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MiR-296-5p inhibits cell invasion and migration of esophageal squamous cell carcinoma by downregulating STAT3 signaling

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Abstract. – OBJECTIVE: Many studies have emphasized the function of microRNA-296 (miR-296) that inhibits tumor formation. To some extent, the role of miR-296 in esophageal squamous cell carcinoma (ESCC) remains misleading. Therefore, the current research was designed to investigate the regulatory mechanisms of miR-296 and signal transducer and activator of transcription 3 (STAT3) in ESCC.

PATIENTS AND METHODS: The mRNA expression of miR-296-5p and STAT3 in ESCC tissues or cell lines was measured via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The protein level of STAT3 was measured by Western blotting assay. The Luciferase reporter assay was used to verify the binding sites between miR-296-5p and STAT3. The transwell assay was employed to identify cell migration and invasion.

RESULTS: Down-regulation of miR-296-5p was detected in ESCC tissues and cell lines ($p<0.01$). Additionally, miR-296-5p was found to target STAT3 directly. Functionally, up-regulation of miR-296-5p or down-regulation of STAT3 significantly inhibited cell migration and invasion in ESCC.

CONCLUSIONS: MiR-296-5p inhibited cell invasion and migration in ESCC by downregulating STAT3. The overexpression of miR-296-5p by targeting STAT3 suppressed tumorigenesis of ESCC cells.

Key Words:
Esophageal squamous cell carcinoma, MiR-296-5p, Migration, Invasion, STAT3.

Introduction

Esophageal cancer is a common gastrointestinal tumor, and about 300,000 people worldwide die from esophageal cancer every year[1]. The incidence and mortality of esophageal cancer are very different between countries, and a higher incidence of esophageal cancer has been found in China[2]. Esophageal cancer mainly includes esophageal squamous cell carcinoma (ESCC) and adenocarcinoma[3]. Although many treatments for ESCC have made great progress, the survival rate of ESCC patients is still low, only 14%-22%[4]. The main reason for the low survival rate of ESCC patients is that ESCC is often diagnosed at an advanced stage[5]. Therefore, finding more effective biomarkers and therapeutic targets for ESCC is invaluable.

In the previous studies[6-10], various microRNAs (miRNAs) have been reported to be involved in the regulatory mechanisms of ESCC by regulating cell proliferation, differentiation and apoptosis. As a tumor suppressor, miR-296 was identified in various human cancers. For example, Li et al[11] reported that miR-296 inhibited cell proliferation and promoted apoptosis by regulating fibroblast growth factor receptor 1 (FGFR1) in human liver cancer. Besides that, miR-296 inhibited the metastasis and epithelial-mesenchymal transition (EMT) of colorectal cancer by targeting S100A4[12]. However, miR-296-5p has been reported to promote cell invasion through the down-regulation of nerve growth factor receptor (NGFR) and Caspase-8[13]. In addition, the carcinogenic function of miR-296 was also identified in laryngeal carcinoma[14]. These studies suggest that the different functions of miR-296 may depend on the types of human cancers.

As a participant in the JAK/STAT signaling pathway, signal transducer and activator of transcription 3 (STAT3) has been reported to mediate the transmission of signals from the cytoplasm to the nucleus. STAT3 has been widely reported to promote tumor metastases in esophageal cancer. Cui et al[15] has found that STAT3 can affect

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EMT induced by hypoxia in ESCC. As a target gene, STAT3 has been found to be regulated by miR-124[16] and miR-874[17] in ESCC. However, the regulatory mechanism between miR-296-5p and STAT3 is still unknown.

Therefore, the abnormal expression of miR-296-5p was investigated in ESCC. We further explored whether miR-296-5p/STAT3 axis inhibited the invasion and migration of ESCC cells. The findings may provide an opportunity to develop effective biomarkers for ESCC.

Patients and Methods

Clinical Tissue Samples

Forty-seven surgical tumor specimens and adjacent tissues were obtained from the Affiliated Hospital of Qingdao University. All patients received no treatment prior to surgery and all participants had signed informed consent. Human tissues were then frozen in liquid nitrogen and stored in a -80°C refrigerator for further use. All tissue samples of the experiment were approved by the Affiliated Hospital of the Qingdao University Institutional Ethics Committee.

