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LncRNA NR2F1-AS1 promotes proliferation and metastasis of ESCC cells *via* regulating EMT

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Abstract. – OBJECTIVE: The aim of this study was to explore the expression of long non-coding ribonucleic acid (lncRNA) nuclear receptor subfamily 2 group F member 1-antisense RNA 1 (NR2F1-AS1) in esophageal squamous cell carcinoma (ESCC) tissues and cells and to investigate its effects on ESCC proliferation and metastasis.

PATIENTS AND METHODS: The expression level of NR2F1-AS1 in 51 pairs of ESCC tissues and corresponding adjacent tissues was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Meanwhile, NR2F1-AS1 expression in ESCC cells was measured *via* qRT-PCR as well. Subsequently, specific interference sequences of NR2F1-AS1 were designed, synthesized, and transiently transfected into ESCC cells. 48 h later, qRT-PCR assay was performed to detect the interference efficiency. The effects of small interfering (si)-NR2F1-AS1 on the proliferation of ESCC cells were determined through cell counting kit-8 (CCK-8) and colony formation assay. Wound healing and transwell assays were conducted to investigate the influences of si-NR2F1-AS1 on the migration and invasion of ESCC cells. Additionally, the changes in the expressions of epithelial-mesenchymal transition (EMT) molecular markers were detected by Western blotting.

RESULTS: QRT-PCR assay revealed that the expression level of NR2F1-AS1 was significantly up-regulated in 42 of 51 cases of ESCC tissues (42/51, 82.4%). Compared with esophageal mucosal epithelial HET-1A cells, NR2FA-AS1 was highly expressed in ESCC cells. CCK-8 and colony formation assay indicated that the proliferation of ESCC cells decreased remarkably after interference in NR2F1-AS1 expression. The results of wound healing and transwell assays showed that the migration and metastasis of cells were significantly lower in si-NR2F1-AS1 group than those in si-NC group. Western blotting demonstrated that the expressions of EMT molecular markers were changed after interfering with NR2F1-AS1 expression.

CONCLUSIONS: NR2F1-AS1 was highly expressed in ESCC tissues and cells. Furthermore, high expression of NR2F1-AS1 promoted the proliferation and metastasis of ESCC cells by modulating EMT.

Key Words:

Esophageal squamous cell carcinoma (ESCC), LncRNA NR2F1-AS1, Proliferation, Metastasis, Epithelial-mesenchymal transition (EMT).

Introduction

Esophageal cancer (EC) ranks sixth in terms of death and eighth in incidence among all cancers worldwide[1]. There are two major types of EC, including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA). Currently, ESCC is more prevalent in China[2]. Since there are no specific symptoms and effectively early diagnosis methods, ESCC patients have already been in the middle or advanced stage when first diagnosed. Therefore, the molecular mechanism of the malignant proliferation and metastasis of ESCC should be comprehensively elaborated to provide biological targets for its early diagnosis, treatment, and prognosis in clinical practice[3].

Long non-coding ribonucleic acids (lncRNAs), a group of RNAs with over 200 nucleotides in length, occupy at least 80% of the human genome, with no potential of protein coding[4]. LncRNAs participate in many cellular processes, such as cell proliferation, metastasis, cell cycle progression, cell growth, and apoptosis. Besides, lncRNAs serve as transcription regulators, post-transcription processing factors, chromatin remodeling factors, and splicing regulators to modulate the expressions of genes during gene modification process[5].

