

MicroRNA-125a-5p enhances the sensitivity of esophageal squamous cell carcinoma cells to cisplatin by suppressing the activation of the STAT3 signaling pathway

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Abstract. Increasing evidence has demonstrated that microRNAs (miRNAs or miRs) play a variety of roles in tumor development, progression and chemosensitivity in a wide range of tumors. In this study, we found that miR-125a-5p exhibited a low expression in esophageal squamous cell carcinoma (ESCC) tissues and cells, and that its low expression was associated with higher tumor staging and shorter a survival time of patients with ESCC. Moreover, miR-125a-5p overexpression contributed to the suppression of cell proliferation, cell cycle arrest, cell apoptosis and a decrease in cell migratory and invasive abilities, whereas the downregulation of miR-125a-5p promoted cell proliferation, accelerated cell cycle progression, suppressed apoptosis and enhanced the migratory and invasive abilities of ESCC EC1 and TE1 cells, which may be tightly associated with the epithelial-mesenchymal transition (EMT) process in ESCC. Importantly, miR-125a-5p enhanced the cytotoxic effects of cisplatin on EC1 and TE1 cells, and co-treatment with miR-125a-5p and cisplatin significantly induced cell apoptosis and reduced the cell migratory and invasive abilities of EC1 and TE1 cells, coupled with an increase in the E-cadherin level and a decrease in the N-cadherin and Vimentin levels. Most notably, signal transducer and activator of transcription-3 (STAT3) was found to be a direct target of miR-125a-5p in ESCC cells, and miR-125a-5p overexpression significantly reduced the protein levels of t-STAT3, p-STAT3 and vascular endothelial growth factor (VEGF) in EC1 and TE1 cells. Furthermore, the combination of miR-125a-5p and

cisplatin markedly inactivated the STAT3 signaling pathway; however, interleukin (IL)-6, a widely reported activator of the STAT3 signaling pathway, reversed the suppressive effects of miR-125a-5p/cisplatin in ESCC cells on the activation of the STAT3 signaling pathway. Of note, we found that IL-6 markedly reversed the altered cell phenotype mediated by the combination of miR-125a-5p and cisplatin in ESCC cells. These findings suggest that miR-125a-5p may play a pivotal role in the development and progression of ESCC, which may be achieved via the manipulation of the STAT3 signaling pathway.

Introduction

Esophageal carcinoma (EC) is one of the most frequently occurring malignances worldwide (1). EC is divided into two main histological types, including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (ECA); ESCC as a main histological type is more common in Africa, Iran and North China (2,3). Despite tremendous advances being made in therapeutic strategies, the 5-year survival rate for patients with ESCC remains markedly poor (4-8). Notably, even though chemotherapy is an effective therapeutic approach for patients with ESCC (9), the development of drug resistance has become the most severe concern, and is the main cause of treatment failure in patients with ESCC (10). Therefore, it is imperative to elucidate the mechanisms responsible for drug resistance in order to improve the survival rate of patients with ESCC.

MicroRNAs (miRNAs or miRs) are a class of small non-coding RNAs comprising of 19-25 nucleotides in length, which regulate gene expression by targeting related genes (11,12). Ample evidence has revealed that miRNAs are involved in a number of cellular processes, such as cell apoptosis, cell cycle, cell invasion and metastasis, the regulation of signaling networks and drug resistance (13-19). Furthermore, miRNAs are tightly associated with tumor initiation, development and progression in a variety of tumors via the modulation of their target gene levels (20-23); hence, miRNAs may function as

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either oncogenes or tumor suppressors in different types of tumors (20,24-27). Therefore, it is imperative to interpret the function of miRNAs in the occurrence and development of a large number of tumors, which suggests that miRNAs, as novel and promising therapeutic targets, may exhibit huge clinical value in the future. In addition, miRNAs may function as molecular markers for early the diagnosis of a number of tumors (28,29), which may aid in the development of a number of diagnostic agents for multiple tumor types. miRNA-125a-5p (miR-125a-5p), a type of newly discovered miRNA molecule, has been verified to be involved in the development and progression of a number of tumors, including laryngeal cancer (30), hepatocellular carcinoma (31-33), lung cancer (34) and prostate carcinoma (35). The tumor suppressive function of miR-125a-5p has also been supported by a number of investigations on a variety of tumor types (32,36,37). Notably, miR-125a has been shown to enhance the sensitivity of paclitaxel-resistant colon cancer cells to paclitaxel (38), suggesting that miR-125a may prove to be a novel ancillary drug for use in chemotherapy for patients with tumors. These data imply that miR-125a-5p has tremendous potential for use in the diagnosis, treatment and prognosis of a wide range of tumors.

In the current study, we examined miRNA-125a-5p expression in ESCC tissues and cell lines, and verified its role in the regulation of the proliferation, the cell cycle, apoptosis, and in the migratory and invasive abilities of ESCC cells. miR-125a-5p was found to enhance the sensitivity of ESCC cells to cisplatin by suppressing the activation of the signal transducer and activator of transcription-3 (STAT3) signaling pathway. Most importantly, interleukin (IL)-6, a widely reported activator of the STAT3 signaling pathway (39), abrogated the inactivated status of the STAT3 signaling pathway elicited by the combined use of miR-125a-5p and cisplatin, which was accompanied by cell phenotypic recovery. Taken together, the data from the current study suggest that the manipulation of miR-125a-5p may be used as a strategy with which to enhance the cytotoxic effects of cisplatin on ESCC via the suppression of the activation of the STAT3 signaling pathway.

Materials and methods

Patients and tissue samples. This study was approved by the Institutional Research Ethics Committee of Zhengzhou University. A total of 56 cases of ESCC tissues and paired normal esophageal epithelial tissues were treated with surgical resection alone from the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China from May, 2010 to August, 2012. The clinical characteristics of the patients with ESCC are summarized in Table I. All tissue samples were confirmed by a pathologist. All samples were obtained with informed written and none of the patients had received any treatments prior to surgery. The tissues were immediately frozen in liquid nitrogen until RNA extraction.

Cell lines and cell culture. The human ESCC cell lines, including Eca109, EC9706, EC1, TE1, KYSE450 and KYSE70, as well as normal esophageal epithelial cells, Het-1A, were maintained in liquid nitrogen in our laboratory. The cell lines

above were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco/Thermo Fisher Scientific, Grand Island, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Sigma-Aldrich, St. Louis, MO, USA) in a humidified 5% CO₂ incubator at 37°C.

Cell transfection. The miR-125a-5p mimic, miR-125a-5p inhibitor and the negative control (NC) (Ribobio, Guangzhou, China) at a final concentration of 30 nM were transfected into the EC1 and TE1 cells using Lipofectamine™ 2000 (Invitrogen/Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. All transfection experiments were performed in triplicate for each treatment group at 24, 48, 72 and 96 h for cell proliferation assay and at 48 h for the other experiments.

Prediction of target genes. The potential target genes of miR-125a-5p were searched using online webpage TargetScan (http://www.targetscan.org/vert_72/), miRanda (<http://www.microrna.org/microrna/home.do>) and miRDB (<http://www.mirdb.org/>).

Plasmid construction and luciferase reporter assay. The human STAT3 3'-UTR-wild-type (STAT3-3'-UTR-WT) region containing the miR-125a-5p binding sequence was amplified by PCR, and the STAT3-3'-UTR-mutation (STAT3-3'-UTR-MUT) region with a substitution of 8 bp in the miR-125a-5p binding region was generated using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The STAT3-3'-UTR-WT and STAT3-3'-UTR-MUT were inserted into the downstream region of the Firefly luciferase gene, respectively. The EC1 and TE1 cells were co-transfected using reporter plasmids (400 per 20 ng internal control *Renilla* luciferase plasmid pRL-SV40) and miR-125a-5p mimic or NC by Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer's instructions. Subsequently, luciferase activity was determined using the Dual Luciferase Assay kit (Promega, Madison, WI, USA) using a Synergy H1 hybrid reader (Biotek, Winooski, VT, USA) at 48 h following transfection. Finally, the luciferase activity was normalized to the *Renilla* luciferase activity.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the tissues and cells, and subjected to miRNA First Strand cDNA Synthesis kit (cat. no. B532453; Sangon Biotech, Shanghai, China) using the specific miR-125a-5p reverse transcription primer, 5'-CTCAACTGGTGTCTGGAGTTCGG CAATTCAGTTGAGTCACAGGT-3' and the U6 gene reverse transcription primer, 5'-GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACGACAAAATA-3'. Quantitative PCR (qPCR; Tiangen Biotech, Beijing, China) was used to determine miR-125a-5p expression using the ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) by the addition of miR-125a-5p specific amplification primers as follows: 5'-ACACTCCAGCTGGGTCCCTGAGACCCTTT AAC-3' (forward) and 5'-TGGTGTCTGGAGTCG-3' (reverse).