Cell Culture and Transfection

The human esophageal cancer cell lines Eca109 and TE-1 and human normal esophageal cell line Het-1A were used in this study. All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). These cells were grown in an incubator at 37°C, with 5% CO_2 in the atmosphere. The medium was replaced every other day depending on the culture state.

The miR-296-5p mimic and inhibitor, STAT3 siRNA were purchased from RiboBio (Guangzhou, China) and transferred into Eca109 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocols.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA containing miRNA to quantify miR-296-5p expression in ESCC tissues and cell lines. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed on an ABI PRISM 7900 Sequence Detection System using Quanti-TectSYBR Green PCR mixture (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-296 and STAT3. The miR-296 and STAT3 levels were analyzed using the $2^{-\Delta\Delta ct}$ method.

Luciferase Assays

The wild or mutant 3'-UTR of STAT3 was inserted into the pGL3 promoter vector (Genscript, Nanjing, China) for Luciferase reporter experiments. Then, the above vector and miR-296-5p mimic were transfected into Eca109 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were then cultured in a 24-well plate for 48 h. Finally, the Dual-Luciferase® Reporter Assay Kit (Promega, Madison, WI, USA) was applied to perform Luciferase assay.

Transwell Migration and Invasion Assay

The migration and invasion abilities of ESCC cells in 24-well plates were assessed using transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA). Then, 5×10^4 ESCC cells without serum were put in the upper chamber with the non-coated membrane, and the lower chamber was filled with 20% FBS to induce ESCC cell migration or invasion into the membrane. In addition, the cells were put in the upper chamber along with the coated membrane for the invasion assay. The cells were then stained with crystal violet (MedChem Express, Shanghai, China).

Western Blot

Protein samples were obtained using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Proteins were separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then incubated with blocked membranes containing 5% non-fat milk at room temperature. Next, we incubated the membranes overnight with anti-STAT3, anti-GAPDH antibodies at 4°C. The membranes were subsequently incubated with matched secondary antibodies. Finally, the protein expression levels were measured by the Bio-Rad Gel imaging system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). Data were shown as mean ± SD (Standard Deviation). The differences were an-

alyzed by Student's *t*-test and Chi-square test. The difference was considered significant at $p<0.05$.

Results

Downregulation of MiR-296-5p in Human ESCC Cell Lines and Tissues

To better understand the role of miR-296-5p in the pathogenesis of ESCC, the abnormal expression of miR-296-5p was detected in ESCC tissues and adjacent normal tissues (n=47, Table I). We found that the downregulation of miR-296-5p was closely correlated with the well and moderately differentiation (p=0.0095) and advanced tumor stage (p=0.0389). Moreover, approximately 69% of ESCC tissues indicated a low expression of miR-296-5p (Figure 1A). Furthermore, the qRT-PCR assay showed that miR-296-5p expression in ESCC tissues was much lower than that in normal tissues (Figure 1B). The low expression of miR-296-5p was also identified in Eca109 and TE-1 cell lines compared with Het-1A cells (Figure 1C). All of these findings suggest that abnormal expression of miR-296-5p may play a pivotal role in the progression of ESCC.

The Cell Migration and Invasion were Inhibited by MiR-296-5p in ESCC

To further explore the role of miR-296-5p in ESCC, we detected cell migration and invasion (Figure 2A) in Eca109 cells. Forty-eight hours after transfection, transwell assay indicated that the abilities of cell migration and invasion in Eca109 cells containing miR-296 mimics were lower than that of miR-NC (Figure 2B, p=0.036 and p=0.023). On the contrary, cell migration and invasion were promoted by the miR-296 inhibitor in Eca109 cells (Figure 2C, p=0.021 and p=0.041). All data revealed that miR-296-5p acted as a tumor suppressor in ESCC by inhibiting cell migration and invasion.

