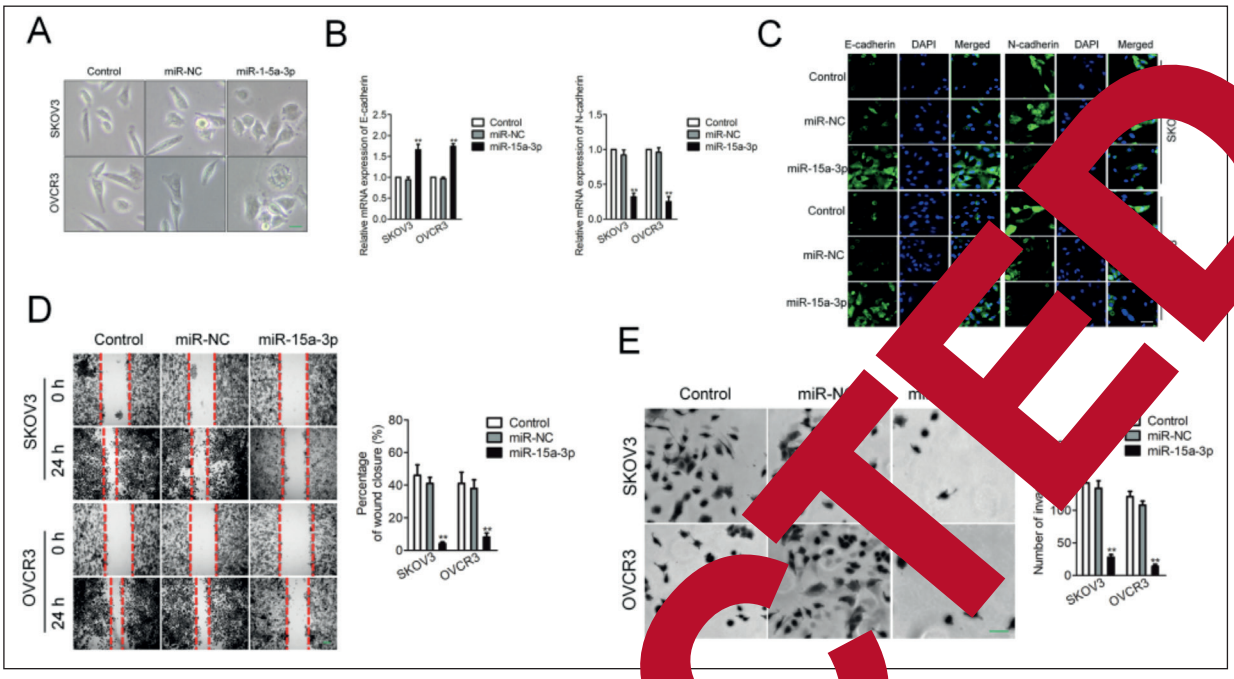

Figure 3. Up-regulation of miR-15a-3p inhibits migration and in of ovarian ca cells *in vitro*. ***(A)*** Cell morphology was captured with optical microscope after transfected with miR-15 ***B)*** Cells w transfected with miR-15a-3p or miR-NC. The mRNA levels of E-cadherin and N-cadherin were assessed b s; ***(C)*** Representative confocal images of immunofluorescence staining; ***(D)*** Wound healing y; ***(E)*** Transwell assay. $^{**}p < 0.01$ as compared to control.

the level of E-cadherin but inhibited the lev f N-cadherin in both SKOV3 and OVCR3 ce Consistently, the immunofluores assay ind cated that up-regulation of mi mproved the expression of E-cadheri d sup sed the expression of N-cadherin ure 3C MET process of cancer cells acc ie of migration and inv cap s. Therefore, we explored the in t of miR-1 on the migration of ovari inoma cells. own in Figure 3D, up ula f miR-15a-3p dramatically supp ed the i on of SKOV3 and OVCR3 c Meanwhile, t asion of SKOV3 and OV cells was also sig cantly inhibited by m 5a-3p fection (Figure 3E).