Western blot analysis. Total proteins were isolated from the ESCC cells using RIPA lysis buffer (Solarbio, Beijing, China). The protein concentration was determined using a Micro BCA

Protein Assay kit (cat. no. 23235; Pierce Biotechnology, Inc., Rockford, IL, USA). The proteins (50 $\mu\text{g}/\text{lane}$) were separated by 12% SDS-PAGE, and then transferred onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). After blocking with skimmed milk, primary antibodies against E-cadherin (cat. no. ab1416, 1:50 dilution), N-cadherin (cat. no. ab98952, 1:500 dilution), Vimentin (cat. no. ab8978, 1:100 dilution), VEGF (cat. no. ab69479, 1:100 dilution), β -actin (cat. no. ab8226, 1:500 dilution) (all from Abcam, Cambridge, MA, USA), total STAT3 (t-STAT3, cat. no. 9139, 1:1,000 dilution) and phosphorylated STAT3 (p-STAT3, cat. no. 4113, 1:1,000 dilution) (all from Cell Signaling Technology, Beverly, MA, USA) were incubated with the PVDF membranes (Millipore Corporation) overnight at room temperature. Subsequently, Horseradish peroxidase (HRP)-labeled secondary antibody (cat. no. SE131, 1:5,000 dilution; Solarbio) was applied to the PVDF membranes. Finally, the protein signal was developed using enhanced chemiluminescence reagents (Beyotime Biotech, Haimen, China). The densitometry of the protein bands was performed using ImageJ software v1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Cell counting kit-8 (CCK-8) assay. The EC1 and TE1 cells (2,000 cells/well) were seeded into a 96-well plate, and these cells were then transfected with miR-125a-5p mimic, miR-125a-5p inhibitor and NC, and treated with cisplatin (0, 1, 2, 5, 10 and 15 $\mu\text{g}/\text{ml}$; Hansoh Pharmaceutical Co. Ltd., Jiangsu, China) or IL-6 (20 $\mu\text{g}/\text{ml}$; PeproTech Inc., Rocky Hill, NJ, USA) in triplicate were applied to the corresponding wells. Cell viability was determined using the CCK-8 kit (Beyotime Biotech) according to the manufacturer's instructions by measuring the absorbance at 450 nm on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Cell cycle detection. Cell cycle assay was conducted as described in a previous study (40). Briefly, the EC1 and TE1 cells were harvested at 48 h following transfection with miR-125a-5p mimic, miR-125a-5p inhibitor or NC, rinsed using PBS and fixed in 70% ethanol overnight at 4°C. After rinsing thrice, propidium iodide (PI; Sigma-Aldrich) was used to treat the cells, and a flow cytometer (BD Biosciences, San Diego, CA, USA) was used to detect the DNA contents.

Cell apoptosis assay. Cell apoptosis assay was performed as described in a previous study (40) using Annexin V FITC/PI (Sigma-Aldrich). In brief, the EC1 and TE1 cells were collected using trypsinase, and Annexin V/PI reagents were added to the cells for 30 min. Finally, a flow cytometer (BD Biosciences) was utilized to determine cell apoptosis.

Wound healing assay. Cell migration was investigated by wound healing migration assay as previously described (41). Briefly, a Ibidi Culture-Insert 2 well (Ibidi Company, Martinsried, Germany) was placed in a 24-well plate, and the EC1 and TE1 cells transfected with miR-125a-5p mimic, miR-125a-5p inhibitor or NC were digested and seeded into 24-well culture plates at a density of 5×10^5 cells/well using RPMI-1640 medium containing 10% FBS. The Culture-Insert 2 well was gently removed 24 h following appropriate cell attachment. At 0, 12 and 24 h, images were obtained at the same position

Table I. Clinical characteristics of the patients with esophageal squamous cell carcinoma.

Characteristic	n=56
Age (years)	
≥ 60	33
<60	23
Sex	
Male	38
Female	18
TNM staging	
I and II	26
III and IV	30
Histological grade	
High differentiation	15
Moderate differentiation	19
Poor differentiation	22
Lymph node metastasis	
Yes	21
No	35

under an inverted microscope (Nikon Instruments, Tokyo, Japan), respectively. The migration distances were quantified by measuring the distances from the wound edges.

Cell invasion assay. Cell invasion assay was performed using a Transwell chamber with Matrigel (BD Biosciences). Briefly, the EC1 and TE1 cells at a density of 1×10^5 were added to the upper layer of the chamber, and 20% FBS was added to the bottom layer of the chamber. Invasive cells were fixed using methanol and stained with crystal violet (Sigma-Aldrich) for 5 min at room temperature 48 h after treatment. Finally, invasive cell numbers were counted under a field of $\times 200$ magnification under an inverted fluorescence microscope (Nikon Instruments).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 software. Data are expressed as the means \pm SD, derived from experiments with at least 3 independently repeats. Comparisons between two groups were made using the Student's t-test, and comparisons between more than two were made using one-way ANOVA followed by Dunnett's test. Values of $P < 0.05$ were considered to indicate statistically significant differences.

Results

Decreased expression of miR-125a-5p in ESCC tissues and cells. Ample evidence has demonstrated that miRNAs are widely involved in tumor development and progression (42,43). In this study, in order to determine whether miR-125a-5p is tightly associated with the occurrence and development of ESCC, we examined its expression patterns in ESCC tissues and cells. We found that relative level of miR-125a-5p in ESCC tissues was significantly lower than that in normal

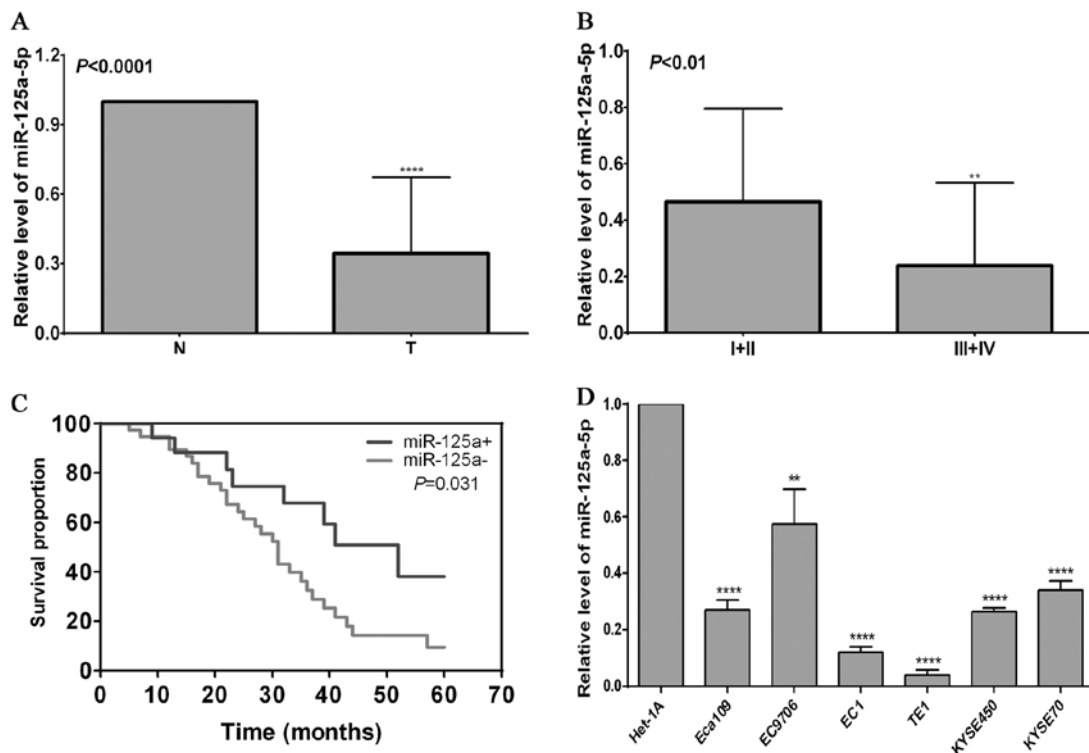


Figure 1. Expression pattern of miR-125a-5p in esophageal squamous cell carcinoma (ESCC) and its association with the prognosis of patients with ESCC. (A) miR-125a-5p level in normal esophageal epithelial tissues (N) and ESCC tissues (T). Total RNA was isolated from 56 ESCC tissues and paired normal esophageal epithelial tissues, and subjected to analysis using the cDNA synthesis kit. RT-qPCR was used to detect the miR-125a-5p level in ESCC tissues and paired normal esophageal epithelial tissues; **** $P < 0.0001$, compared with normal tissues. (B) Expression of miR-125a-5p is tightly associated with tumor TNM staging. TNM staging in a variety of ESCC tissues was confirmed by pathology, and RT-qPCR was used to detect the miR-125a-5p level in ESCC tissues; ** $P < 0.01$, compared with ESCC with I and II staging. (C) Association of miR-125a-5p level with the prognosis of the patients with ESCC. Kaplan-Meier analysis was used to evaluate the association of miR-125a-5p with the overall survival rate, and the log-rank (Mantel-Cox) test was used to determine the difference between positive miR-125a-5p and negative miR-125a-5p expression. (D) Expression of miR-125a-5p in various ESCC cell lines (Eca109, EC9706, EC1, TE1, KYSE450 and KYSE70 cells) and the normal esophageal epithelial cell line, Het-1A; * $P < 0.01$ and **** $P < 0.0001$, compared with the Het-1A cells.