MiR-296-5p Directly Targeted STAT3 in ESCC Cells

Furthermore, STAT3 was found to be one of the target genes of miR-296-5p in TargetScan database (http://www.targetscan.org/vert_71/) (Figure 3A). In addition, the Luciferase reporter assay was performed to verify whether miR-296-5p targets STAT3 directly. We found that Luciferase activity of Eca109 cells containing miR-296-5p mimics and wild STAT3 was markedly suppressed by 53% (p<0.01) in comparison with the control. However, there was almost no change in the cells containing miR-296-5p

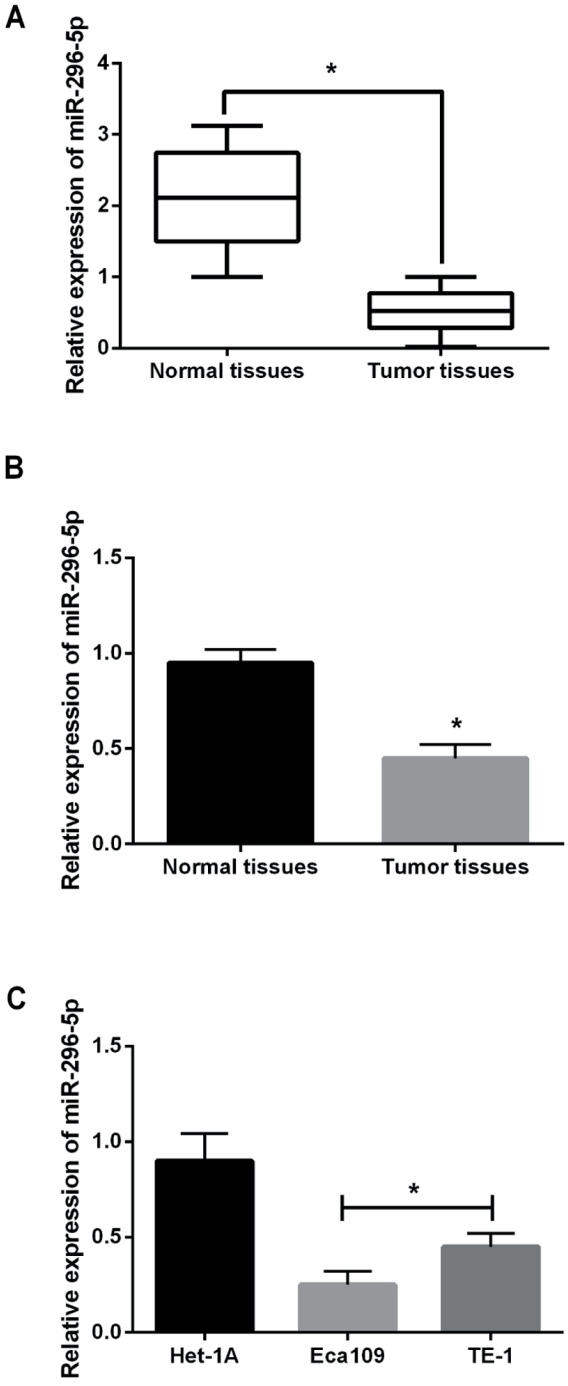

Figure 1. Down-regulation of miR-296-5p in human ESCC cell lines and tissues. ***A,*** The miR-296-5p expression was down-regulated in tumor tissues (n = 47). ***B,*** Expression of miR-296-5p in tumor tissues and corresponding non-tumor tissues. ***C,*** The miR-296-5p expression in Eca109, TE-1, and Het-1A cells (control). **p<0.01.

Table I. Relationship between miR-296-5p expression and their clinicopathological characteristics in 47 esophageal cancer patients.

Characteristics	Number of cases (No. = 47)	miR-296-5p		*p*-value
		High	Low	
Age (years)				0.1175
≥ 60	27	12	15	
<60	20	3	17	
Gender				0.5224
Male	25	7	18	
Female	22	8	14	
Tumor size (cm)				0.0889
≥ 4	19	7	12	
<4	28	8	20	
Degree of differentiation				0.0095*
Well and moderately Poorly	29	5	24	
	18	10	8	
Tumor invasion				0.0389*
T1+T2	28	9	19	
T3+T4	19	6	13	
TNM stage				0.1175
I + II	27	7	20	
III + IV	20	8	12	
Lymph-node metastasis				
Negative	26	10	16	0.2752
Positive	21	5	16	

