The LncRNA AL161431.1 targets miR-1252-5p and facilitates cellular proliferation and migration *via* MAPK signaling in endometrial carcinoma

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Abstract. – **OBJECTIVE:** The aim of this study was to determine the expression profile and the underlying mechanism of the long intergenic non-protein coding RNA AL161431.1 in EC (endometrial carcinoma).

MATERIALS AND METHODS: In this study, the expression data for the lncRNA AL161431.1 in EC was downloaded from The Cancer Genome Atlas (TCGA) database and used to examine its expression profile. quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis were used to detect gene and protein expression, respectively. A subcellular fractionation assay was used to determine the location of AL161431.1. Cell Counting Kit-8 (CCK-8) and colony formation assays were used to evaluate cellular proliferation. Cell migration and wound healing assays were used to detect the effects on cell migration. RNA pull-down and Luciferase reporter assays were used to confirm the interaction between AL161431.1 and miR-1252-5p.

RESULTS: High expression levels of AL161431.1 were observed in EC patients, tissues, and cells. Loss-of-function experiments validated the carcinogenic role of AL161431.1. Based on the determined cytoplasmic location of AL161431.1, we investigated the ceRNA network and its relation to AL161431.1, miR-1252-5p, and MAPK (mitogen-activated protein kinase) signaling in EC. The molecular mechanism of the interaction between AL161431.1 and miR-1252-5p, and its effects on the MAPK signaling pathway was validated using rescue experiments in Ishikawa cells.

CONCLUSIONS: Our novel results indicate that AL161431.1 targets and binds to miR-1252-5p, resulting in the de-repression of MAPK signaling in EC cells. This highlights the potential for AL161431.1 to be targeted as a potent therapeutic strategy in the treatment of EC.

Key Words:
 Endometrial carcinoma (EC), LncRNAs, AL161431.1, MiR-1252-5p, MAPK signaling.

Introduction

Endometrial carcinoma (EC) is one of the most frequent carcinomas among women and the second reason for gynaecological cancer-related mortality[1-3]. Patients with early-stage EC have a higher survival rate than those with advanced-stage EC[4]. Despite the increasing incidence of EC, the comprehension of the molecular mechanism underlying EC progression remains poorly understood[5]. Hence, it is imperative to investigate the mechanism and identify potential molecular targets for EC intervention.

Long non-coding RNAs (lncRNAs) are a large class of transcribed RNA molecules greater than 200 nucleotides (nt) in length that are not translated into proteins[6,7]. LncRNAs can modulate gene expression to affect corresponding biological processes in human tumors *via* various mechanisms, which include acting as a sponge for miRNAs so as to regulate the expression of the downstream genes[8,9]. Importantly, the lncRNA HCP5 promotes the progression of triple negative breast cancer by acting as a competing endogenous RNA (ceRNA) in order to regulate BIRC3 expression by acting as a molecular sponge for miR-219a-5p[10]; the lncRNA NORAD facilitates the occurrence and development of non-small cell lung cancer by adsorbing miR-656-3p[11]; the lncRNA DLX6-AS1 accelerates the proliferation, invasion, and migration of non-small cell lung cancer through its action upon the miR-27b-3p/GSPT1 pathway[12]; the level of the lncRNA NEF is low in osteosarcoma and represses cancer cell migration and invasion by downregulating miRNA-21[13]. LncRNA H19/miR-612/HOXA10 axis[14] or lncRNA SNHG8/miR-152/c-MET axis[15] was reported to participate in the development of endometrial

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carcinoma. The above-mentioned articles highlight the importance of lncRNAs in various cancers and emphasize the need to investigate the role of lncRNAs in EC. In this research, the lncRNA AL161431.1 was selected for further investigation. According to The Cancer Genome Atlas (TCGA) database, AL161431.1 is found to be at increased levels in UCEC (uterine corpus endometrial carcinoma) tissues in comparison to normal tissues. AL161431.1 has not been previously implicated in oncogenesis and the specified role of AL161431.1 in EC is unclear. The aim of this study is to evaluate the expression and investigate the regulatory mechanism of AL161431.1 in EC. First, the expression of AL161431.1 in EC patients in the TCGA database was observed to be higher in tumor tissues in comparison to adjacent normal tissue and cell lines. Loss-of-function experiments revealed a carcinogenic role for AL161431.1 in the proliferation and migration abilities of EC cells. Based on the location of AL161431.1 in the cytoplasm, we evaluated the effects of the ceRNA network, including AL161431.1, miR-1252-5p, and MAPK (mitogen-activated protein kinase) signals. The identified mechanism was then validated using rescue assays in Ishikawa cells.

