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ORIGINAL RESEARCH

Long Noncoding RNA LINC00261 Reduces Proliferation and Migration of Breast Cancer Cells via the NME1-EMT Pathway

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Objective: Long noncoding RNAs (lncRNAs) are emerging as a class of important biological regulators. lncRNAs participate in diverse biological functions and disease processes, especially those leading to tumorigenesis. In this study, we investigate the role of linc00261 in the pathogenesis of breast cancer.

Methods: linc00261 and *NME1* expression levels were determined in breast cancer tissue and adjacent normal tissue using qRT-PCR. Cell proliferation and migration were analyzed using MTT and transwell assays, respectively. Epithelial–mesenchymal transition markers were examined via Western blotting assay. RNA pull-down was used to examine the interaction between linc00261 and the *NME1* mRNA transcript.

Results: linc00261 is expressed in lower levels on breast cancer tissues than in para-carcinoma tissues. Reintroduction of linc00261 can inhibit the migration of breast cancer cells and arrest their proliferation. Additionally, linc00261 knockdown is sufficient to cause breast carcinoma tumorigenesis. We also found that linc00261 interacts with *NME1* mRNA, protecting it from degradation. This protection leads to increased cellular levels of NME1, which functions as suppressor of tumor metastasis.

Conclusion: Taken together, these data demonstrate detailed mechanistic links between the linc00261/NME1 axis and tumorigenesis and show that linc00261 might serve as a novel therapeutic target.

Keywords: linc00261, NME1, breast cancer, E-cadherin, N-cadherin

Introduction

Worldwide, breast cancer is one of the most frequently diagnosed cancers in women. Although advances have been made in breast cancer diagnosis and clinical treatment, the disease remains a major health problem.[1–3] In the United States, the average age of patients with breast cancer is lower than that of patients with other commonly occurring cancers including colorectal, lung, and prostate cancers. This is partly because the median age at diagnosis for patients with breast cancer is lower than that for other commonly diagnosed cancers.[4,5] Therefore, it is both important and necessary to investigate the etiological mechanisms underlying the onset, development, and progression of breast carcinoma.

Long noncoding RNAs (lncRNAs) are transcripts with more than 200 bps that have no, or minimal, protein coding capacities.[6] lncRNAs play important roles in various biological processes.[7–9] Increasing evidence indicates that many lncRNAs are dysregulated in cancer and play roles in the development of many features of

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cancer.[10–14] PVT1 is an oncogenic lncRNA, and the PVT1 promoter functions as a tumor-suppressor in breast cancer.[15,16] The HOTAIR lncRNAs paly active roles in reprogramming the chromatin state to promote breast cancer metastasis.[17] Linc00261, a newly discovered lncRNA, acts as a suppressive regulator in several cancers, such as hepatocellular carcinoma,[18] gastric cancer,[19] non-small-cell lung cancer,[20] and colon cancer[21] through various molecular mechanisms. To date, the function of linc00261 in breast cancer has not been reported. In this study, we found that the expression of linc00261 in breast cancer tissue was significantly downregulated compared to para-carcinoma tissues. Functional assays were used to elucidate the influence of linc00261 on cell proliferation and migration. Our results show that linc00261 suppresses the progression of breast cancer by enhancing *NME1* mRNA stability.

Patients and Methods

Clinical Samples

This study was approved by the Ethics Committee of the People's Hospital of Ganzhou. Written informed consent was obtained from each patient, and clinical specimens were collected in accordance with the Declaration of Helsinki. A total of 60 pairs of breast cancer tissues and adjacent non-tumor tissues were harvested from patients at the People's Hospital of Ganzhou between May 2017 and July 2018. The mean patient age was 49.58 ± 16.59 years. None of the enrolled subjects underwent relevant chemotherapy, radiotherapy, biological therapy, or drug-targeted therapy before surgery. Tissues were stored in liquid nitrogen until required for use.

Cell Cultures and Treatments

MCF7 and MDA-MB-231 cells were obtained from the Chinese Academy of Sciences Cell Bank and cultured in 1640 and MEM medium (Gibco, USA) supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. Where indicated, cells were treated with 50 μM α-amanitin (Sigma-Aldrich, USA) for 0–24 h.