Statistical analyses were performed by the χ^2-test. TNM, tumor-node-metastasis. *p<0.05 was considered significant.

mimic and mutant STAT3 (Figure 3B). According to the Luciferase reporter assay, we can deduce that miR-296-5p directly targets STAT3 in ESCC cells. To further verify the above results, we transfected miR-296-5p mimics or inhibitor into Eca109 cells. MRNA and protein expression levels of STAT3 were significantly reduced in Eca109 cells containing miR-296-5p mimics. Increased expression of STAT3 was identified in cells with miR-296-5p inhibitor in comparison with the control (Figure 3C, 3D). Briefly, miR-296-5p targeted STAT3 directly and suppressed STAT3 expression.

The Function of STAT3 and Interaction with MiR-296-5p in ESCC

To investigate the effect of STAT3 on cell migration and invasion in ESCC, STAT3 siRNA or corresponding control siRNA was transfected into Eca109 cells. First, STAT3 was detected to be upregulated in Eca109 and TE-1 lines compared to Het-1A cells (Figure 4A). Next, STAT3 expression was found to be decreased in Eca109 cells containing STAT3 siRNA versus the control (Figure 4B). Functionally, cell migration and invasion were impaired by STAT3 siRNA (Figure 4C). The findings proved that STAT3 promoted cell migration and invasion in ESCC.

Based on the above results, miR-296-5p was speculated to suppress the function of STAT3 in ESCC. To verify the prediction, the negative control or STAT3 expression vector was transfected into Eca109 cells with miR-296-5p mimics. As we predicted, the decreased STAT3 expression induced by miR-296-5p mimics was recovered by STAT3 expression vector (Figure 5A, 5B). As shown in Figure 5C, STAT3 weakened the effect of miR-296-5p on inhibiting cell migration and invasion in Eca109 cells. Combined with all of the results, miR-296-5p was found to suppress cell migration and invasion