Materials and Methods

Cell Culture

Human endometrial carcinoma cell lines, including HEC1-B, KLE, HEC1-A, and Ishikawa, along with the EMC cell line, which was used as a normal control, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; PAN-Biotech, Adenbach, Bagolia, Germany) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) were utilized for cell culture at 37° C in 5% CO_2.

Cell Transfection

Ishikawa or HEC1-A cells were transiently transfected with two specific shRNAs against AL161431.1 (shAL161431.1#1 and shAL161431.1#2) or a non-specific control (NC). MiR-1252-5p mimics, NC mimics, miR-1252-5p inhibitor, and NC inhibitor were synthesized by RiboBio (Guangzhou, Guangdong, Chi-

na). The pcDNA3.1 vector targeting MAP2K1, MAPK1, and an empty vector were constructed by Genechem (Shanghai, China). Cells were collected 48 h post transfection. Lipofectamine 2000 (Invitrogen, CA, USA) was used for each transfection.

RNA Extraction and Quantitative Analysis

TRIzol reagent (Invitrogen, CA, USA) was used in the extraction of RNA. Total RNA was reverse transcribed into complementary DNA (cDNA) *via* cDNA Reverse Transcription Kit (Invitrogen, CA, USA). quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was then carried out using the SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). All the primers used in the present study are listed in the **Supplementary Table I**. The following program was used for the qRT-PCR: 10 s at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Ct values were used to calculate the expression of RNA levels. Target gene expression ($2^{-\Delta\Delta Ct}$) was normalized and GAPDH or U1 was used as the endogenous control. The $2^{-\Delta\Delta CT}$ method was used for transcript quantification.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was utilized to test cellular proliferation. Transfected HEC1-A or Ishikawa cells were placed in 96-well plates with culture medium. The reaction mixture (10 μL) from the CCK-8 kit was then added. Next, cellular proliferation was examined by measuring the absorbance at a wavelength of 450 nm.

Colony Formation Assay

Transfected HEC1-A or Ishikawa cells were added into 6-well cell culture plates and propagated for approximately 2 weeks for their use in the colony formation assays. The cells were subsequently stained by crystal violet (Sangon Biotechnology, Shanghai, China). After colonies formed, they were counted manually.

Cell Migration Assay

Briefly, Ishikawa or HEC1-A cells (1×10^4 cells per well) were harvested after transfection and then, added to the upper chamber along with culture medium. Meanwhile, DMEM with 10% FBS (Thermo Fisher Scientific, Waltham, MA,

USA) was added to the lower compartment. Cotton swabs were used to remove cells on the upper surface of the transwell chambers. Next, the migrated cells were fixed, stained, and then, imaged using a photomicroscope (×10 fields per chamber; Olympus, Tokyo, Japan). Five random fields were selected and averaged.

Wound Healing Assay

Transfected HEC1-A or Ishikawa cells were added into 6-well plates. Artificial wounds for a live cell analysis were made on the cell monolayer using culture-inserts. Migratory cells, in addition to wound healing, were monitored at 0 and 24 h. Three artificial wounds were immediately photographed for each group at the indicated time points following the wound formation. Cell migration was assessed by measuring the differences in the wound areas.

Subcellular Fractionation Assay

RNA was isolated from the nuclear or cytoplasmic fraction using the Nuclear/Cytosol Fractionation Kit (Biovision, San Francisco Bay, CA, USA) and was evaluated with qRT-PCR analysis. U6 or GAPDH acted as the identifiers for the nuclear or cytoplasmic fractions, respectively. Nuclear and Cytoplasmic Extraction Reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Luciferase Reporter Assay

AL161431.1-WT and its respective mutant (AL161431.1-Mut) were synthesized by GenePharma (Shanghai, China). They were both co-transfected into Ishikawa or HEC1-A cells with miR-1252-5p mimics or NC mimics. A Dual-Luciferase Report Assay System (Promega, Madison, WI, USA) was adopted to monitor and calculate the Luciferase activity 48 h post transfection.

RNA Pull-Down Assay

Biotin-labeled miRNAs (Bio-miR-1252-5p-WT and Bio-miR-1252-5p-WUT) and their NC (Bio-NC) were transcribed with the Biotin RNA Labeling Mix (Roche, Mannheim, Germany) and T7 RNA polymerase (Roche, Mannheim, Germany). They were subsequently treated with RNase-free DNase I (Roche, Mannheim, Germany), and then, purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The specific procedures were carried out according to the manufacturers' specifications.