Abnormal expressions of lncRNAs have been detected recently in malignant tumors, including lung cancer, breast cancer, and liver cancer. *In*

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vitro and *in vivo* experiments have demonstrated that lncRNAs facilitate or inhibit the progression of tumors and play vital roles in the diagnosis and treatment of tumors[6-8]. Moreover, lncRNAs act as important regulators to accelerate ESCC proliferation and metastasis. Pan et al[9] have found that knockdown of lncRNA cancer susceptibility candidate 9 (CASC9) overtly suppresses the migration and invasion of ESCC cells. Further mechanism experiments have proved that CASC9, serving as an oncogene, recruits EZH2 to negatively modulate the expression of PDCD4 and change the level of H3K27me3 in the promoter region. Lin et al[10] have discovered that lncRNA homeobox A transcript at the distal tip (HOTTIP) is significantly up-regulated in ESCC tissues and cells. Further *in vitro* experiments have confirmed that HOTTIP is able to regulate the expression of HOXA13 by adsorbing miR-30b, thus facilitating ESCC metastasis. However, there are no reports on the expression and biological function of lncRNA nuclear receptor subfamily 2 group F member 1-antisense RNA 1 (NR2F1-AS1) in ESCC tissues and cells. In this study, we verified, for the first time, that NR2F1-AS1 was highly expressed in ESCC tissues and cells, and promoted the proliferation and metastasis of ESCC *in vitro*.

Patients and Methods

Tissue Specimens

A total of 51 patients undergoing radical surgery for EC in the Esophageal Department of The First Hospital of Jilin University from January 2015 to December 2017 were enrolled as research subjects. Collected surgical specimens were definitely diagnosed as ESCC by the Pathology Department of the hospital. No radiotherapy, chemotherapy or targeted therapy was performed for any patient before surgery. All surgically collected specimens were immediately stored in liquid nitrogen at –180°C within 0.5 h after resection. This investigation was approved by the Medical Ethics Committee of the hospital. Informed consent was obtained from patients before the study.

Cell Culture

Human ESCC cell lines (ECA109, TE-1, ECA7906, KYSE-30, KYSE-70) and human esophageal mucosal epithelial cell line (HET-1A) were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, passaged and digested with 0.25% trypsin in a 5% CO_2 incubator at 37°C.

Cell Transfection

ESCC cells in the logarithmic growth phase were taken, digested, and seeded into 6-well plates at a concentration of 5×10^5 cells/well. Then, the cells were cultured in a 37°C incubator overnight. Next, small interfering (si)-NR2F1-AS1 was transiently transfected into ESCC cells according to the instructions of Lip2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 6 h, the medium was replaced. Interference sequences were as follows: Si-NR2F1-AS1 #1: F 5'-GCACAGAGAUAAUGGCAAAUU-3', R 5'-UUUGCCAUUAUCUCUGUGCU U-3'. si-NR2F1-AS1 #2: F 5-GAAGAAAGAUCCAGAGGAAUU-3', R 5'-GAAGA AAGAUCCAGAGGAAUU-3', si-NR2F1-AS1 #3:F 5'-GAAGAAAGAUCCAGAGGAAUU-3', R 5'-GAAGAAAGAUCCAGAGGA AUU-3'

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA in tissues and cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNAs were reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a RT kit (Toyobo, Osaka, Japan). Real-time fluorescent qPCR kit (Toyobo, Osaka, Japan) was used for PCR in accordance with the instructions. Primer sequences used in this study were as follows: lncRNA NR2F1-AS1: F 5'-AGGCGTTGTGCGTAGAGGA-3', R 5'-GGATTTTTAGCAAGGCGAGCG-3'. GAPDH: F 5'-GCACCGTCAAGGCTGAGAAC-3', R 5'-GCCTTCTCCATGGTG GTGA A-3'.

Cell Counting Kit-8 (CCK-8) Assay

One day before the assay, ESCC cells in the logarithmic growth phase were taken from experimental group and control group, respectively. ESCC cells were inoculated into 96-well plates at a density of 3×10^3 cells/well. After culture in a 37°C incubator for 0, 24, 48, 72, and 96 h,

respectively, 10 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, followed by incubation at 37°C for 2 h in the dark. Optical density (OD) at 450 nm was detected by a microplate reader. This assay was repeated for three times.

Clone Formation Assay

ESCC cells in experimental group and control group were first trypsinized and re-suspended in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% FBS. Then, the cells were inoculated into 6-well plates, with 800 cells per well. Next, the cells were cultured in a 5% CO_2 incubator at 37°C for 14 d. After washing with phosphate-buffered saline (PBS), formed colonies were fixed with methanol for 30 min and stained with 1% (1 mg/mL) crystal violet for 20 min. With >50 cells counted as one clone, the number of formed clones was counted.