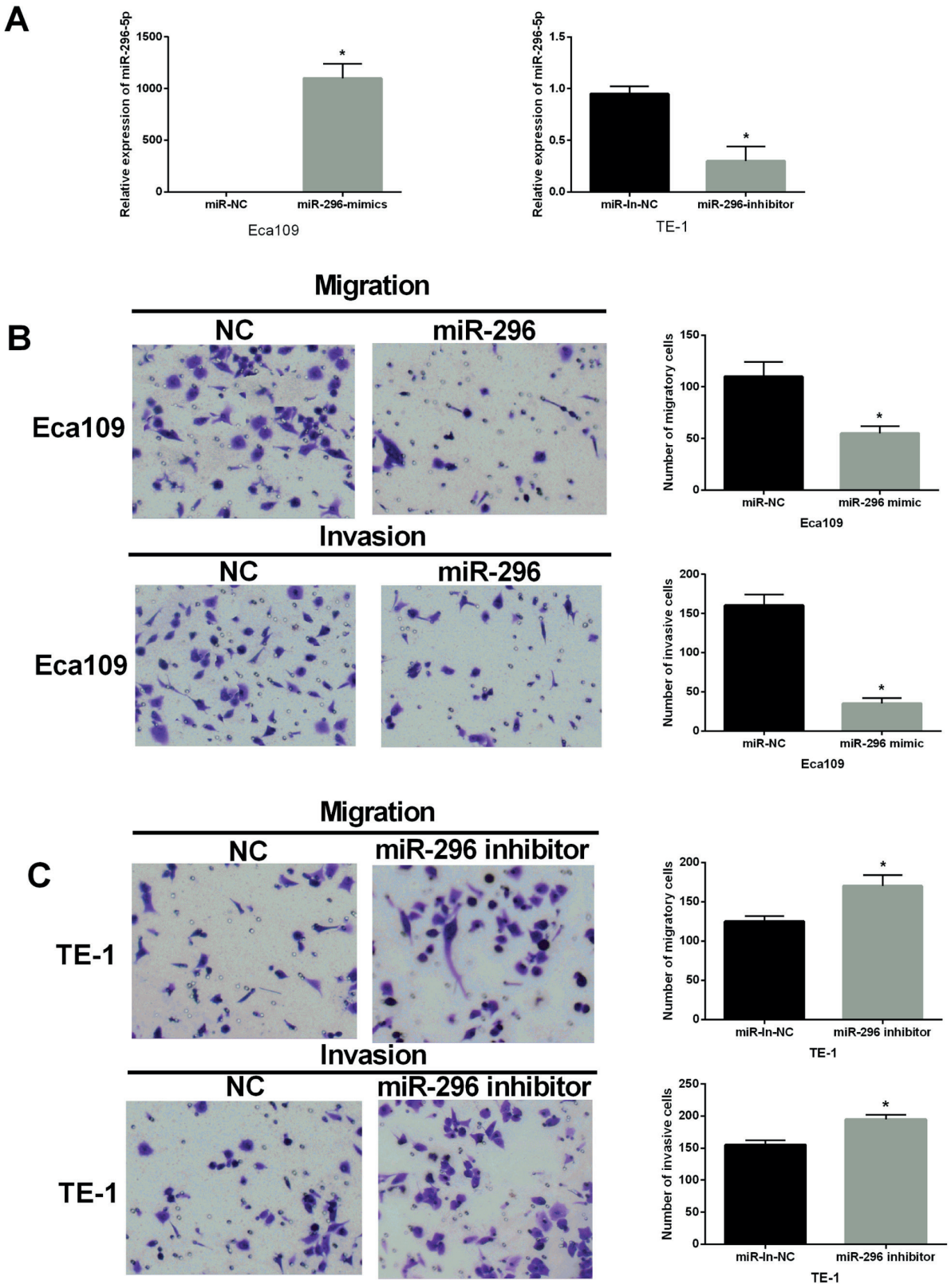

Figure 2. MiR-296-5p inhibited ESCC cell migration and invasion. ***A,*** MiR-296-5p mimic or inhibitor was transfected into Eca109 cells, and miR-296-5p expression was detected *via* qRT-PCR. ***B,*** Overexpression of miR-296-5p significantly decreased migratory and invasive abilities in Eca109 cells (magnification × 40). ***C,*** The miR-296-5p inhibitor increased migratory and invasive abilities in Eca109 cells (magnification × 40). **$p<0.01$.