Up-r n of m 15a-3p Suppresses the th a Lung Metastasis of ian C lls In Vivo

T , we i tigated whether miR-15a-3p aff the growth and metastasis of ovarian c s *in vivo*. SKOV3 cells that were nsfected with miR-NC or miR-15a-3p were utaneously implanted into nude mice. The tu was monitored and measured every week. As s own in Figure 4A, up-regulation of miR-15a-3p markedly inhibited the tumour growth 3 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

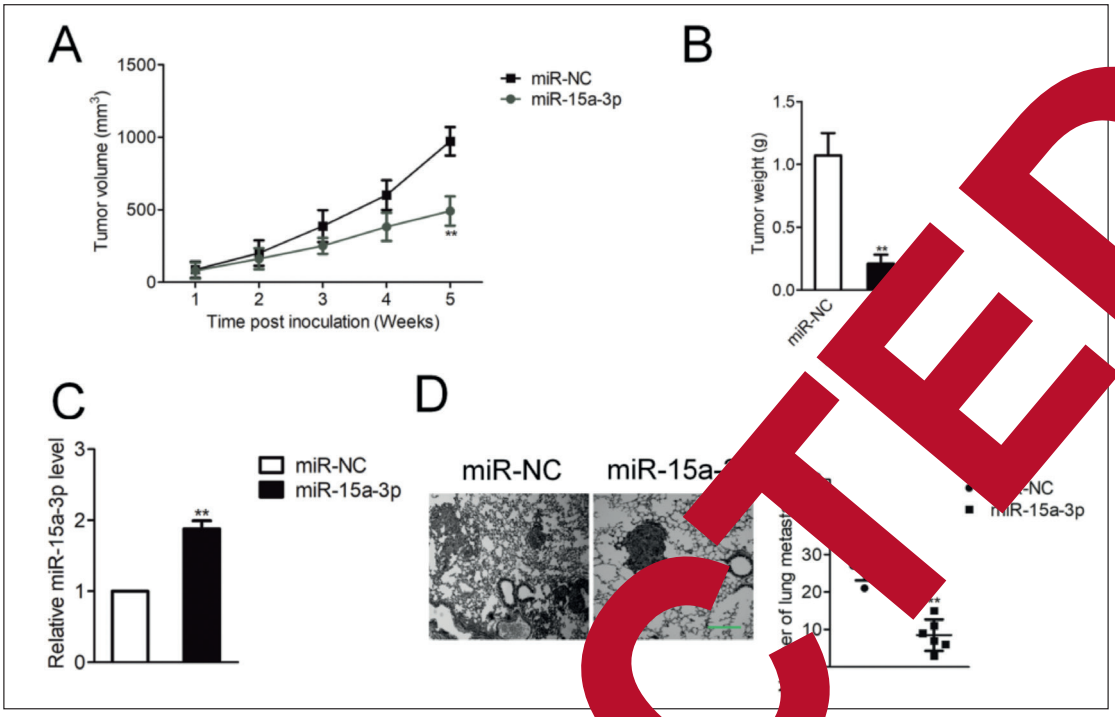

Figure 4. Up-regulation of miR-15a-3p inhibits the tumor growth [illegible] SKOV3 cells *in vivo*. ***(A)*** miR-NC or miR-15a-3p transfected Cskov3 cells were implanted into nude mice. [illegible] of tumor was measured once a week; ***(B)*** The tumors of two group were taken out at [illegible] experiment, and the tumor images (*left*) and tumor weight (*right*) are shown; ***(C)*** The levels of miR-15a-3[illegible] analyzed using qRT-PCR assays; ***(D)*** Representative pictures of hematoxylin-eosin (H&E) staining u[illegible]ungs [illegible]at were injected with miR-NC or miR-15a-3p transfected SKOV3 cells. Numbers of lung metast[illegible]were [illegible]nd showed by each data point. $^{**}p < 0.01$ as compared to miR-NC.