Would Healing Assay

Before the assay, a marker pen was utilized to draw horizontal lines evenly and straightly cross the perforated core on the back of 6-well plates. ESCC cells in logarithmic growth phase were collected, grouped, and transfected as described above. After 24 h of transfection, scratches were made again using a micropipette tip perpendicular to pre-drawn horizontal lines. The cells were then carefully rinsed with PBS for 3 times to remove the falling cells. Next, 3 fields of view were randomly selected for each sample to record location and take photos. 6-well plates were put in a 5% CO_2 incubator at 37°C and saturated humidity for 48 h of culture, followed by photographing under a microscope.

Transwell Assay

The surface of the bottom membrane of an upper transwell chamber was coated with 50 mg/L Matrigel diluted at 1:50 before cell invasion assay, followed by standing at 37°C for 30 min. Next, the cells were cultured in serum-free medium for 12-24 h, followed by the preparation of cell suspension, digestion with trypsin, and centrifugation. After discarding the medium, the cells were washed once or twice with PBS and re-suspended in serum-free medium containing bovine serum albumin (BSA). Cell density was adjusted to 1×10^5/100 cells/μL. 100 μL of cell suspension was added to the upper transwell chamber. Meanwhile, RPMI-1640 medium or DMEM containing 20% FBS was added to the lower chamber. The transwell chamber was placed in a 5% CO_2 incubator for incubation at 37°C for 12 h. Next, Matrigel and cells in the upper chamber were wiped off with cotton swabs. Invasive cells were stained with 1% crystal violet for 20 min. For migration assay, except for the coating of the basement membrane with Matrigel, the remaining steps were the same as those in invasion assay.

Western Blotting Analysis

Total proteins in tissues and cells were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Protein concentration and purity were detected using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Subsequently, proteins samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) through semi-dry transfer method. After blocking with 5% skimmed milk powder at room temperature for 2 h, the membranes were incubated with primary antibody (diluted at a certain ratio) at 4°C overnight. On the next day, the membranes were washed with Tris Buffered Saline and Tween-20 (TBST) for 3 times, and incubated with corresponding secondary antibody at room temperature for 1 h. Immunoreactive bands were finally exposed by the enhanced chemiluminescence (ECL) method using a gel imaging system.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Each assay was repeated for 3 times. Measurement data were expressed as ($\bar{x} \pm s$). Differences between two groups were analyzed by using the Student's *t*-test. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ or $p<0.01$ was considered statistically significant.

Results

Up-regulated Expression of NR2F1-AS1 in ESCC

QRT-PCR assay was first used to detect the expression level of NR2F1-AS1 in 51 cases of ESCC tissues. The results uncovered that NR2F1-AS1

expression increased significantly in 42 cases of ESCC tissues when compared with adjacent tissues (Figure 1A). Subsequently, qRT-PCR assay was used to measure the expression level of NR2F1-AS1 in ESCC cells. The results showed that NR2F1-AS1 expression level was significantly up-regulated (Figure 1B). To investigate the biological function of NR2F1-AS1 in ESCC cells, two ECSS cell lines with the highest NR2F1-AS1 expression were selected for subsequent experiments. NR2F1-AS1 specific interference sequences were designed and transiently transfected into ESCC cells. After 48 h, qRT-PCR assay was conducted to determine the interference efficiency (Figure 1C and 1D).

Si-NR2F1-AS1 Inhibited Proliferation of ESCC Cells

ESCC cells were transiently transfected with si-NR2F1-AS1 and si-NC. CCK-8 assay was performed to detect the effect of si-NR2F1-AS1 on the proliferation of ESCC cells. The results revealed that interference in NR2F1-AS1 expression significantly repressed the proliferation of ESCC cells (Figure 2A and 2B). Next, the effect of si-NR2F1-AS1 on the proliferation ESCC cells was investigated *via* colony formation assay as well. Similarly, the results were consistent with CCK-8 assay (Figure 2C and 2D).