esophageal epithelial tissues ($P < 0.0001$) (Fig. 1A). Further analysis revealed that the relative level of miR-125a-5p in the patients with I + II stage disease was markedly higher than in those with III + IV stage disease ($P < 0.01$) (Fig. 1B), suggesting that miR-125a-5p is tightly associated with the tumor clinical staging in ESCC. To further determine the prognostic value of miR-125a-5p, Kaplan-Meier survival analysis was used to evaluate the association of the miR-125a-5p expression level with the prognosis of patients with ESCC. The results revealed that a high expression of miR-125a-5p contributed to a better overall survival of patients with ESCC (Fig. 1C). Furthermore, an *in vitro* analysis demonstrated that the relative level of miR-125a-5p in ESCC cells (Eca109, EC9706, EC1, TE1, KYSE450 and KYSE70) was evidently lower than that in the normal esophageal epithelial cell line, Het-1A ($P < 0.01$) (Fig. 1D), which further supported the data obtained from ESCC tissues. These findings suggest that miR-125a-5p is involved in the development, progression and prognosis of ESCC and that its upregulation contributes to an improved prognosis of patients with ESCC. Therefore, it is very imperative to examine the function of miR-125a-5p in the occurrence and development of ESCC.

Role of miR-125a-5p in the regulation of the proliferation, cell cycle and apoptosis of ESCC cells. To preliminarily elucidate the underlying function of miR-125a-5p in ESCC, we transfected

NC, miR-125a-5p mimic and miR-125a-5p inhibitor into EC1 and TE1 ESCC cells, and RT-qPCR was employed to examine miR-125a-5p expression in the ESCC cells. We found that relative level of miR-125a-5p in the miR-125a-5p mimic group was significantly higher than that in the control group and NC group in the EC1 and TE1 cells ($P < 0.05$), whereas transfection with the miR-125a-5p yielded opposite results in the EC1 and TE1 cells (Fig. 2A). Further analysis using CCK-8 assay confirmed that miR-125a-5p overexpression contributed to the suppression of the proliferation of the EC1 and TE1 cells at various time points, including 24, 48, 72 and 96 h, compared with the control group and NC group ($P < 0.05$), and opposite data were observed in the miR-125a-5p inhibitor group (Fig. 2B). Cell cycle analysis revealed that the cell numbers in the G0/G1 phase in the miR-125a-5p mimic group were markedly higher than those in the control group and NC group and opposite results were observed with the cells in the S phase ($P < 0.05$); however, transfection with miR-125a-5p inhibitor markedly decreased the cell numbers in the G0/G1 phase and increased the cell numbers in the S phase compared with the control group and NC group (Fig. 2C and D). Moreover, cell apoptosis assay revealed that miR-125a-5p upregulation markedly promoted the apoptosis of the EC1 and TE1 cells, and miR-125a-5p downregulation evidently suppressed cell apoptosis, compared with the control group and NC group (Fig. 2E and F). These

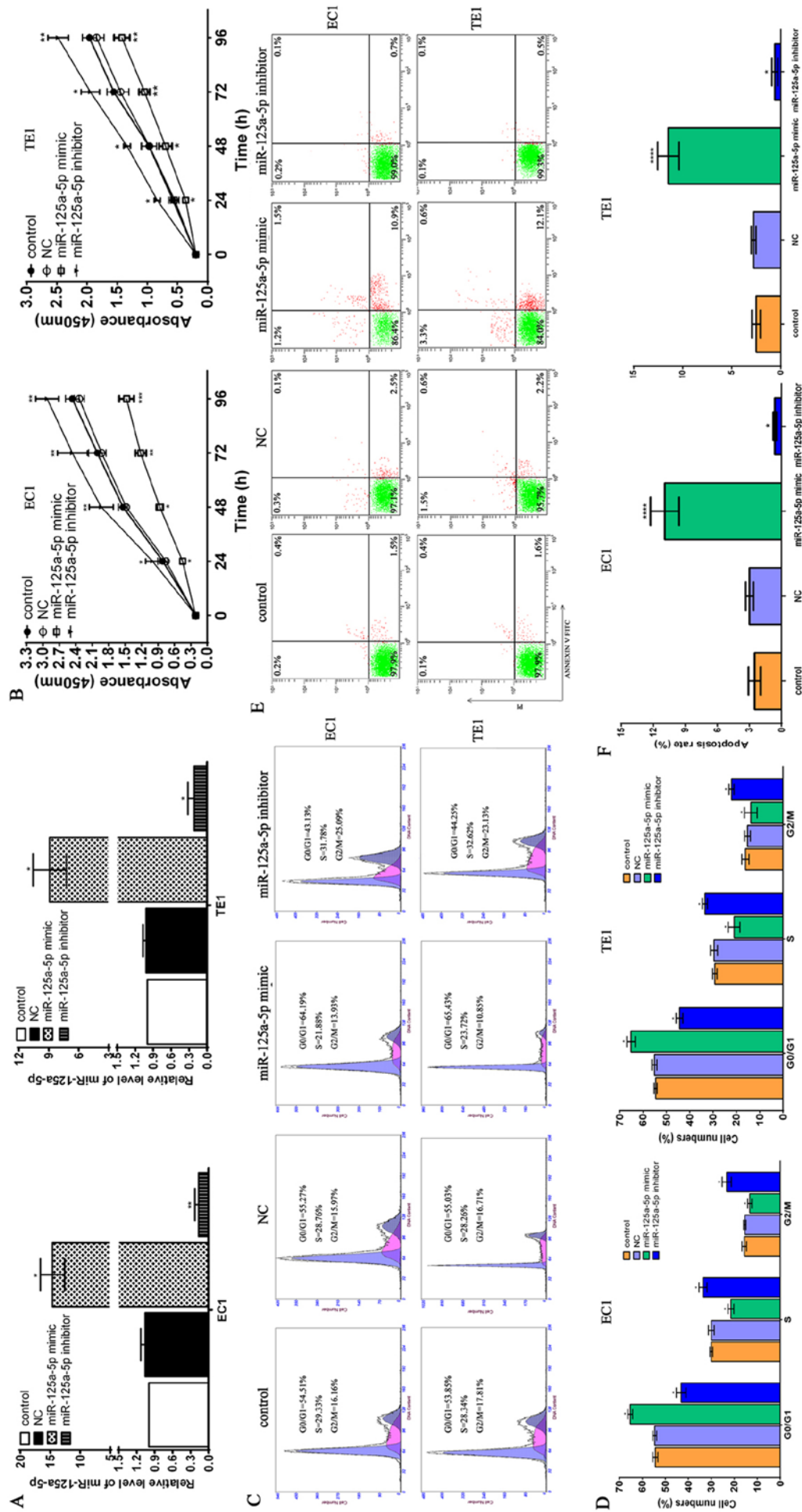


Figure 2. The potential role of miR-125a-5p in the proliferation, cell cycle progression and apoptosis of esophageal squamous cell carcinoma (ESCC) cells. (A) miR-125a-5p mimic or inhibitor triggered the marked upregulation or downregulation miR-125a-5p of ESCC ECI and TE1 cells, respectively. NC, miR-125a-5p mimic and inhibitor were transfected into ECI and TE1 ESCC cells, and total RNA was extracted from the ECI and TE1 cells using TRIzol reagent at 48 h after transfection. Subsequently, cDNA was synthesized using the cDNA synthesis kit and RT-qPCR was used to determine the miR-125a-5p level in the ECI and TE1 cells; * $P < 0.05$, compared with the control group and NC group. (B) Effects of miR-125a-5p upregulation or downregulation on the proliferation of ESCC ECI and TE1 cells. The CCK-8 kit was used to determine cell proliferation according to the standard protocol, and the absorbance value at 450 nm was obtained using a microplate reader; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the control group and NC group. (C) miR-125a-5p upregulation or downregulation altered the cell cycle distribution in the G0/G1 and S phase in the ESCC ECI and TE1 cells. (D) Statistical analysis of cell cycle distribution in ESCC cells; * $P < 0.05$, compared with the control group and NC group. (E) Effects of miR-125a-5p upregulation or downregulation on the apoptosis of ESCC cells. (F) Statistical analysis of the number of apoptotic ESCC cells; * $P < 0.05$ and *** $P < 0.0001$, compared with the control group and NC group.