binding sites between the 2[illegible]R of [illegible]st1 and miR-15a-3p (Figure 5A). [illegible]nvestig[illegible]ther miR-15a-3p was bound to [illegible]'-U[illegible], the wild-type (wt) [illegible]utan[illegible] 3'-UTR of Twist1 was inser[illegible]downstrea[illegible] the luciferase reporter. [illegible]ells were [illegible]sfected with the 3'-U[illegible]of [illegible] and miR-15[illegible]-3p. The luciferase a[illegible]ty assay [illegible]ated that miR-15a-3p marke[illegible] reduced the [illegible]rase activity of cells th[illegible]ere transfected wi[illegible] the wt 3'-UTR of T[illegible], whe[illegible] miR-15a-3p transfection had no [illegible]fican[illegible]hibitory effect on the luciferase activi[illegible]s that [illegible]e transfected with the [illegible]3'-U[illegible]f Tw[illegible] (Figure 5B). Then, both [illegible]CR a[illegible]oblotting indicated that the leve[illegible] Twist1 [illegible] decreased in ovarian cancer cel[illegible]pared to human primary cervical epi-t[illegible]CerEpiC (Figure 5C-D). We also [illegible]lyzed the relationship between miR-15a-3p [illegible]Twist1 in ovarian cancer tissues. As shown i[illegible]re 5E-F, the mRNA level of Twist was sign[illegible]cantly 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