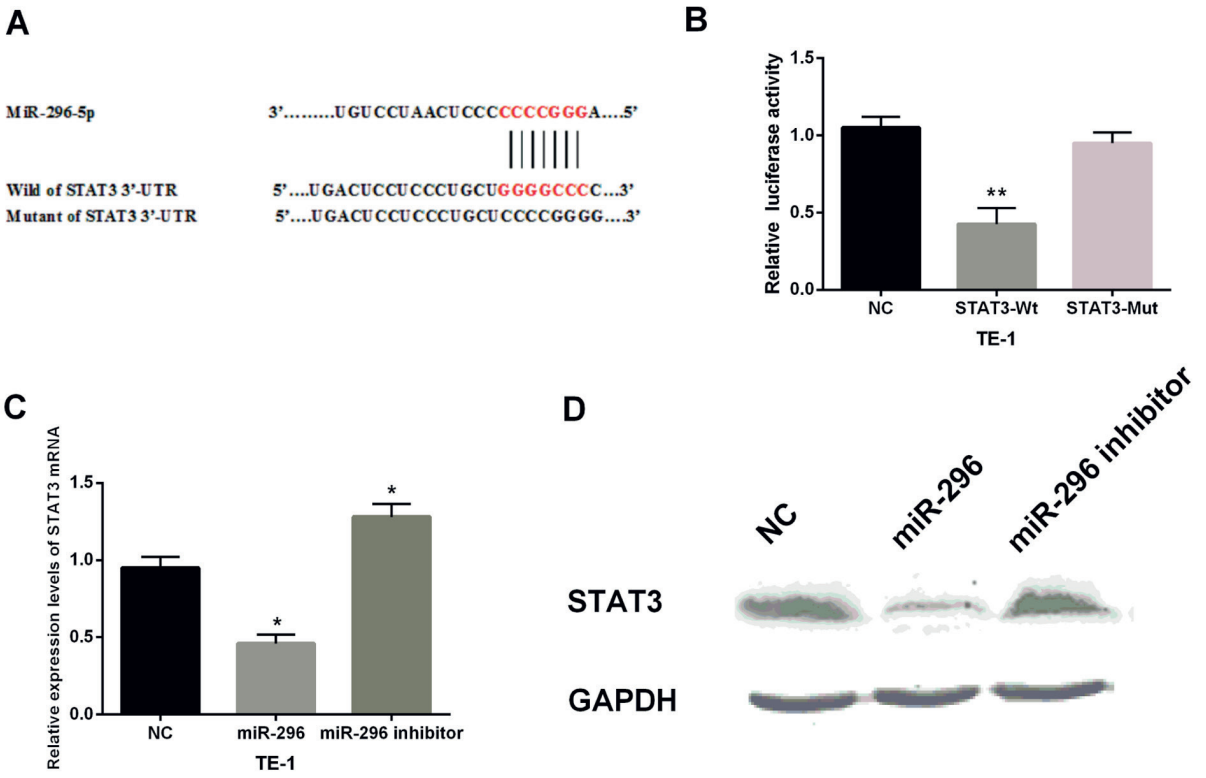

Figure 3. MiR-296-5p directly targeted STAT3 in ESCC cells. ***A,*** Binding sites between STAT3 and miR-296-5p. ***B,*** Luciferase reporter assay. ***C-D,*** The mRNA and protein expressions of STAT3 were analyzed in cells transfected with miR-296-5p mimic or inhibitor. $^{**}p<0.01$.

in ESCC by downregulating STAT3. These results may have an impact on the tumorigenesis of ESCC.

Discussion

Zhang et al[18] have demonstrated that miRNAs are involved in tumorigenesis of various cancers. Moreover, numerous miRNAs that act as tumor promoters or suppressors were found to regulate ESCC[19-24]. Particularly, miR-296 acting as a tumor suppressor was commonly identified in a variety of cancers, such as lung cancer[25], glioblastoma[26], and laryngeal carcinoma[27]. The inhibitory effect of miR-296-5p was also detected in ESCC in this work. STAT3 has been reported to function as an oncogene in several cancers[28]. Additionally, STAT3 has been found to promote cell invasion and metastasis[29]. In this work, the carcinogenesis of STAT3 was also identified in ESCC. However, no studies have reported the interaction between miR-296-5p and STAT3 in ESCC. We showed that miR-296-5p inhibited cell migration and invasion in ESCC *via* down-regulating STAT3.

Generally, dysfunction of cell proliferation, apoptosis, migration and invasion is a signal of tumorigenesis. MiRNAs mediate these cellular processes by binding to different target sites and further regulate the expression of downstream target genes[30]. Previous reports have revealed that miR-296 affected tumor formation by targeting some genes, such as S100A4 in colorectal cancer[12], CX3CR1 in lung cancer[25], and NF2 in glioblastoma[26]. In this work, miR-296-5p was confirmed to target STAT3 directly. Furthermore, we demonstrated that miR-296-5p suppressed the expression of STAT3 in ESCC.

Besides that, we also analyzed the effects of miR-296-5p and STAT3 on cell migration and invasion in ESCC. We found that the upregulation of miR-296-5p inhibited cell migration and invasion in ESCC. He et al[12] also identified the inhibitory effect of miR-296 in colorectal cancer. In addition, the silence of STAT3 was found to inhibit cell migration and invasion in ESCC in this