NR2F1-AS1 Promoted Migration of ESCC cells by Regulating EMT

To explore the effect of NR2F1-AS1 on the metastasis of ESCC cells, si-NR2F1-AS1 and si-NC were first transiently transfected into ESCC cells. Wound healing assay showed that interference in the expression of NR2F1-AS1 significantly repressed the migration of ESCC cells (Figure 3A and 3B). Transwell assay manifested that si-NR2F1-AS1 group exhibited remarkably reduced migration and invasion of cells in comparison with si-NC group (Figure 3C and 3D). Thereafter, the

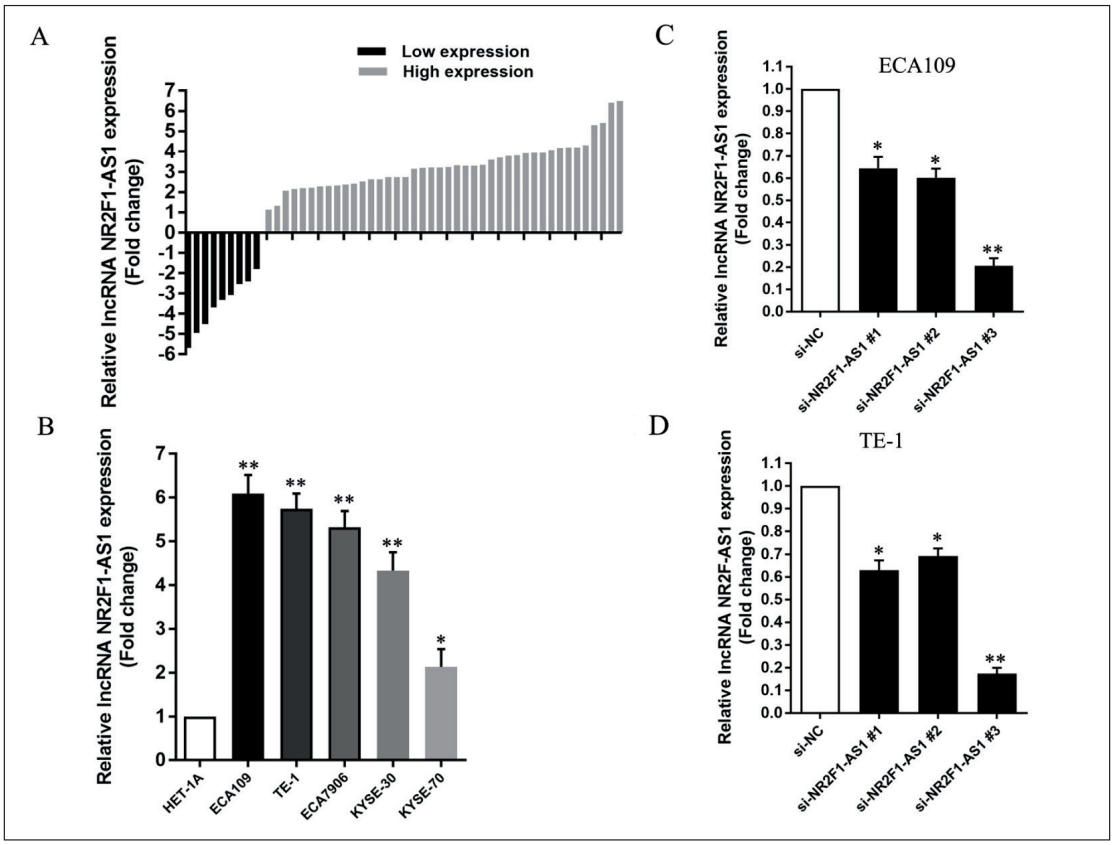

Figure 1. NR2F1-AS1 expression is up-regulated in ESCC. **A,** NR2F1-AS1 expression level in 51 pairs of ESCC tissues and corresponding adjacent tissues detected by qRT-PCR assay. The expression level of NR2F1-AS1 was significantly elevated in 42 ESCC cases. **B,** Expression level of NR2F1-AS1 in ESCC cells was measured through qRT-PCR assay. **C,** and **D,** Interference efficiency determined *via* qRT-PCR assay 48 h later after transient transfection of si-NR2F1-AS1 and si-NC in ESCC cells.