Western Blot Assay

Transfected HEC1-A or Ishikawa cells were trypsinized and lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA). After being centrifuged for 30 min, the protein concentrations were determined using a Pierce Bicinchoninic acid (BCA) Protein Detection Kit (Bio-Rad Laboratories, Hercules, CA, USA). The lysates were then transferred into polyvinylidene difluoride membranes (PVDF; Bio-Rad Laboratories) and treated with 5% skim milk. After incubation with the following primary antibodies, anti-AKT2 (1/1000, ab175354, Abcam, Cambridge, USA) or anti-AKT3 (1/1000, ab152157, Abcam, Cambridge, USA) or anti-MAP2K1 (MEK1) (1/1000, ab96379, Abcam, Cambridge, USA) or anti-MAPK1 (ERK1) (1/1000, ab32537, Abcam, Cambridge, USA) or anti-GAPDH (1/10000, ab8245, Abcam, Cambridge, USA), the membranes were washed before being incubated with a secondary antibody. The blots were visualized *via* enhanced chemiluminescence (ECL; Amersham Pharmacia Biotechnology, Buckinghamshire, UK). GAPDH was used as an internal control.

Statistical Analyses

All experiments were repeated a minimum of three times. Data were presented as the mean ± SD. The Student's *t*-test was utilized to identify independent groups or ANOVA with a Tukey's post-hoc test, which identified more groups using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). A p value < 0.05 was set as the threshold of statistical significance.

Results

Silencing of AL161431.1 Decreased Cell Proliferation and Migration