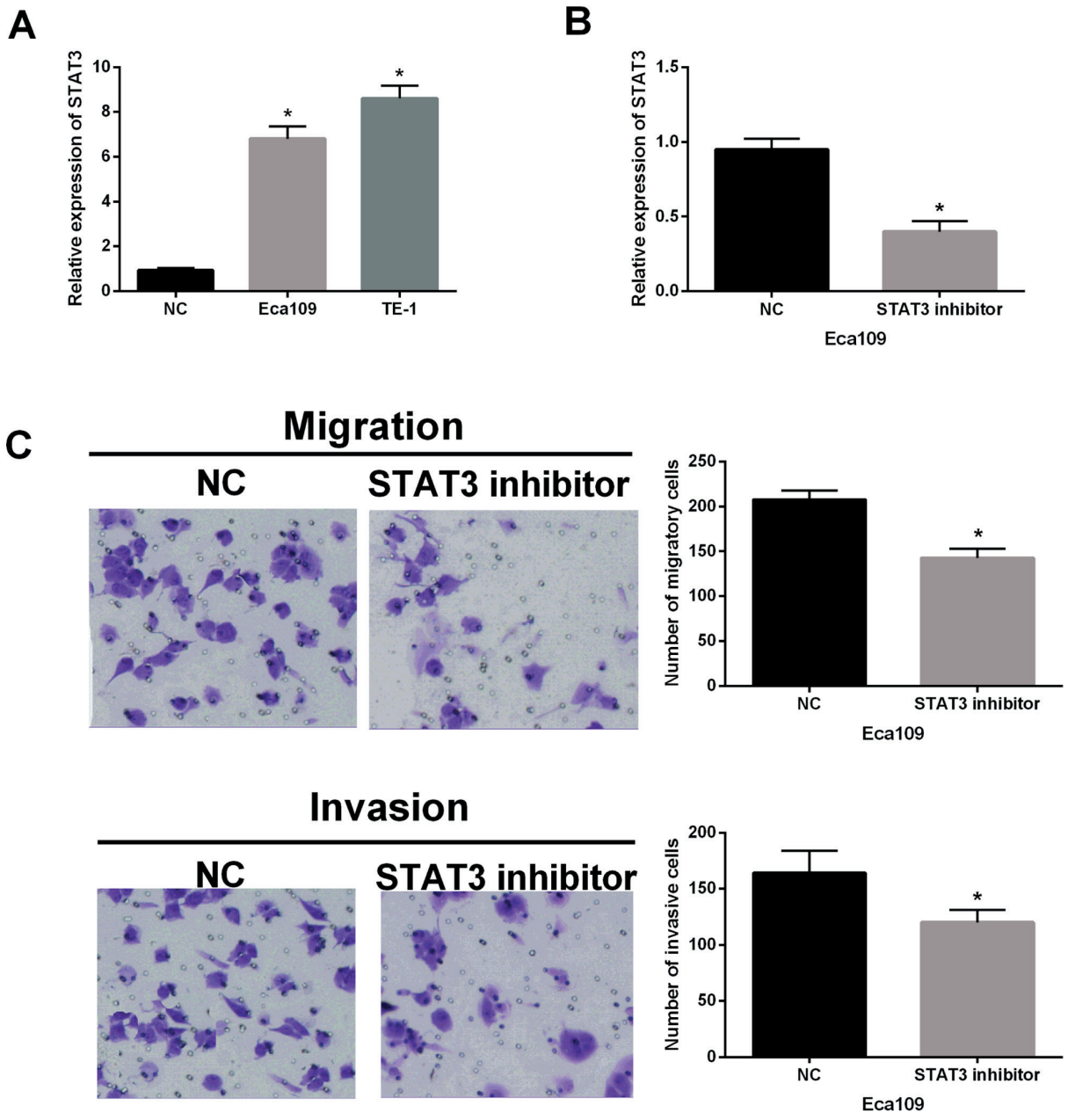

Figure 4. STAT3 promoted ESCC cell migration and invasion. ***A,*** The STAT3 expression in ESCC was detected by qRT-PCR. ***B,*** The STAT3 expression in cells containing si-STAT3 was confirmed *via* qRT-PCR. ***C,*** Transwell assays in Eca109 cells containing si-STAT3 (Magnification × 40). **$p<0.01$.