data suggest that miR-125a-5p functions as a tumor suppressor in ESCC.

miR-125a-5p suppresses the migratory and invasive abilities of ESCC cells via the regulation of the epithelial-mesenchymal transition (EMT) process. Accumulating evidence has demonstrated that miRNAs are widely involved in the migration and invasion in a wide range of tumors (44,45). However, whether miR-125a-5p is tightly associated with the invasion and metastasis of ESCC remains undetermined. Thus, in this study, we examined the effects of miR-125a-5p mimic or inhibitor on the migratory and invasive abilities of EC1 and TE1 cells. The results from wound healing assay and Transwell chamber assay revealed that miR-125a-5p overexpression significantly suppressed the cell migratory and invasive abilities; conversely, miR-125a-5p downregulation markedly promoted the migratory and invasive abilities of the EC1 and TE1 cells, compared with the control group and NC group ($P<0.01$) (Fig. 3A-D). A number of studies have revealed that EMT plays an important role in invasion, metastasis, and carcinogenesis in a variety of tumors (46,47). Thus, in this study, to elucidate the underlying mechanisms responsible for the suppressive effects of miR-125a-5p on the invasive ability of ESCC cells, we detected the expression levels of EMT-related molecular markers in ESCC cells. We found that miR-125a-5p overexpression led to E-cadherin upregulation, and the downregulation of N-cadherin and Vimentin in the EC1 and TE1 cells, whereas miR-125a-5p downregulation decreased the E-cadherin level, and enhanced the levels of N-cadherin and Vimentin, compared with the control group and NC group (Fig. 3E and F), suggesting miR-125a-5p inhibits the EMT process in ESCC. These findings indicate that miR-125a-5p mediates the suppression of the migration and invasion of ESCC cells via the inhibition of the EMT process.

miR-125a-5p upregulation enhances the cytotoxic effects of cisplatin, induces cell apoptosis and reduces the migratory and invasive abilities of ESCC cells. Chemoresistance is the main cause of tumor treatment failure, and aberrant miRNA levels are closely linked to chemosensitivity and chemoresistance in a wide range of tumors. To further investigate whether miR-125a-5p is tightly implicated in chemosensitivity and chemoresistance in ESCC cells, in this study, we investigated the effects of various concentrations of cisplatin combined with NC or miR-125a-5p transfection on the proliferation, apoptosis, migration and invasion of ESCC cells. We found that miR-125a-5p significantly enhanced the cytotoxic effects of cisplatin on EC1 and TE1 cells, compared with NC group treated with cisplatin (Fig. 4A). Further analysis revealed that transfection with miR-125a-5p mimic alone or treatment with cisplatin alone markedly induced cell apoptosis and reduced the migratory and invasive abilities of the EC1 and TE1 cells, compared with the NC group ($P<0.05$) (Fig. 4B-G). However, miR-125a-5p in combination with cisplatin was the most effective in the induction of cell apoptosis and the decrease in the migratory and invasive abilities of the EC1 and TE1 cells (Fig. 4B-G). To further elucidate the underlying mechanisms of the combined effects of miR-125a-5p and cisplatin, we further examined the levels of the EMT-related proteins,

E-cadherin, N-cadherin and Vimentin. We found that transfection with miR-125a-5p alone, and treatment with cisplatin alone or their combination significantly promoted E-cadherin expression, and suppressed the expression levels of N-cadherin and Vimentin, compared with the NC group ($P<0.05$) in the EC1 and TE1 cells. However, the combined use of miR-125a-5p and cisplatin was the most effective in suppressing the EMT process (Fig. 4H and I). These data indicate that miR-125a-5p plays a pivotal role in enhancing the cisplatin-mediated chemosensitivity of ESCC cells via suppressing the EMT process.

STAT3 is a direct target of miR-125a-5p in ESCC. To clarify the possible molecular mechanisms of the chemosensitivity triggered by miR-125a-5p in ESCC, we performed a search for the potential target genes of miR-125a-5p using TargetScan, miRanda and miRDB. We found that STAT3 was a potential target gene of miR-125a-5p (Fig. 5A), and corresponding STAT3-3'-UTR-WT and STAT3-3'-UTR-MUT plasmids were constructed (Fig. 5A). Subsequently, these vectors, along with NC or miR-125a-5p were co-transfected into the EC1 and TE1 cells, and the luciferase activity was determined by measuring the relative luciferase intensity. We found that miR-125a-5p significantly decreased the luciferase activity in the STAT3-3'-UTR-WT group, but it did not affect the luciferase activity in the cells in the STAT3-3'-UTR-MUT group (Fig. 5B and C), suggesting that miR-125a-5p can directly bind to the 3'UTR region of STAT3. To validate the results mentioned above, we further performed western blot analysis to detect the expression levels of t-STAT3, p-STAT3 and its downstream target gene, VEGF, in the EC1 and TE1 cells. The results demonstrated that miR-125a-5p overexpression significantly decreased the protein levels of t-STAT3, p-STAT3 and VEGF in the EC1 and TE1 cells, compared with the control and NC group ($P<0.01$) (Fig. 5D-G). These findings suggest that STAT3 is a direct target gene of miR-125a-5p, and that miR-125a-5p suppresses the invasive ability of ESCC cells, and enhances chemosensitivity and that these effects may be mediated via the suppression of the activation of the STAT3 signaling pathway in ESCC.

Combination of miR-125a-5p with cisplatin inactivates the STAT3 signaling pathway in ESCC cells. To further explore the underlying mechanisms of the antitumor effects mediated by miR-125a-5p in combination with cisplatin in ESCC, we examined the protein expression levels of t-STAT3, p-STAT3 and VEGF in ESCC cells subjected to different treatments. We found that miR-125a-5p alone or cisplatin alone significantly downregulated the protein levels of t-STAT3, p-STAT3 and VEGF, compared with the NC group ($P<0.01$) (Fig. 6A and B). However, the combination of miR-125a-5p with cisplatin exerted the most prominent inhibitory effect on the expression levels of these proteins (Fig. 6A and B). These findings suggest that miR-125a-5p and cisplatin play a synergistic antitumor role in ESCC via suppressing the activation of the STAT3 signaling pathway.

IL-6 attenuates the inhibitory effects of miR-125a-5p combined with cisplatin on the activation of the STAT3 signaling pathway in ESCC cells. To determine whether the re-activation of the STAT3 signaling pathway evoked by IL-6 (a widely