AL161431.1 is a lncRNA presumed to be overexpressed in uterine corpus endometrial carcinoma (UCEC) patients and tissues due to its high level of expression, as noted in the TCGA dataset (Figure 1A-C). The mRNA expression of AL161431.1 in endometrial carcinoma (EC) cell lines showed that AL161431.1 was highly expressed (Figure 1D). Two different shRNAs targeting AL161431.1 (shAL161431.1#1 and shAL161431.1#2) were used to effectively silence AL161431.1 (Figure 1E). In CCK-8 and EdU assays, the proliferative ability was sup-
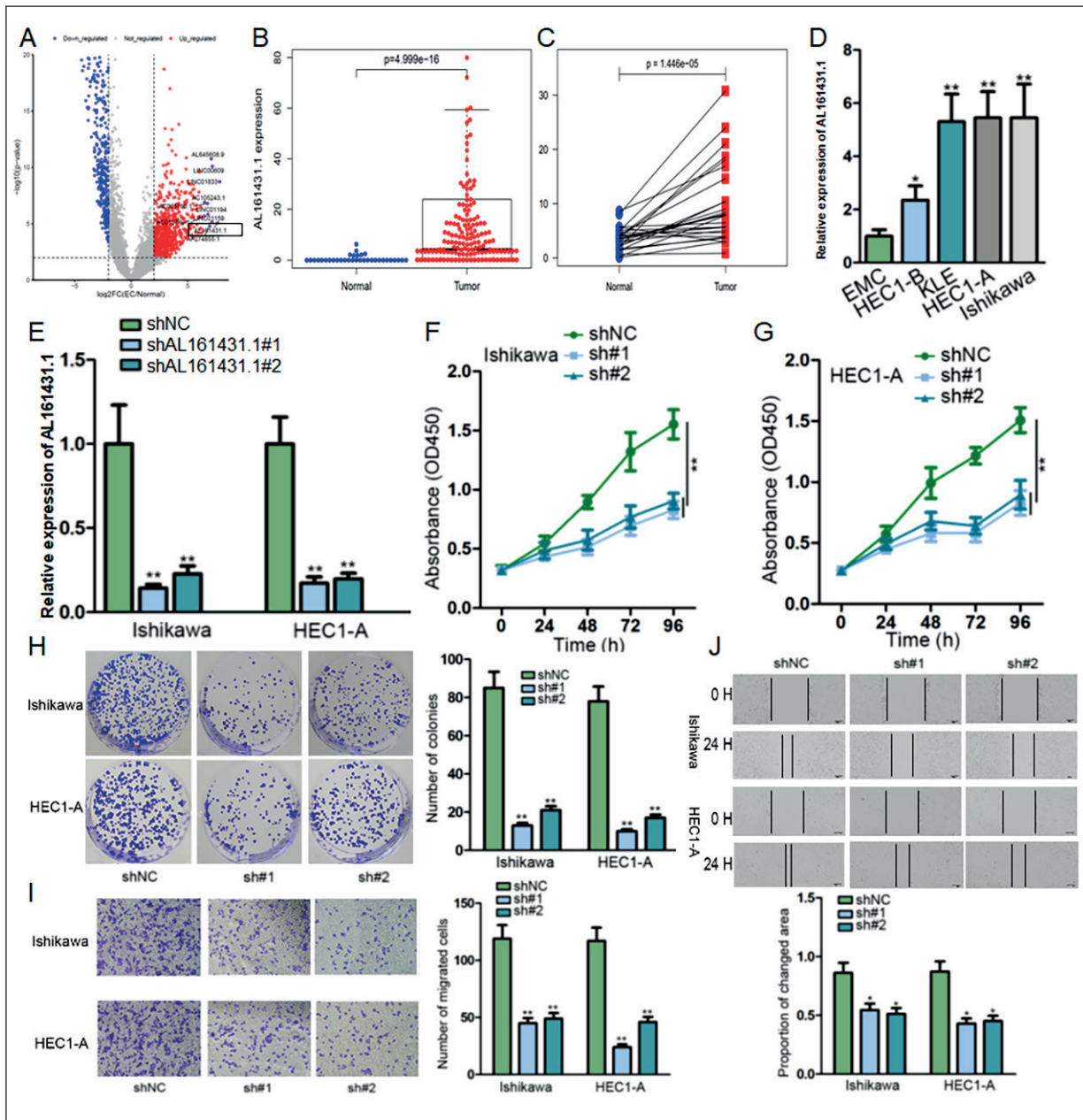
Figure 1. Knockdown of AL161431.1 repressed EC cell proliferation and migration. **A,** Volcano plot of the TCGA_lncRNA profile: the lncRNA AL161431.1 was marked with a box. **B,** The expression pattern of AL161431.1 in UCEC patients and normal patients from the TCGA dataset. **C,** The expression pattern of AL161431.1 in matched EC and NON-EC tissues from the TCGA dataset. **D**, AL161431.1 expression in EC cells (HEC1-B, KLE, HEC1-A, Ishikawa) and normal EMC cells was evaluated by qRT-PCR. **E,** AL161431.1 expression was silenced by shRNAs in Ishikawa and HEC1-A cells. **F-H,** The biological effect of AL161431.1 knockdown on EC proliferation was examined by CCK-8 and colony formation assays. **I-J,** Transwell migration assays and wound-healing assays were conducted in order to evaluate EC cell migration in response to AL161431.1 depletion. Each experiment was carried out at least 3 times. *$p < 0.05$, **$p < 0.01$. 10× magnifications (**H** and **I**). Scale bars = 100 μm (J).