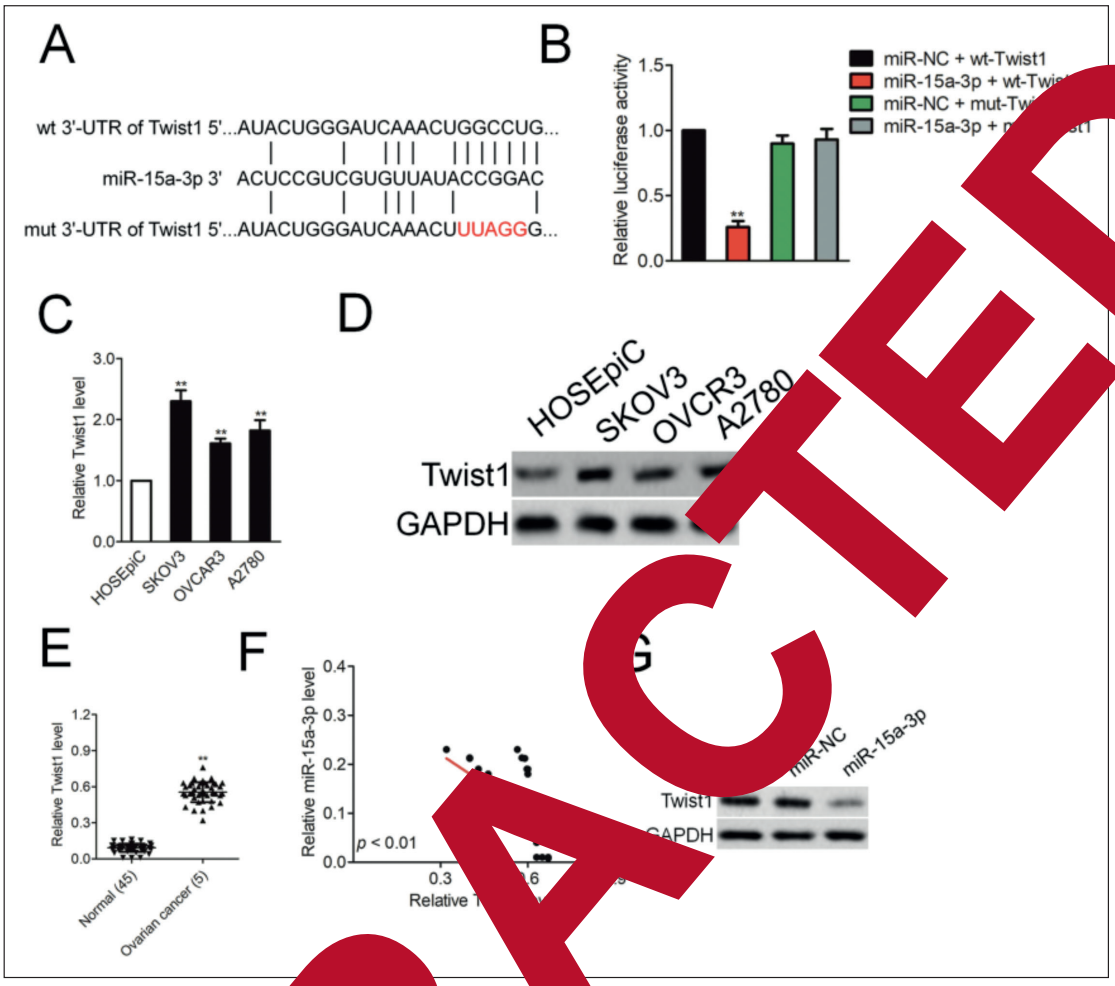

Figure 5. Twist1 is a direct and ...tional t... of miR-15...p. ***(A)*** Predicted miR-15a-3p target sequences in 3'-UTR of Twist1; ***(B)*** Luciferase report... ay. **p <... ...pared to miR-NC + wt-Twist1; ***(C)*** The level of Twist1 in ovarian cancer cell lines was detected b... PCR ... ...1 as compared to HOSEpiC cell; ***(D)*** The expression of Twist1 in ovarian cancer cell lines ... dete... ...stern blot assay; ***(E)*** RNA was extracted from patients' ovarian cancer tissues, and the mRNA level of ...t1 was m... ...l by qRT-PCR. **$p < 0.01$ as compared to normal; ***(F)*** The correlation analysis of miR-15a-3p and T... expressions. ... ...evel of Twist1 in SKOV3 cells that were transfected with miR-15a-3p was analyzed by weste... ...assay.

demon... ...e that miR-15a-3p i... ...bits the growth, migr... ...l and i... ...ion of ovarian cancer cells by do... ...gulat... ...Twist1, and that the suppressive effect... ...a-3p ... ...be rescued by Twist1 ...expre...

Kn... ...down ... ...wist1 Mimics the In... ...ry Impacts of miR-15a-3p on ... ...and Metastasis of Ovarian ...ncer Cells

...p-regulation of miR-15a-3p suppresses the g... ...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-