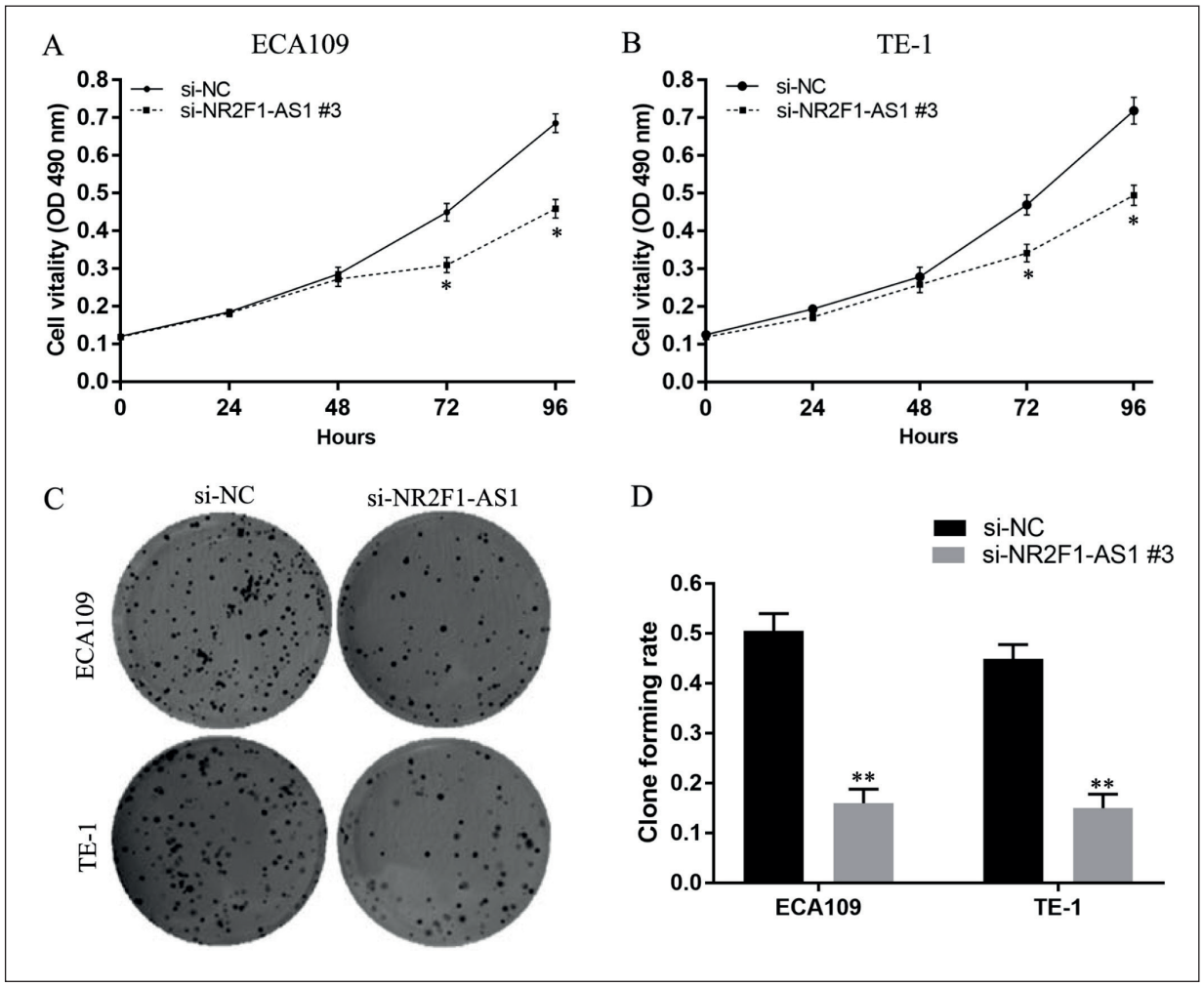

Figure 2. Si-NR2F1-AS1 inhibits proliferation of ESCC cells. **A,** and **B,** Effect of si-NR2F1-AS1 on ESCC cell proliferation detected through CCK-8 assay. **C,** and **D,** Changes in the proliferation of ESCC cells determined *via* colony formation assay after transfection of si-NR2F1-AS1 and si-NC (magnification 40×).

possible mechanism of NR2F1-AS1 in affecting the migration and invasion of ESCC cells was explored. Western blot analysis indicated that the protein expressions of E-cadherin, N-cadherin, and Vimentin, molecular markers of the EMT pathway, were significantly altered after interfering in NR2F1-AS1 expression (Figure 3E and 3F).

Discussion

As one of the most common malignancies of the digestive system, EC has shown a markedly upward incidence rate in China in recent years. Currently, it has become the third most malignant tumor, only second to lung cancer and gastric cancer[11]. Surgery, chemotherapy, radiotherapy, biologically targeted therapy, and other treatment means have greatly improved the survival rate of EC patients. However, local recurrence, and distant metastasis remain the leading causes of tumor-related deaths[12].

As RNAs with a length of >200 nt and no protein coding ability, lncRNA are widely distributed in mammals[13]. They can mainly repress the expressions of target genes by binding to chromatin silencing complex or histone silencing complex to concentrate chromatin. Meanwhile, lncRNAs serve as "ceRNAs" to regulate post-transcriptional expressions of genes, and modulate the activity of tumor suppressor and mRNA processing and translation[14].

Huang et al[15] first revealed that NR2F1-AS1 was highly expressed in hepatocellular carcino-