pressed, due to the limited cellular viability and the formation of small colonies caused by the downstream effects of transfecting sh#1 and 2 (Figure 1F-H). Transwell and wound healing experiments revealed that the migra-

tory capacity was impaired after AL161431.1 had been silenced (Figure 1I-J). These findings demonstrated that silencing AL161431.1 decreased the cellular proliferation and migration of EC cells.

AL161431.1 is Located in the Cytoplasm and Interacts with MiR-1252-5p

To investigate the mechanism by which AL161431.1 exerts its function in EC, first we determined its cellular location. A subcellular fractionation assay showed that AL161431.1 is predominantly located in the cytoplasm (Figure 2A). Based on this finding, we considered that AL161431.1 may play a role in ceRNA crosstalk, a famous mechanism comprised of lncRNAs/circRNAs, miRNAs, and mRNAs[16,17]. The DIANA and StarBase websites were used to identify three possible miRNA targets of AL161431.1: miR-1252-5p, miR-3118, and miR-134-5p (Figure 2B). The expression levels of miR-1252-5p, miR-3118, and miR-134-5p were measured by qRT-PCR. These results indicated that miR-1252-5p appeared to be downregulated in EC cells (Figure 2C). MiR-3118 expression was shown to be relatively high in EC cells, while miR-134-5p expression was similar in both tumor cells and normal cells (data not shown). In order to validate the interaction between AL161431.1 and miR-1252-5p, we performed Luciferase reporter and RNA pull-down experiments. The binding sites for AL161431.1 and miR-1252-5p, and the mutant binding sites, are shown in Figure 2D. MiR-1252-5p was increased after the transfection of miR-1252-5p mimics (Figure 2E). The Luciferase activity of AL161431.1-WT was impeded by miR-1252-5p mimics, whereas the Luciferase activity of AL161431.1-Mut was unaffected (Figure 2F).
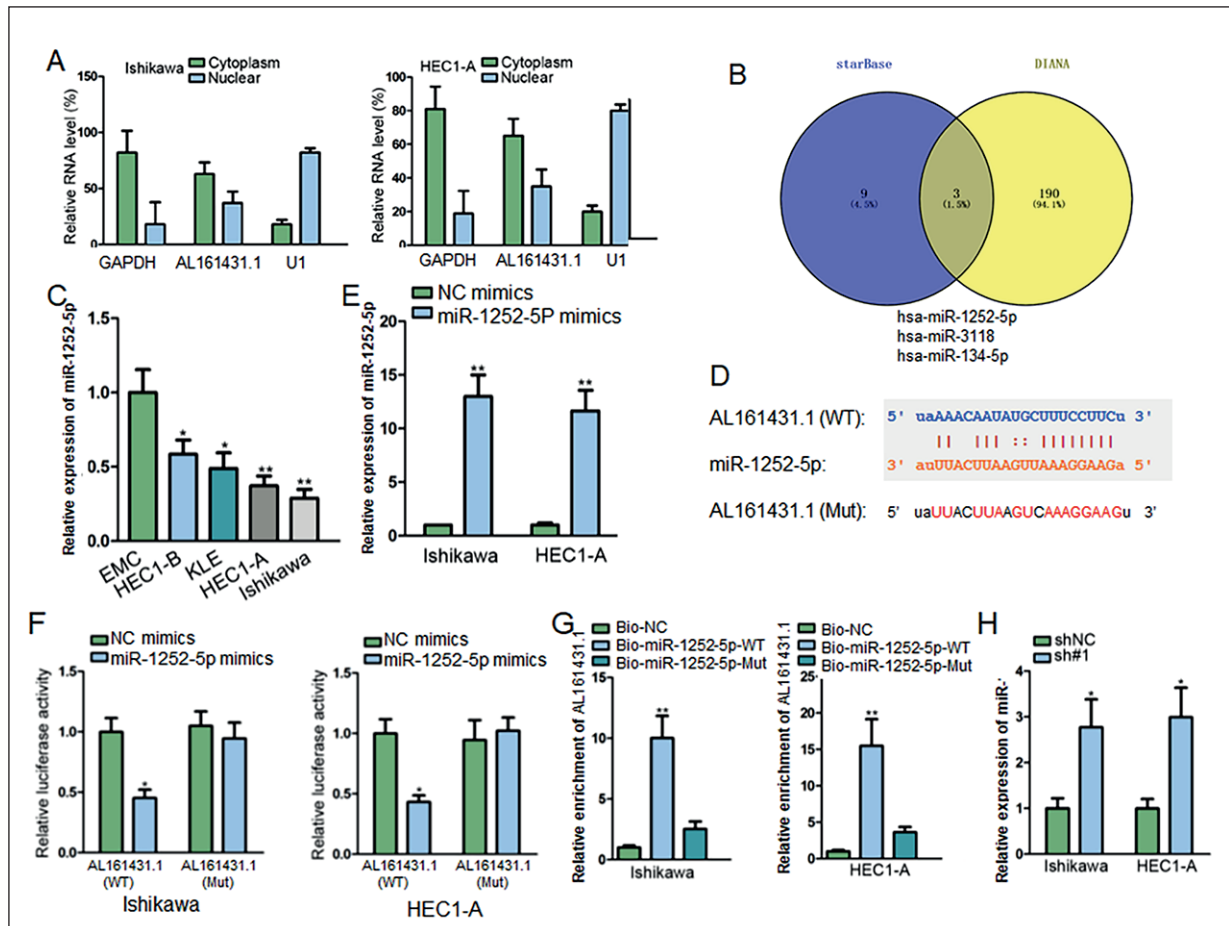

Figure 2. AL161431.1 acted as a sponge for miR-1252-5p in EC cells. **A,** A subcellular fractionation assay was performed to identify the location of AL161431.1 in Ishikawa and HEC1-A cells. **B,** Three miRNAs targeting AL161431.1 were screened and are depicted in the Venn diagram. **C,** The expression levels of miR-1252-5p in EC cells and control cells were detected using qRT-PCR. **D,** The potential binding sites of miR-1252-5p in the sequence of AL161431.1. **E,** Transfection efficiency of miR-1252-5p mimics in Ishikawa and HEC1-A cells. **F-G,** Luciferase reporter assays and RNA pull-down assays were carried out to evaluate the interaction between miR-1252-5p and AL161431.1. **H,** The expression changes of miR-1252-5p were measured in response to AL161431.1 knockdown. Each experiment was carried out at least 3 times. *$p < 0.05$, **$p < 0.01$.

RNA pull-down assays indicated that AL161431.1 was pulled down by the bio-miR-1252-5p-WT probes (Figure 2G). Additionally, we detected the upregulation of miR-1252-5p by sh#1 (Figure 2H). These data indicate that miR-1252-5p is targeted by AL161431.1 in the cytoplasm.