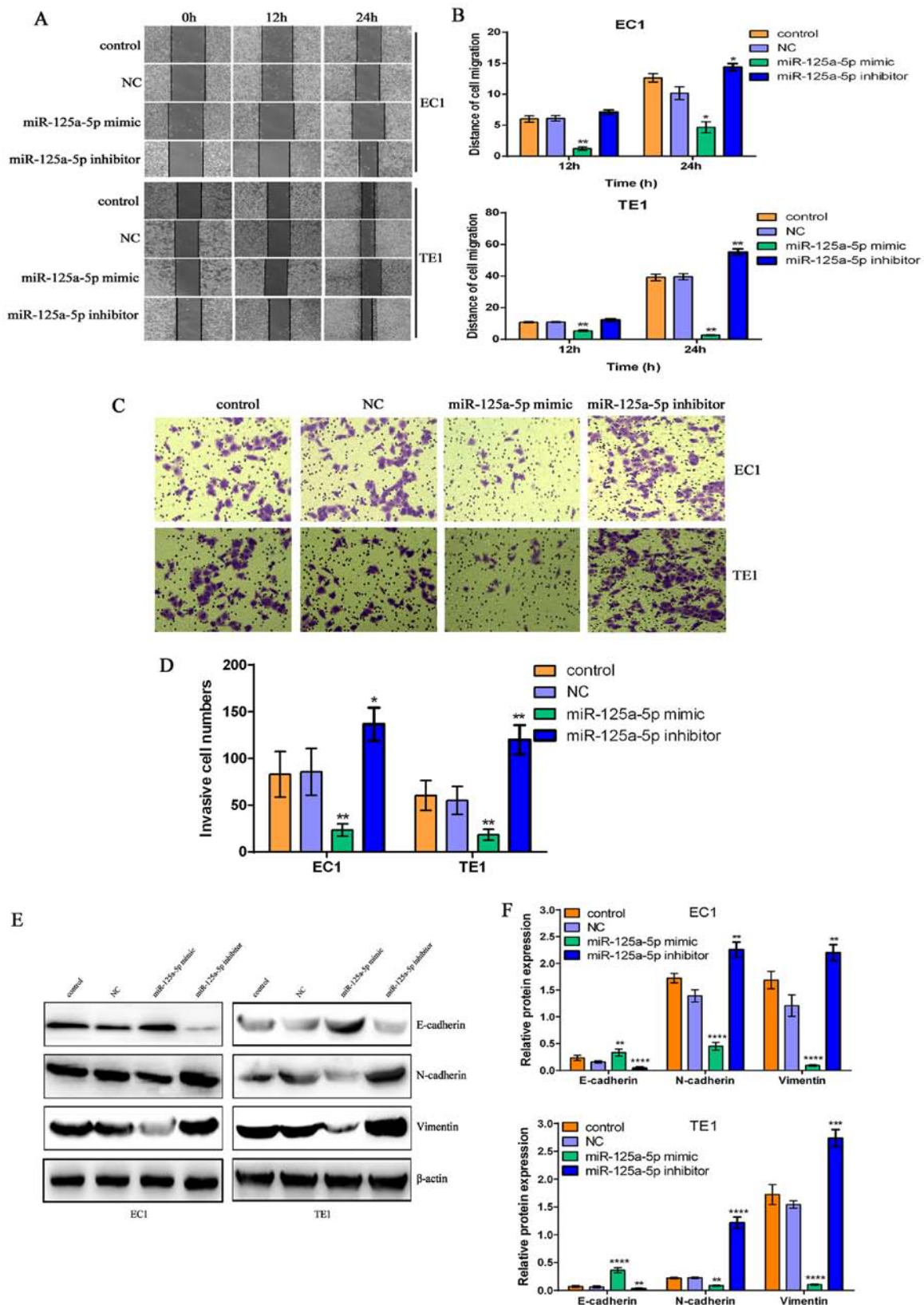


Figure 3. The miR-125a-5p-mediated changes in the migratory and invasive abilities of esophageal squamous cell carcinoma (ESCC) cells are tightly associated with the epithelial-mesenchymal transition (EMT) process. (A) miR-125a-5p overexpression or downregulation evidently reduced or promoted the migratory ability of EC1 and TE1 ESCC cells, respectively. (B) Migration distance was counted to evaluate the migratory ability of EC1 and TE1 cells; * $P < 0.05$ and ** $P < 0.01$, compared with the control group and NC group. (C) miR-125a-5p overexpression or downregulation markedly suppressed or enhanced the invasive ability of EC1 and TE1 ESCC cells. (D) Statistical analysis of the invasive numbers of EC1 and TE1 ESCC cells; * $P < 0.05$ and ** $P < 0.01$, compared with the control group and NC group. (E) miR-125a-5p overexpression suppressed the protein expression levels of N-cadherin and Vimentin, and promoted E-cadherin protein expression; miR-125a-5p downregulation promoted the expression levels of N-cadherin and Vimentin proteins, and suppressed E-cadherin protein expression in EC1 and TE1 ESCC cells. Western blot analysis was utilized to investigate the expression levels of the EMT-related molecular markers, E-cadherin, N-cadherin and Vimentin, and β -actin was used as a loading control. (F) Relative levels of E-cadherin, N-cadherin and Vimentin in ESCC EC1 and TE1 cells; ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$, compared with the control group and NC group.

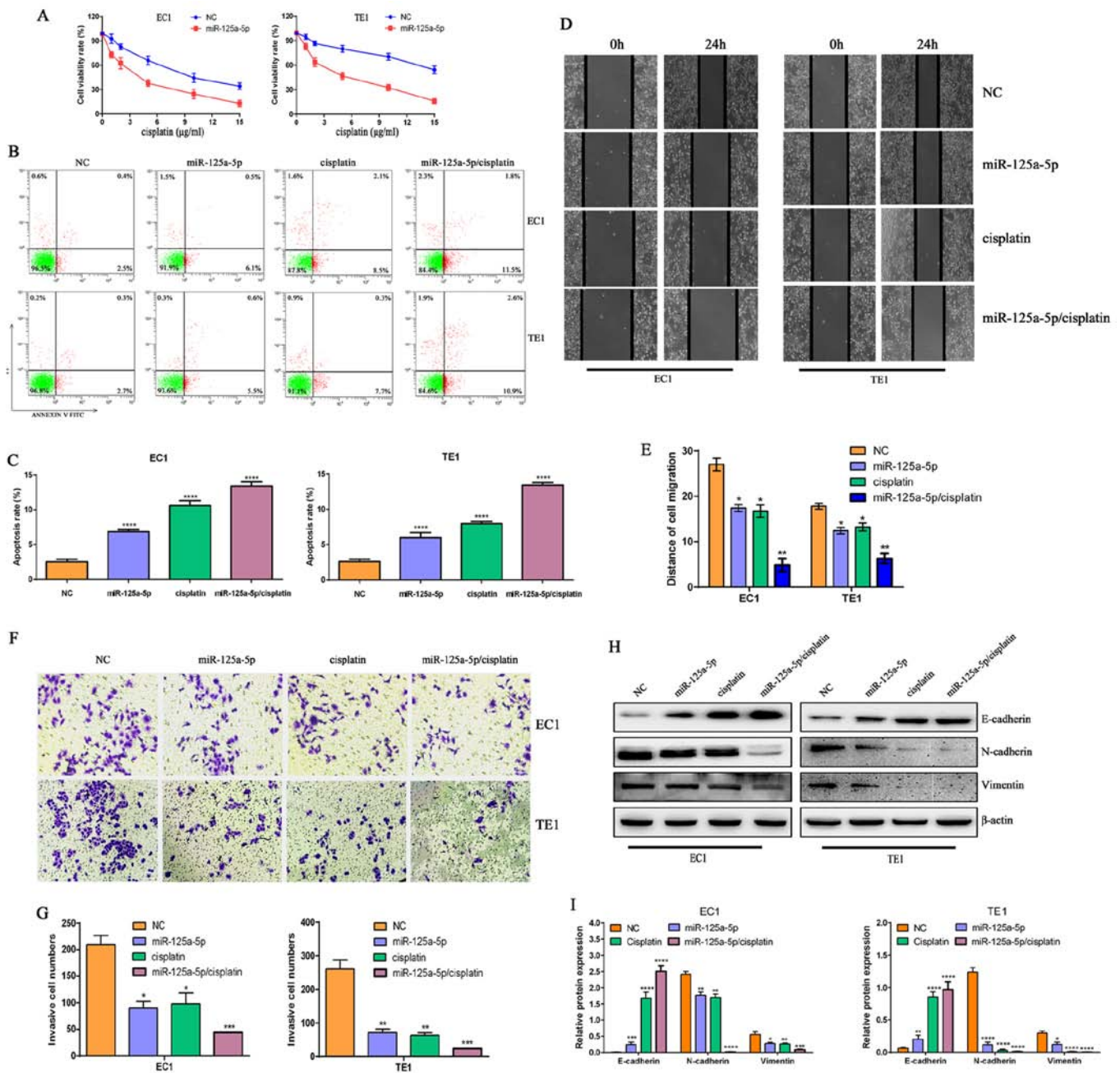


Figure 4. miR-125a-5p enhances the sensitivity of esophageal squamous cell carcinoma (ESCC) cells to cisplatin. (A) miR-125a-5p increased the cell-killing effects of cisplatin on EC1 and TE1 ESCC cells. (B) miR-125a-5p combined with cisplatin significantly promoted the apoptosis of EC1 and TE1 ESCC cells. (C) Statistical analysis of the numbers of apoptotic EC1 and TE1 ESCC cells subjected to the different treatments; **** $P < 0.0001$, compared with NC group. (D) miR-125a-5p in combination with cisplatin significantly inhibited the migratory ability of EC1 and TE1 ESCC cells. (E) Migration distance was counted to evaluate the migratory ability of EC1 and TE1 cells; * $P < 0.05$ and ** $P < 0.01$, compared with the NC group. (F) miR-125a-5p in combination with cisplatin significantly inhibited the invasive ability of EC1 and TE1 ESCC cells. (G) Statistical analysis of the invasive numbers of EC1 and TE1 ESCC cells subjected to various treatments; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the NC group. (H) miR-125a-5p in combination with cisplatin promoted E-cadherin expression, and suppressed the expression levels of N-cadherin and Vimentin in EC1 and TE1 ESCC cells. Western blot analysis was utilized to examine the expression levels of the EMT-related molecular markers, E-cadherin, N-cadherin and Vimentin, and β -actin was used as a loading control. (I) Relative levels of E-cadherin, N-cadherin and Vimentin in EC1 and TE1 ESCC cells, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$, compared with the NC group.

reported activator of the STAT3 signaling pathway) can abolish the inactivation of the STAT3 signaling pathway induced by miR-125a-5p in combination with cisplatin in ESCC cells, western blot analysis was employed to examine the activation status of the STAT3 signaling pathway in the EC1 and TE1 cells. The results indicated that IL-6 markedly increased the protein expression levels of t-STAT3, p-STAT3 and VEGF, compared with the miR-125a-5p/cisplatin group ($P < 0.01$) in the

EC1 and TE1 cells (Fig. 6C and D), suggesting that IL-6 can re-activate the STAT3 signaling pathway in ESCC cells treated with a combination of miR-125a-5p and cisplatin.