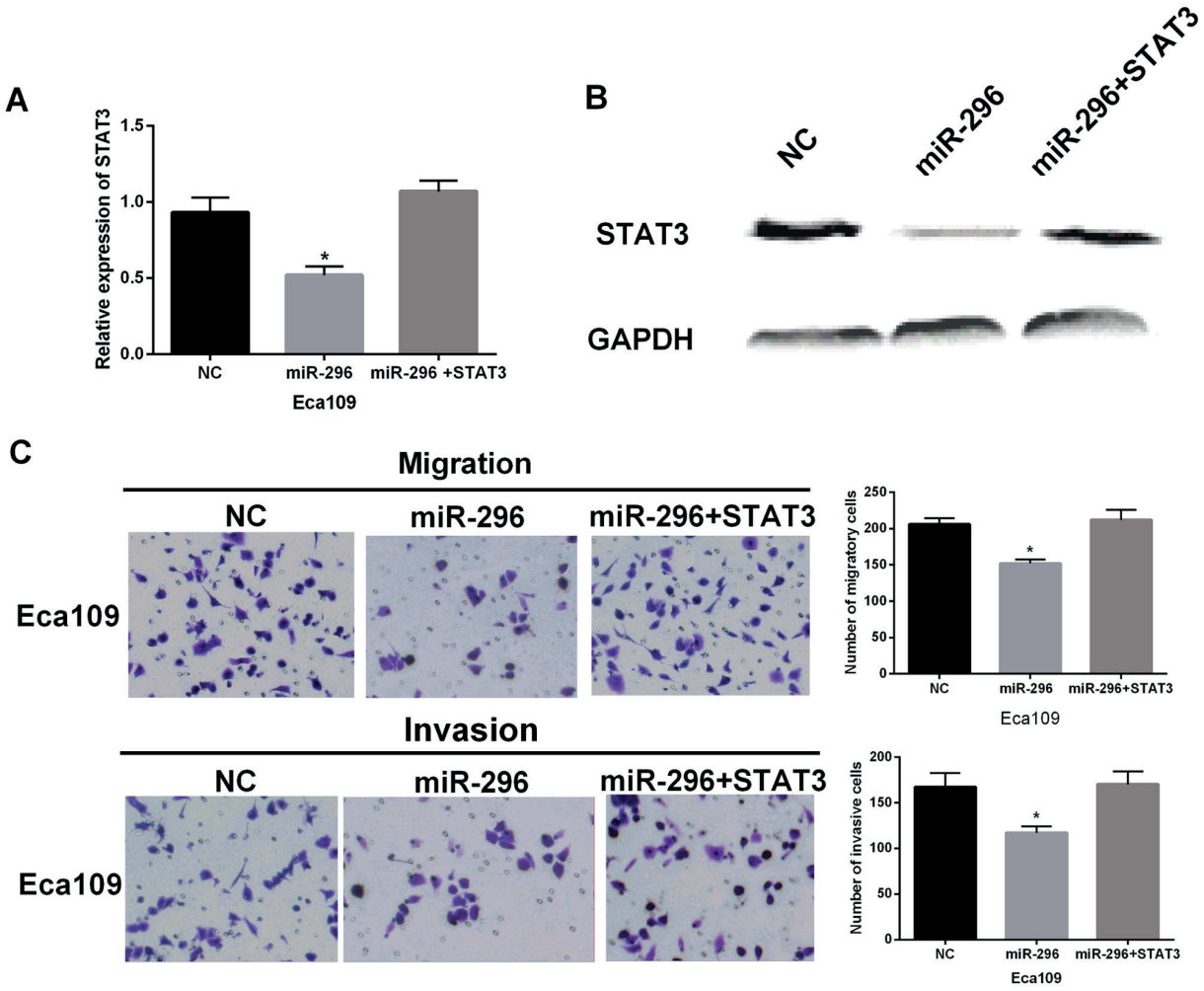

Figure 5. STAT3 mediated the inhibitory effects of miR-296-5p in ESCC cells. ***A,*** The relative mRNA expression of STAT3 was identified in Eca109 cells with miR-296 mimics and STAT3 vector. ***B,*** The protein expression in cells contained miR-296-5p mimics and STAT3 vector. ***C,*** The cell migration and invasion were detected in cells containing miR-296-5p mimics and STAT3 vector (magnification × 40). $^{**}p<0.01$.

work. Previously Cheng et al[16] also showed the same results of STAT3 in esophageal cancer cells as well, but with miR-124. The evidence revealed that miR-296-5p/STAT3 axis may play an important role in the pathogenesis of ESCC.

Conclusions

We demonstrated that miR-296-5p inhibited cell invasion and migration of ESCC by downregulating STAT3. Moreover, STAT3 was verified as a direct target gene of miR-296-5p in ESCC. The overexpression of miR-296-5p by targeting STAT3 suppressed tumorigenesis of ESCC cells.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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