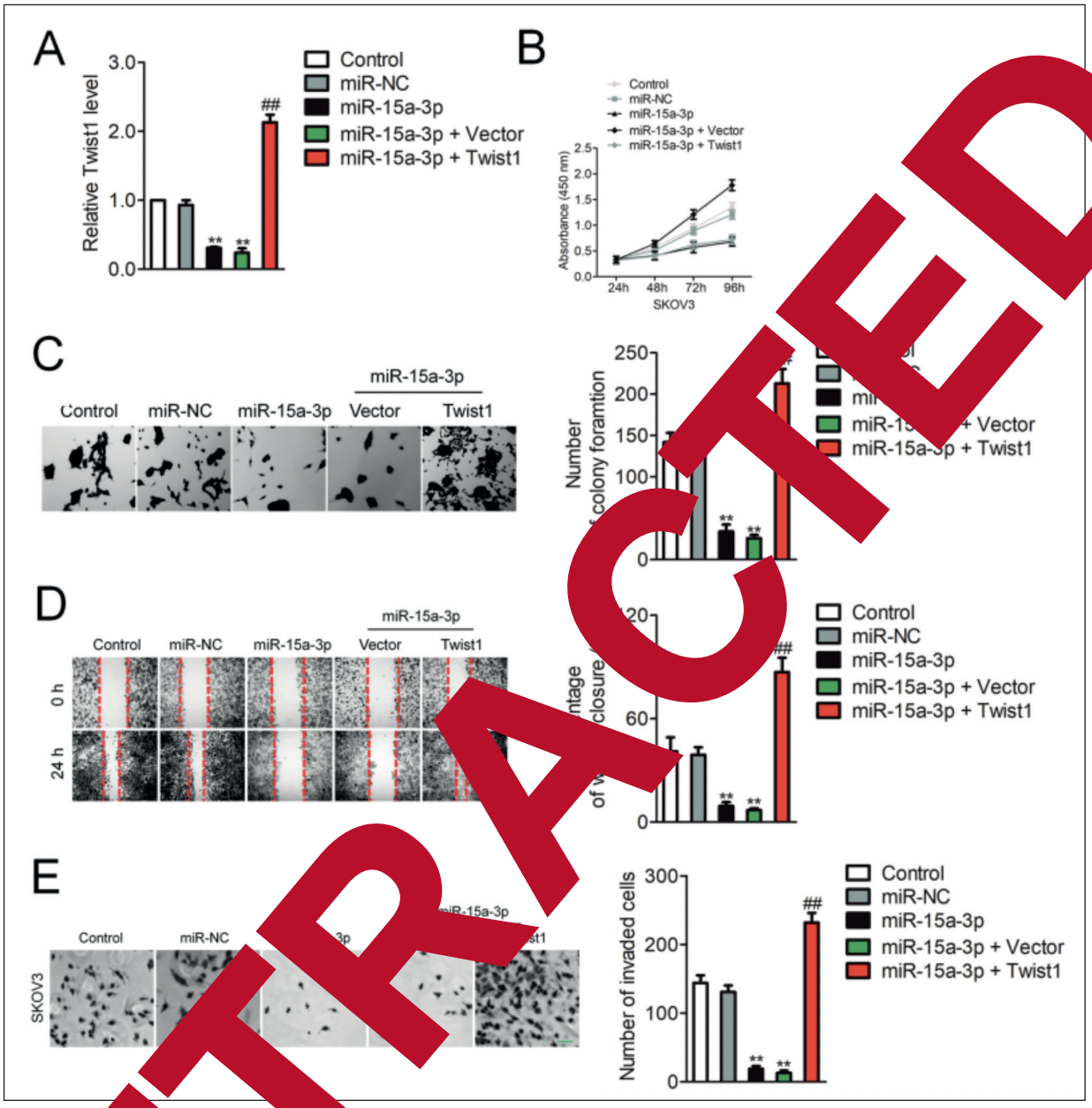

Figure 6 effect of miR-15a- ovarian cancer can be rescued by Twist1. ***(A)*** SKOV3 cells were transfected with miR-15 alone o cotransfected with miR-15a-3p and Twist1. The level of Twist1 was detected using qRT-PCR; ***(B)*** Cell eration ity was measured by MTT; ***(C)*** Colony formation assay; ***(D)*** SKOV3 cells were transfected with mi p alo cotrans cted with miR-15a-3p and Twist1. Wound healing assay was conducted; ***(E)*** Cell invasion ability ed by th nswell invasion assay. $^{**}p < 0.01$ as compared to control, $^{\#\#}p < 0.01$ as compared to miR-NC + Ve

egulation of Twist1 in SKOV3 s inhibited the migration and invasion of V3 cells (Figure 7D-7E). Altogether, these r demonstrate that Twist1 knockdown has simi r 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-