STAT3 activation evoked by IL-6 attenuates the suppressive effects mediated by miR-125a-5p/cisplatin on the proliferation and invasion of ESCC cells and also attenuates the pro-apoptotic effects. To further determine whether STAT3 activation

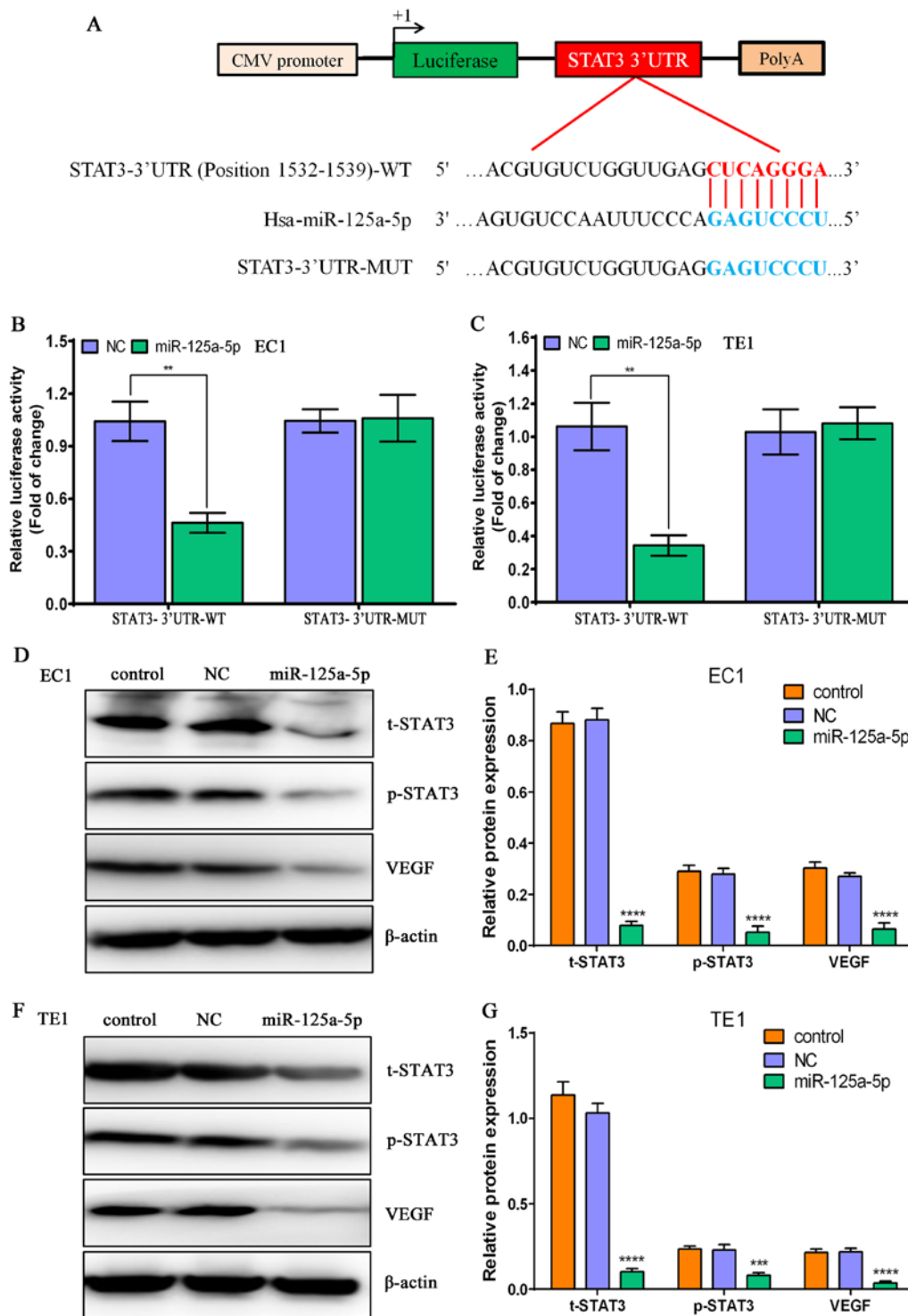


Figure 5. STAT3 is a direct target of miR-125a-5p in esophageal squamous cell carcinoma (ESCC) cells. (A) The signal transducer and activator of transcription-3 (STAT3) 3'-UTR sequences including wild-type (WT) or mutant (MUT) were inserted into the downstream of luciferase reporter vector according to diagrammatic presentation. (B) The luciferase activity was determined by co-transfecting the vectors (STAT3 3'-UTR-WT and MUT) combined with NC or miR-125a-5p mimic into EC1 ESCC cells; ** $P < 0.01$, compared with the NC group. (C) The luciferase activity was determined by co-transfecting the vectors (STAT3 3'-UTR-WT and MUT) combined with NC or miR-125a-5p mimic into TE1 ESCC cells; ** $P < 0.01$, compared with the NC group. (D) Western blot analysis of the protein expression levels of t-STAT3, p-STAT3 and vascular endothelial growth factor (VEGF) in the EC1 ESCC cells subjected to various treatments, and β -actin was used as a loading control. (E) Relative protein levels of t-STAT3, p-STAT3 and VEGF in EC1 ESCC cells subjected to different treatments; **** $P < 0.0001$, compared with the control group and NC group. (F) Western blot analysis of the protein expression levels of t-STAT3, p-STAT3 and VEGF in TE1 ESCC cells subjected to various treatments, and β -actin was used as a loading control. (G) Relative protein levels of t-STAT3, p-STAT3 and VEGF in TE1 ESCC cells subjected to various treatments; *** $P < 0.001$ and **** $P < 0.0001$, compared with the control group and NC group.

can partially recover the phenotype of ESCC cells, which had been altered by miR-125a-5p/cisplatin, IL-6 was used to treat the ESCC cells, and CCK-8, flow cytometry and Transwell

chamber assay were used to examine the status of proliferation, apoptosis and invasion of EC1 and TE1 cells, respectively. We found that miR-125a-5p/cisplatin/IL-6 treatment significantly