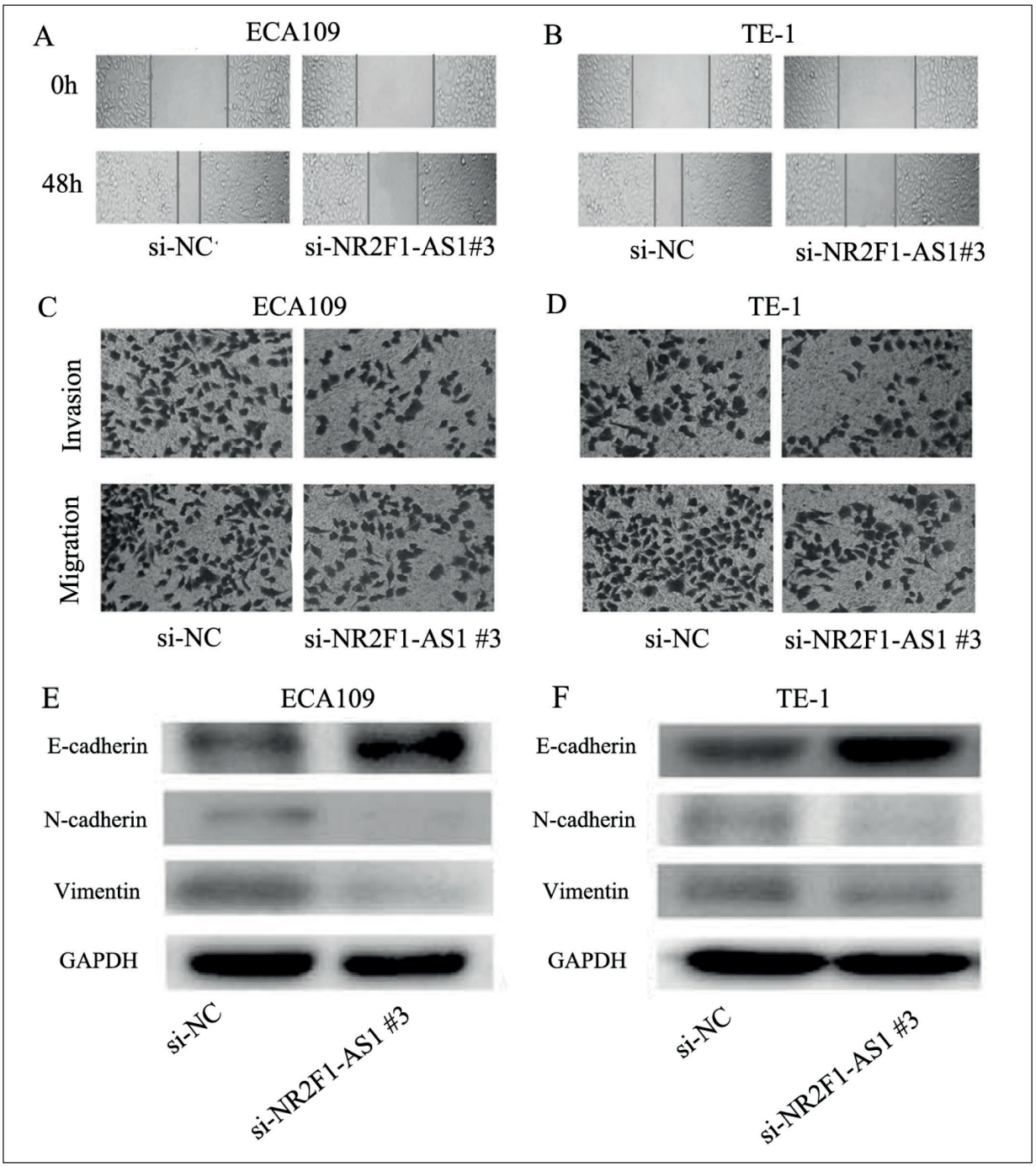

Figure 3. NR2F1-AS1 promotes metastasis of ESCC cells by modulating EMT. **A,** and **B,** Migration of ESCC cells detected *via* wound healing assay after knocking down the expression of NR2F1-AS1. **C,** and **D,** Effects of interference in NR2F1-AS1 on migration and invasion of ESCC cells investigated through transwell assay (magnification 40×). **E,** and **F,** Changes in the protein expressions of EMT molecular markers in ESCC cells in experimental and control groups detected through Western blotting analysis after transfection of si-NR2F1-AS1 and si-NC.

ma. It can also adsorb miR-363 to regulate the expression of ABCC1, thus promoting the resistance of hepatocellular carcinoma to oxaliplatin. In thyroid cancer, NR2F1-AS1 regulates the expression of CCND1 after transcription, thereby promoting the proliferation and metastasis of thyroid cancer cells[16]. In this study, we discovered for the first time that NR2F1-AS1 expression was

significantly up-regulated in ESCC cells. High expression of NR2F1-AS1 significantly facilitated the proliferation and metastasis of ESCC cells.

EMT refers to the transition of cell morphology to mesenchymal cells due to decreased endothelial adhesion factors (E-cadherin) of endothelial-derived tumor cells and increased proteins contracting the cytoskeleton (Vimentin). This process detaches epithelial cells from the basement membrane, thereby preparing for the migration of tumor cells[17,18]. It has been reported that lncRNAs can be involved in EMT as crucial regulatory factors. Huang et al[19] have pointed out that homeobox transcript antisense intergenic RNA (HOTAIR) is highly expressed in colorectal cancer. High expression of HOTAIR indicates poor prognosis of patients. *In vitro* experiments have also verified that silencing HOTAIR expression leads to increased expression of E-cadherin and decreased expression of Vimentin. Meanwhile, HOTAIR down-regulation enhanced characteristics of epithelial cells and notably reduced movement, invasion, and metastasis of colorectal cancer cells. Furthermore, highly expressed LOC440040 triggers PI3K/Akt/mTOR signal transduction pathway and EMT, accelerating the growth, invasion, and metastasis of prostate cancer cells[20].

Conclusions

We showed that NR2F1-AS1 promotes the invasion and migration of ESCC cells by regulating EMT.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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