AL161431.1 Targeting of MiR-1252-5p Facilitates Increased MAPK Signaling

The potential downstream mRNAs affected by the interaction of AL161431.1 and miR-1252-5pm was investigated. The starBase, TargetScan, and miRmap websites were used to identify overlapping targets. A total of 2005 mRNAs (Figure 3A) were selected for KEGG enrichment in the Human Diseases category of the KEGG PATHWAY database. In the EC signaling pathway, a total of 11 candidate mRNAs were shown to be enriched (Figure 3B). qRT-PCR was used to determine whether they are regulated by miR-1252-5p and AL161431.1. In Ishikawa and HEC1-A cells, AKT2, AKT3, MAP2K1, and MAPK1 mRNA and protein levels were shown to be significantly reduced by miR-1252-5p mimics (Figure 3C and E). Similarly, the mRNA and protein levels of MAP2K1 and MAPK1 were overtly reduced following the downregulation of AL161431.1, however, the mRNA and protein levels of AKT2 and AKT3 were not significantly decreased (Figure 3D and F). Taken together, AL161431.1 targeted miR-1252-5p to de-repress MAP2K1 and MAPK1, which are central signal-
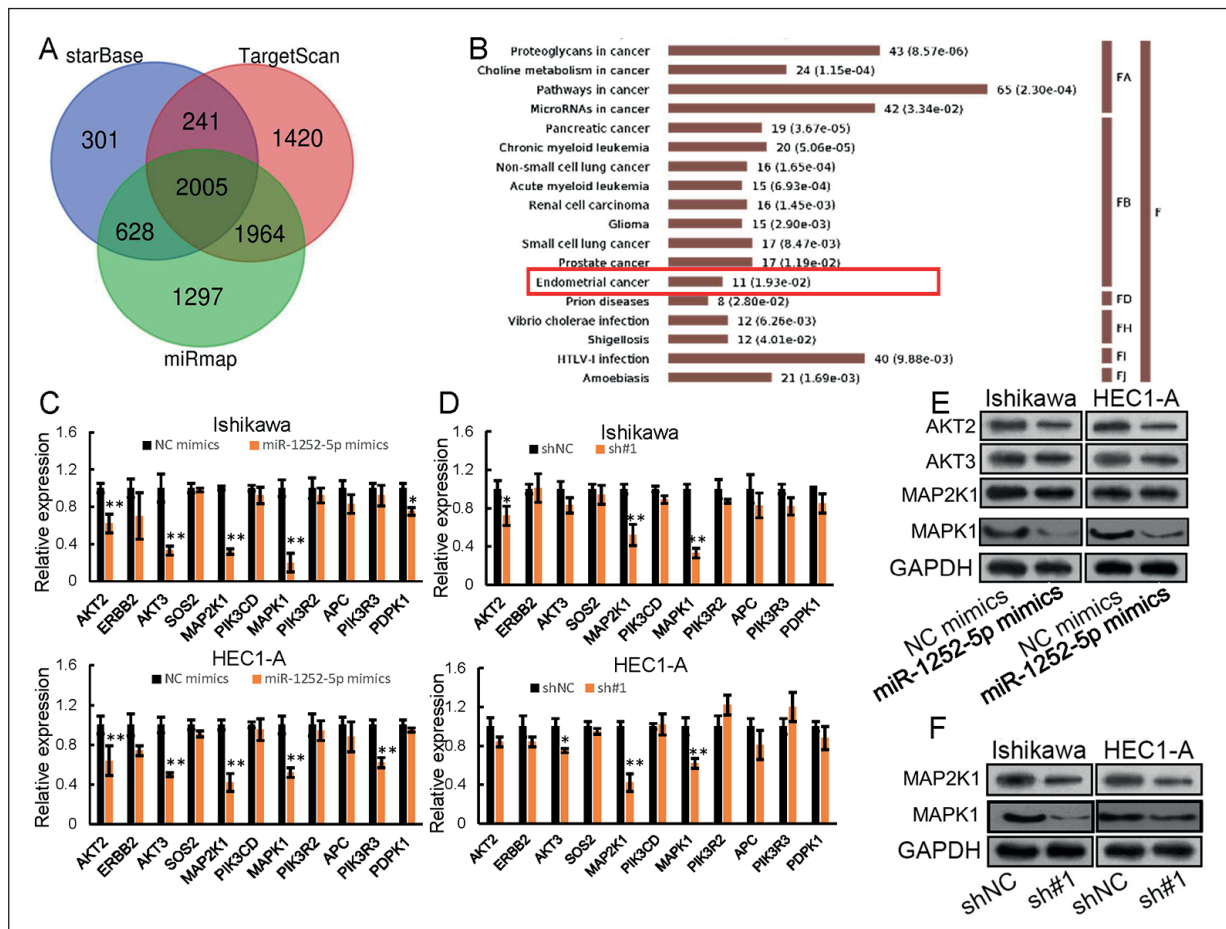