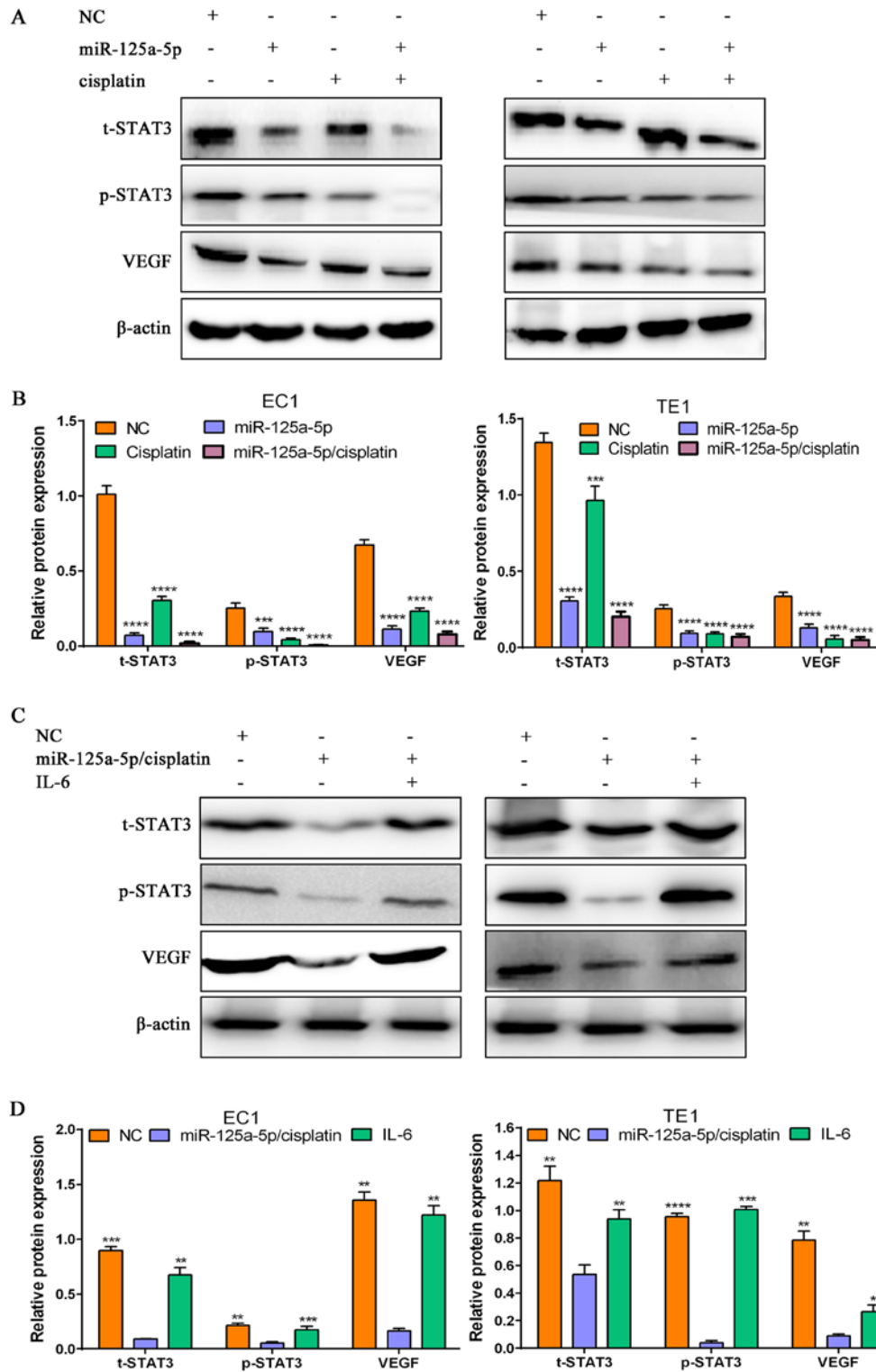


Figure 6. miR-125a-5p/cisplatin suppresses signal transducer and activator of transcription-3 (STAT3) activation and vascular endothelial growth factor (VEGF) expression, and the STAT3 activator, interleukin (IL)-6 re-activates the STAT3 signaling pathway in esophageal squamous cell carcinoma (ESCC) cells treated with miR-125a/cisplatin. (A) Western blot analysis of the protein expression levels of t-STAT3, p-STAT3 and VEGF in the NC group, miR-125a-5p alone group, cisplatin alone group and miR-125a-5p/cisplatin combination group, and β -actin was employed as a loading control. (B) The relative protein levels of t-STAT3, p-STAT3 and VEGF in the NC group, miR-125a-5p alone group, cisplatin alone group and miR-125a-5p/cisplatin combination group; *** P <0.001 and **** P <0.0001, compared with the NC group. (C) Western blot analysis of the protein expression levels of t-STAT3, p-STAT3 and VEGF in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group, and β -actin was employed as a loading control. (D) The relative protein levels of t-STAT3, p-STAT3 and VEGF in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group; * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001, compared with the miR-125a-5p/cisplatin combination group.

increased the viability of the EC1 and TE1 cells at 24, 48 and 72 h, compared with the miR-125a-5p/cisplatin group (Fig. 7A).

Cell apoptosis assay revealed that miR-125a-5p/cisplatin/IL-6 treatment markedly decreased the apoptosis of the EC1 and

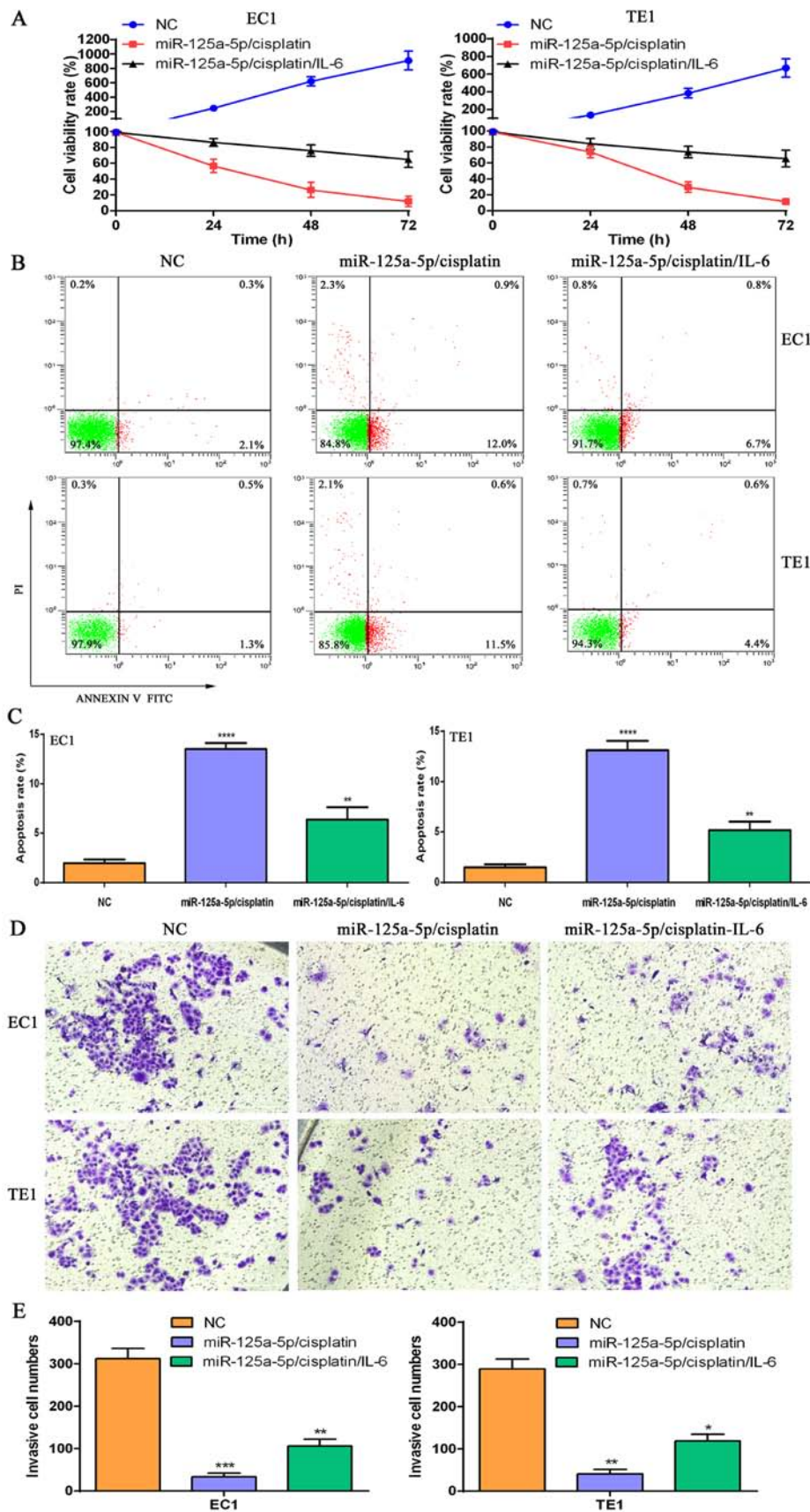


Figure 7. The signal transducer and activator of transcription-3 (STAT3) activator, interleukin (IL)-6 reverses the alteration in the cell phenotype mediated by miR-125a-5p/cisplatin in esophageal squamous cell carcinoma (ESCC) cells. (A) CCK-8 kit assay for cell proliferation in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group. (B) Flow cytometry of cell apoptosis in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group. (C) Statistical analysis of the apoptotic cell numbers in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group; **P<0.01 and ****P<0.0001, compared with the NC group. (D) Transwell chamber assay for cell invasive ability in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group. (E) Statistical analysis of the invasive cell numbers in the NC group, miR-125a-5p/cisplatin combination group and miR-125a-5p/cisplatin/IL-6 combination group, *P<0.05, **P<0.01 and ***P<0.001, compared with the NC group.

TE1 cells, compared with the miR-125a-5p/cisplatin group ($P < 0.01$) (Fig. 7B and C). Notably, we found that miR-125a-5p/cisplatin/IL-6 treatment evidently restored the invasive ability of the EC1 and TE1 cells, compared with the miR-125a-5p/cisplatin group ($P < 0.05$) (Fig. 7D and E). These data suggest that miR-125a-5p/cisplatin exerts antitumor effects by inhibiting the activation of the STAT3 signaling pathway.

Discussion

Mounting evidence has demonstrated that miRNAs function as either oncogenes or tumor suppressor genes in various type tumors, which may bring forth new challenges or may open up novel opportunities for the use of miRNAs as novel molecular targets for a myriad of tumors (48,49). Moreover, miRNAs have been verified as a therapeutic tool in the management of pancreatic adenocarcinoma in clinical studies (50,51), suggesting that miRNAs have important clinical value in many different types of tumors. Therefore, the identification of key miRNA molecules implicated in the development and progression of ESCC may provide new diagnostic and prognostic markers, and may aid in the development of more effective treatment strategies for patients with ESCC. In the current study, we found that miR-125a-5p was downregulated in ESCC tissues and cells, implying that miR-125a-5p may function as a tumor suppressor in ESCC. Further analysis revealed that a decreased miR-125a-5p expression was tightly associated with a higher tumor staging and a lower survival rate of patients with ESCC, suggesting that miR-125a-5p participates in tumor development and progression; thus, miR-125a-5p may be a molecular marker for the malignant degree and prognosis of patients with ESCC.