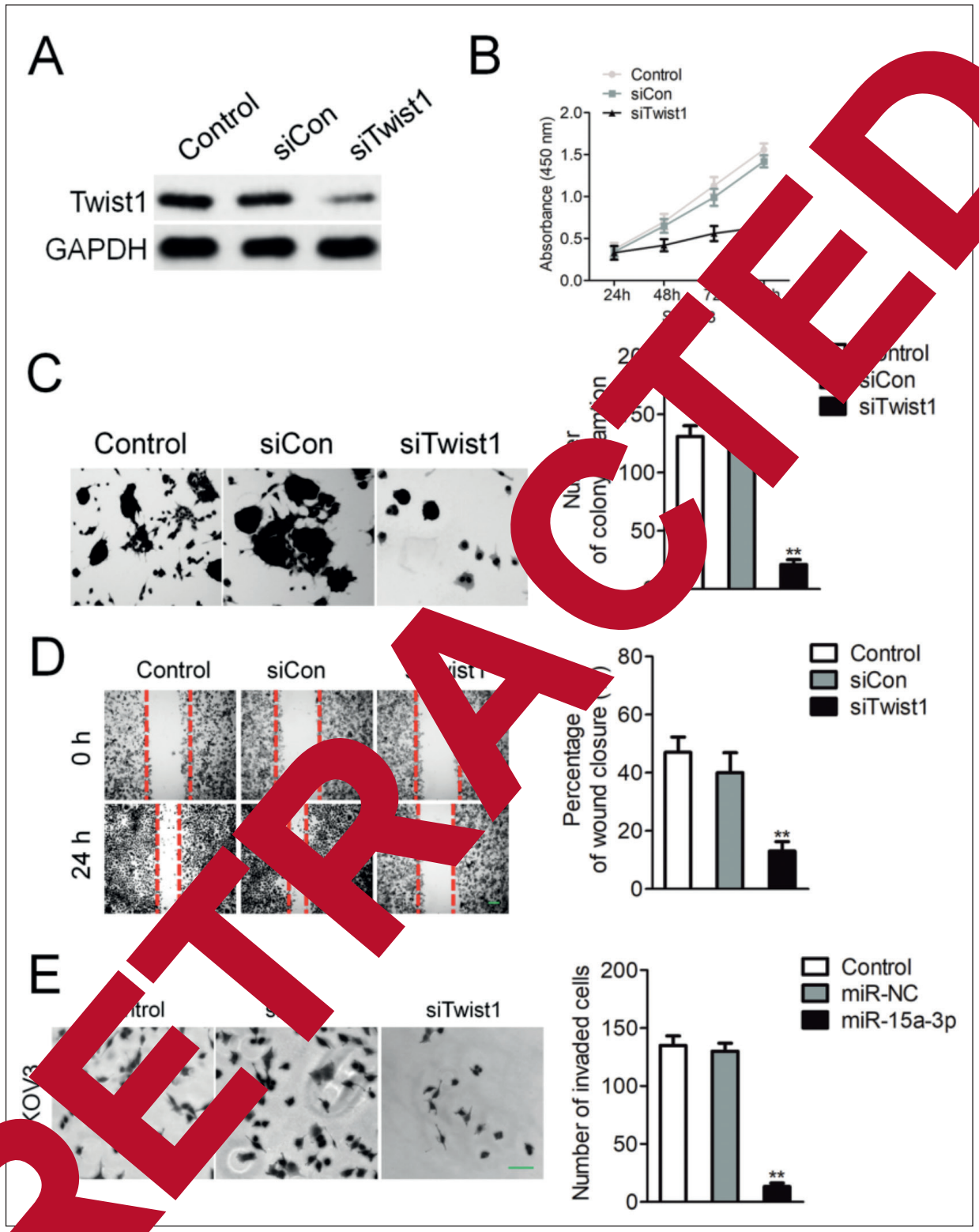

Figure 7. Twist1 knockdown mimics the effects of miR-15a-3p on ovarian cancer cells. ***(A)*** siTwist1 and siCon was transfected SKOV3 cells. The level of Twist1 was detected by western blot; ***(B)*** Cell proliferation activity was measured by MTT; ***(C)*** Colony formation assay; ***(D)*** SKOV3 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*. In chronic myeloid leukemia, miR-15a-5p ne regulates the survival and metastasis of leu ia cells by targeting the C-X-C motif chemo 10 (CXCL10)[30]. Recently, miR-15a-3p has b demonstrated to negatively reg the expre sion of Twist1 and inhibit inva etastasis of gastric cancer cells[31]. N neless e effect of miR-15a-3p in ovarian noma l been well investigated. Our st ho 15a-3p was down-re ed i cancer and that up-regulation miR-15a- ibited the growth and met f ovarian ca a cells *in vitro* and *i* .

In our re ch, the sion of Twist1 was remarkab ecreased in 3 and OVCR3 cells t were transfected w miR-15a-3p. It was rted th wist1 is a regulator of EMT an been ven to e targeted by miR-15a-3p i cancer ovarian cancer cells, reve at th xpression of Twist1 was dly de fter cells were transfected wit iR-15a-3 ombined with the luciferase rep assay all these findings indicate that T irect target gene of miR-15a-3p. thermore, the level of Twist was higher in ian cancer tissues and was negatively cor- 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 limitati in a subcutaneous tumor model, transfected cells were injected i ude mice. In fact, the cells that were s transfected with miR-15a-3a were more conv Second, according to the *in vitro* r lts, the lation between miR-15a-3p and ant metast prognosis of patients w ovaria cancer to be investigated in t ture. ly, the down-stream pathway tha by T t1 in ovarian cancer i yet

clusions

We onstrate tha 15a-3p is down-regul ovarian carcin a. Up-regulation of 15a-3p inhibits the growth, migration, and sion as well nduces the mesenchymal-ep- ial transition ET) process of ovarian cancer l. In add , miR-15a-3p suppresses the tum d lung metastasis of ovarian cancer *vivo*. Twist1, which we identified, the direct target of miR-15a-3p, which exerts role in the inhibitory impact of miR- a-3p n 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.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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