Figure 3. AL161431.1 functioned as a ceRNA by regulating miR-1252-5p and MAPK signaling. **A,** 2005 possible mRNAs regulated by miR-1252-5p were selected and depicted in the Venn diagram. **B,** Human disease of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification and functional enrichment of the 2005 possible mRNAs. **C-D,** The mRNA of 11 possible mRNAs was assessed after the overexpression of miR-1252-5p (**C**) or silencing of AL161431.1 (**D**). **E-F,** The protein levels of the downregulated protein in response to the overexpression of miR-1252-5p (**E**) or silencing of AL161431.1 (**F**). *p < 0.05, **p < 0.01.

ing molecules in the EC-related MAPK signaling pathway. In addition, miR-1252-5p may also relieve the repression of AKT2 and AKT3, but this role is independent of its association with AL161431.1.

MiR-1252-5p Repression or MAPK Signals Overexpression Reversed the Obstructive Impacts of AL161431.1 Silencing on Cell Proliferation and Migration

Rescue assays were conducted in which Ishikawa cells were separately transfected with shNC, sh#1 and sh#1+miR-1252-5p inhibitor or sh#1+MAP2K1 or sh#1+MAPK1. The transfection efficacy was determined using qRT-PCR and

Western blotting (data not shown). According to the analysis of CCK-8 assay, the decreased cellular proliferation observed after silencing AL161431.1 was rescued *via* the inhibition of miR-1252-5p or the promotion of MAP2K1 or MAPK1, which was also dissected by EdU assay (Figure 4A-B). Additionally, cellular migration was inhibited after silencing AL161431.1, but was recovered with miR-1252-5p repression or MAP2K1 or MAPK1 addition, as observed from the transwell and wound healing experiments (Figure 4C-D). Overall, these results provide evidence to support that AL161431.1 regulates cellular proliferation and migration through its interaction with miR-1252-5p and subsequent effects on the MAPK signaling pathway.
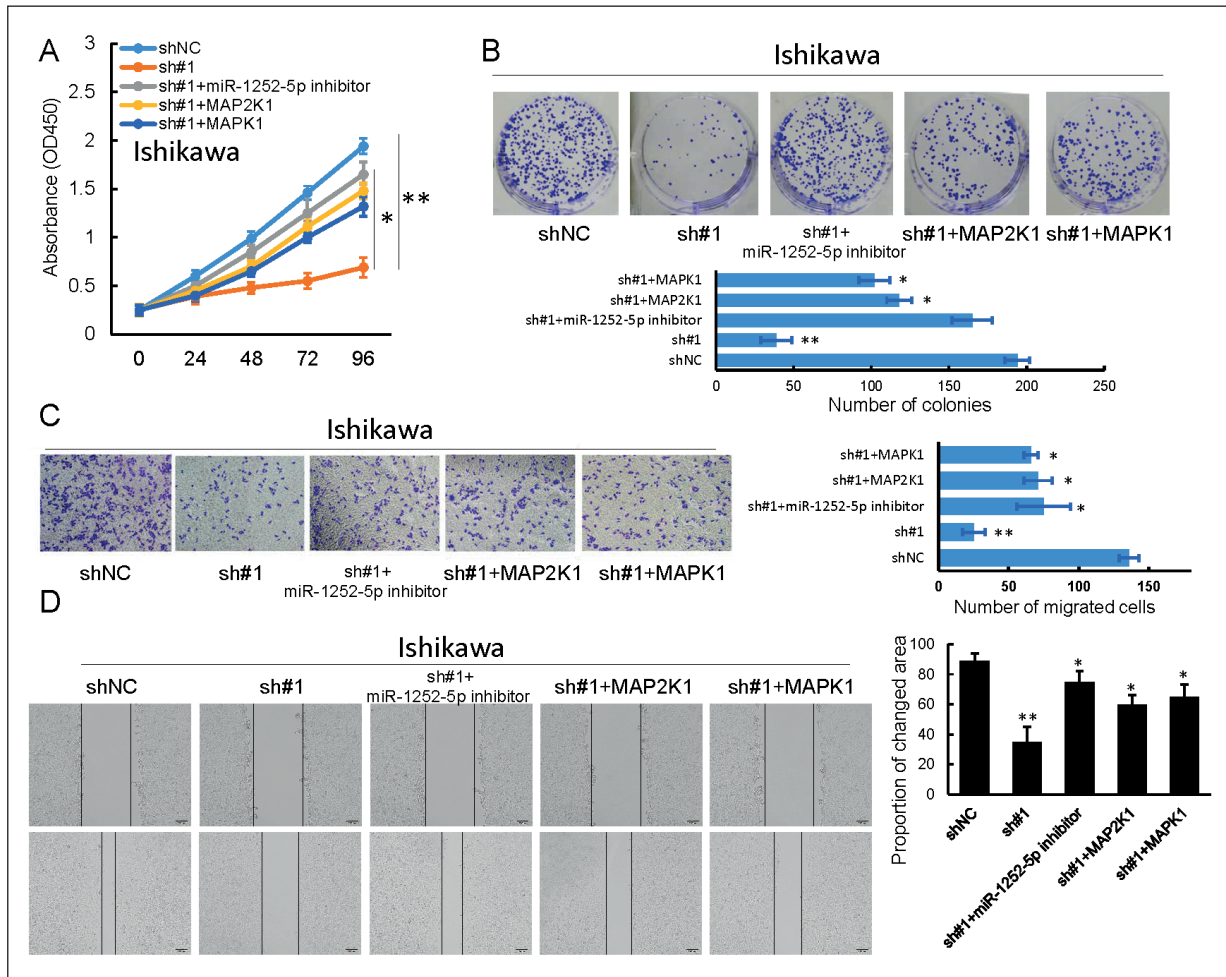