There is strong evidence that miRNAs are tightly implicated in a mass of complex regulatory networks of crucial genes in a variety of tumors (52-54). It has been indicated that target genes of miRNAs are direct regulators of the hallmarks of cancer, including proliferation, cell cycle and cell apoptosis (52). Tao *et al* found that a decreased miR-125a expression in osteosarcoma tissues, and its overexpression contributed to growth suppression in osteosarcoma cells by downregulating E2F2 expression (55). Moreover, miR-125a-5p overexpression has been shown to markedly inhibit cell proliferation and tumor formation in retinoblastoma, exerting antitumor effects by suppressing the transcriptional co-activator with PDZ binding motif (TAZ) (36), a critical downstream component of the Hippo signaling pathway; TAZ overexpression has been shown to markedly accelerate tumor initiation and progression (56,57). Qin *et al* confirmed that miR-125a-5p overexpression significantly suppressed the proliferation and migratory ability of cervical cancer cells through the direct targeted inhibition of ABL2 expression (37). Converse results from leukemia have revealed that miR-125a overexpression induces daunorubicin resistance by suppressing the apoptosis of HL-60, K562 and THP-1 cells, which was further verified to be directly correlated with the downregulation of GRK2 and Puma (58). These findings fully highlighted the essential role of miR-125a-5p in the regulation of proliferation and apoptosis in a plethora of tumors. In this study, we found that miR-125a-5p overexpression significantly inhibited the proliferation, arrested the cell cycle in the G0/G1 phase and

induced the apoptosis of ESCC cells, whereas miR-125a-5p downregulation markedly promoted the proliferation and cell cycle progression, and inhibited the apoptosis of ESCC cells. These findings suggest that miR-125a-5p may be an important regulator of cell proliferation, cell cycle and apoptosis in ESCC, and thus the manipulation of miR-125a-5p may be a novel molecular target for ESCC.

Understanding the possible mechanisms of tumor invasion and metastasis remains a formidable challenge for a large number of tumors. Several studies have focused on the involvement of miRNAs in tumor invasion and metastasis (59-62). The EMT process has been reported to be involved in tumor progression and metastasis, which may be a pivotal mechanism of tumor invasion and metastasis (63,64). Recently, increasing evidence has demonstrated that miRNAs implicated in tumor invasion and metastasis are tightly associated with the EMT process (65-68). To further interpret the possible role of miR-125a-5p in invasion and metastasis of ESCC, we further examined the alterations in the migratory and invasive abilities of ESCC cells triggered by miRNA-125a-5p. We found that miR-125a-5p overexpression significantly reduced the migratory and invasive abilities of ESCC cells, whereas its downregulation promoted the migratory and invasive abilities of ESCC cells. Further analysis revealed that transfection with miR-125a-5p mimic markedly upregulated the protein expression levels of N-cadherin and Vimentin, and downregulated the E-cadherin protein level in ESCC, and converse results were observed in the miR-125a-5p inhibitor group, implying that the involvement of miR-125a-5p in tumor invasion and metastasis may be partly achieved through the modulation of EMT-related signaling pathways. These findings potentiate miR-125a-5p as a potential predictor for the invasion and metastasis of ESCC.

Chemoresistance is a major hurdle in the treatment of many tumor patients. Recent studies have revealed that aberrant miRNA levels are tightly implicated in chemoresistance or chemosensitivity in a host of tumors (69-74), suggesting that targeting miRNAs to eradicate chemoresistance or improve chemosensitivity may be a novel therapeutic strategy for the therapy of tumor patients. Nishida *et al* found that miR-125a-5p evidently suppressed cell proliferation via targeting ERBB2, and miR-125a-5p markedly enhanced the sensitivity of gastric carcinoma cells to trastuzumab (75). Another study revealed that miR-200a increased the chemoresistance of breast cancer cells to chemotherapeutic agents; by contrast, miR-200a downregulation promoted the sensitivity of resistant cancer cells to gemcitabine (70). Furthermore, the re-introduction of miR-31 has been shown to significantly promote clonogenic resistance to cisplatin and carboplatin in NCI-H2452 cells without miR-31 expression (76). Another study revealed that miR-125a overexpression enhanced the sensitivity of paclitaxel on paclitaxel-resistant colon cancer cells (38). These findings highlight the crucial role of miRNAs in potentiating chemosensitivity in the process of tumor therapy. However, whether miR-125a-5p is implicated in the chemosensitivity of ESCC cells remains unknown. In this study, as expected, miR-125a-5p significantly elevated the killing efficacy of cisplatin on ESCC cells, and miR-125a-5p/cisplatin significantly promoted the apoptosis and reduced the migratory and invasive abilities of ESCC cells. Further analysis revealed that co-treatment with miR-125a-5p

and cisplatin markedly increased the E-cadherin level and reduced the levels of N-cadherin and Vimentin, suggesting that the miR-125a-5p-mediated enhancement of the cisplatin sensitivity of ESCC cells may be tightly associated with the suppression of the EMT process. The data presented herein suggest that miR-125a-5p markedly improved the therapeutic efficacy of cisplatin in ESCC, and thus miR-125a-5p may be a novel ancillary drug of cisplatin for use in the treatment of patients with ESCC in the future.

Several studies have demonstrated that the STAT3 signaling pathway is implicated in drug resistance in a variety of tumors (77,78), and its activation not only promotes tumor growth rapidly, but also imparts therapeutic resistance in cancer cells (77,79-81). In this study, we determined whether the involvement of miRNA-125a-5p in the sensitivity of ESCC cells to cisplatin may be tightly associated with the activated status of the STAT3 signaling pathway. Therefore, we performed a search for the potential target genes of miR-125a-5p using TargetScan, miRanda and miRDB, and found that STAT3 was the direct target gene of miR-125a-5p by luciferase reporter assay. Further analysis demonstrated that miR-125a-5p overexpression significantly reduced the protein levels of t-STAT3, p-STAT3 and its downstream target gene, VEGF, ESCC cells. These findings suggest that the miR-125a-5p-mediated chemosensitivity may be tightly associated with the inactivation of the STAT3 signaling pathway in ESCC. Correspondingly, whether the re-activation of the STAT3 signaling pathway can reverse the chemosensitivity mediated by miR-125a-5p overexpression in ESCC, was examined. IL-6 [an activator of the STAT3 signaling pathway (39)] was used to re-activate the the STAT3 signaling pathway in ESCC cells. We found that IL-6 significantly recovered the t-STAT3 and p-STAT3 levels which were suppressed by miR-125a-5p/cisplatin in the ESCC cells, and further analysis revealed that the re-activation of the STAT3 signaling pathway evoked by IL-6 significantly recovered cell viability, decreased cell apoptosis and promoted the invasion of ECSS cells co-treated with miR-125a-5p and cisplatin. The data presented herein suggest that miR-125a-5p enhances the sensitivity of ESCC cells to cisplatin via suppressing the activation of the STAT3 signaling pathway, which may be an underlying molecular mechanism through which miR-125a-5p exerts antitumor effects on ESCC.

In conclusion, the data from the present study suggest that a low level of miR-125a-5p is tightly associated with a higher tumor staging and a poor prognosis of patients with ESCC. The overexpression of miR-125a-5p significantly suppressed the proliferation, arrested the cell cycle in G0/G1 phase, induced the apoptosis and reduced the migratory and invasive abilities of ESCC cells, and converse results were observed in miR-125a-5p inhibitor group, which may be closely associated with the EMT process. Further analysis indicated that miR-125a-5p enhanced the sensitivity of ESCC cells to cisplatin, which may be achieved by the inactivation of the STAT3 signaling pathway. Most importantly, the re-activation of the STAT3 signaling pathway triggered by IL-6 prominently led to a recovery of the viability, decreased cell apoptosis and an increased cell invasive ability of ESCC cells. The data presented herein may provide a novel therapeutic strategy for the therapy of patients with ESCC by the combined use of miR-125a-5p with cisplatin.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QF supervised the whole project. QF, YZ and KM designed the study. YZ, KM and SY performed the majority of the experiments; XZ contributed to plasmid construction and luciferase reporter assay; FW performed the CCK-8 experiment; XZ and HL participated in the design and interpretation of some of the experiments; YZ and QF interpreted all the results and wrote the manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Research Ethics Committee of Zhengzhou University. All samples were obtained with informal written and none of the patients had received any treatments prior to surgery.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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