Figure 4. AL161431.1 affected EC cell proliferation and migration in a ceRNA manner. **A-B,** The decreased cellular proliferation mediated by shAL161431.1#1 was increased following the transfection of a miR-1252-5p inhibitor or MAP2K1 and MAPK1 expression vector. **C-D,** The migratory ability of Ishikawa cells was reduced as a result of AL161431.1 depletion but restored after miR-1252-5p was silenced or MAP2K1 and MAPK1 was enhanced. Each experiment was carried out at least 3 times. *$p < 0.05$, **$p < 0.01$. 10× magnifications (**B** and **C**). Scale bars = 100 μm (**D**).

Discussion

Long non-coding RNAs have been reported to be involved in the initiation and progression of diverse carcinomas, including endometrial carcinoma (EC)[8,14,18-20]. Notably, lncRNA MEG3 restricts endometrial carcinoma tumorigenesis and development through its effects on the PI3K pathway[21]; the lncRNA TDRG1 boosts tumorigenicity in endometrial carcinoma through the binding and targeting of the VEGF-A protein[22]; the lncRNA DLEU1 promotes the tumorigenesis and progression of EC by targeting mTOR[23]. In this study, the lncRNA AL161431.1, which has been shown to be highly expressed in uterine corpus endometrial carcinoma (UCEC) tissues, attracted our attention. AL161431.1 has not been previously reported in any disease, including cancer. The function of AL161431.1 and whether or not it plays a role in the initiation or progression of EC remains unknown. This work is the first to demonstrate the elevated expression and tumor-promoting role of AL161431.1 in EC.

Subcellular fractionation assays were adopted for investigating the possible underlying mechanism of AL161431.1. Considering the predominant cytoplasmic location of AL161431.1, we speculated that AL161431.1 may be involved in the ceRNA network[24,25]. A thorough search of the internet resulted in the discovery of three common miRNAs that are targeted by AL161431.1. Of the three, miR-1252-5p was the only one chosen to be used in follow-up assays as only miR-1252-5p was shown to have a low expression in EC cells. Several studies[26-28] demonstrated the tumor-suppressive role of miR-1252-5p in tumors. MiR-1252-5p was shown to suppress the proliferation capacity and metastasis of non-small cell lung cancer by targeting FOXR2[29]. MiR-1252-5p mediates the progression of laryngeal carcinoma through the PRR4 gene[30]. Decreased miR-1252-5p is associated with an unfavorable prognosis of non-small cell lung cancer[26]. The targeting of miR-1252-5p by AL161431.1 was validated in this study. Furthermore, we demonstrated the effects of both AL161431.1 and miR-1252-5p on downstream mRNAs. Next, we showed that either miR-1252-5p overexpression or AL161431.1 silencing inhibited MAP2K1 and MAPK1 protein expression the most. Moreover, an increasing number of studies have used MAP2K/MEK (mitogen-activated protein kinase kinase) inhibitors (PD98059 or U0126) to induce apoptosis and autophagic cell death in many cancer cell lines

by regulating MAP2K-MAPK1/3 signaling pathway[31-33]. Our current study was the first to define the relationship between AL161431.1/miR-1252-5p and the expression of MAP2K-MAPK1/3 signaling. The molecular mechanism of AL161431.1/miR-1252-5p/MAPKs axis was validated using rescue experiments.

Conclusions

The results of our study firstly, showed that the lncRNA AL161431.1 targeted miR-1252-5p, which resulted in the upregulation of MAPK signaling in EC cells. These data highlight the potential use of AL161431.1 as a promising therapeutic strategy in the treatment of EC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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