RNA Extraction and Real-Time qPCR Analysis

Total RNA was isolated using Trizol reagent (Invitrogen; Thermo Fisher Scientific, USA) following the manufacturer's instructions. RNA was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, USA). Real-time qPCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) and SYBR Green (Takara). β-actin expression was measured as an internal control. The primer sequences used were:

Linc00261 forward primer: 5′-TCAGATTGCTCCTGGACACTT-3′,

reverse primer: 5′-GGACCATTGCCTCTTGATTAG-3′;

Snail forward primer: 5′- CTTCGCTGACCGCTCCAACC-3′,

reverse primer: 5′- GGAGCAGGGACATTCGGGAGA-3′;

NME1 forward primer: 5′- CGAGGGGCCTCCTATCTCA-3′,

reverse primer: 5′- ACCAACAAGGCGGAATCCTT-3′;

Slug forward primer: 5′- GGCTCATCTGCAGACCCATT −3′,

reverse primer: 5′- TGCTACACAGCAGCCAGATT −3′;

β-actin forward primer: 5′- AGGCCAACCGCGAGAAGATG-3′,

reverse primer: 3′- CACACGGAGTACTTGCGCTCAG-5′.

Vector Construction and Small Interfering RNA (siRNA/shRNA) Synthesis and Transfection

The cDNA encoding the LINC00261 CDS or antisense CDS was amplified using the Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) and subcloned into the EcoRI and XhoI sites of the pcDNA3.1 vector. The sense and antisense constructs were dubbed pcDNA3.1-LINC00261, pcDNA3.1-Antisense, respectively.

The MS2-12× fragment was amplified from pSL-MS2-12× (Addgene) using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) and subcloned into the EcoRV and XhoI sites of pcDNA3.1 and pcDNA3.1-LINC00261. The MS2 constructs were named pcDNA3.1-MS2, pcDNA3.1-MS2- LINC00261. SiRNAs and shRNAs specifically targeting LINC00261, and control siRNA/shRNA, were synthesized by GenePharma (Table 1). ShRNA was subcloned into the EcoRI and NotI sites of the plv-shRNA vector. Breast cancer cell line was transfected with the plasmids and siRNAs using Lipofectamine 2000, RNAiMAX (Invitrogen) following the manufacturer's protocol.

Cell Proliferation

Cell viability was measured using MTT assays (Sigma-Aldrich, St. Louis, MO, USA). A total of 2000 breast

Table 1 siRNAs/shRNAs Used in This Study

siRNA	Sense 5′-3′	Antisense 5′-3′
siLinc00261-1	CCAAUAGACCAACAGCCAU	AUGGCUGUUGGUCUAUUGG
siLinc00261-2	GAAAGCUGUAGCCAUUCAA	UUGAAUGGCUACAGCUUUC
siLMNA	AUCUCAUCCUGAAGUUGCUUC	GAAGCAACUUCAGGAUGAGAU
siNC	ACGUGACACGUUCGGAGAA	UUCUCCGAACGUGUCACGU
shLinc00261-1	GCAATTAATTCAGGACACT	AGTGTCCTGAATTAATTGC
shLinc00261-2	GCAAGGGCACAAACAATGT	ACATTGTTTGTGCCCTTGC
shNC	ACGTGACACGTTCGGAGAA	TTCTCCGAACGTGTCACGT

cancer cells (MCF-7 or MDA-MB231 cell line) were seeded into 96-well plates and cultured for 12 h. Cell proliferation was assessed following the manufacturer's protocol. The absorbance at each time point was plotted to generate cell proliferation curves. These experiments were performed in triplicate.

Transwell Cell Migration Assay

Experiments were performed using a membrane with 8 μm pores (Millipore, Bedford, USA). After transfection, breast cancer cells (MCF-7 or MDA-MB231 cell line) were suspended in serum-free medium, and 2×10^4 cells were seeded into the upper chamber and cultured at 37°C for 24 h. The lower compartments contained medium supplemented with 10% FBS. After incubation for 24 h, the cells that had migrated to the bottom surface of the membrane were stained with 0.5% crystal violet and examined by an inverted microscope (Olympus, Tokyo, Japan). We randomly selected six fields (magnification 100×) from each sample to calculate the number of cells that had passed through the membrane. These experiments were performed in triplicate.

Western Blotting

Total cell and tissues lysates were collected, lysed using the RIPA reagent (Beyotime, Nanjing, China), and prepared in a 1× sodium dodecyl sulfate buffer. Samples were loaded onto 8% sodium dodecyl sulphate-polyacrylamide gels (20 μg per well) for electrophoresis and then transferred onto nitrocellulose filter membranes. After incubation with E-Cadherin and N-Cadherin (Catalog number 1702–1 and 2447–1, respectively, 1: 1000 dilution, Epitomics, USA) and β-actin (1: 5000; 66,009-1-Ig, Proteintech) primary antibodies, membranes were incubated with secondary antibodies. Secondary antibodies used were goat anti-rabbit IgG and goat anti-mouse IgG (Catalog numbers ZB-2301 and ZB-2305, respectively, 1: 2000 dilution, ZSGB-BIO, China). β-actin was used as a loading control for Western blots.

RNA Immunoprecipitation (RIP-MS2) Assays

pcDNA3.1-MS2 or pcDNA3.1-MS2-Linc00261 constructs were co-transfected into MDA-MB-231 cells with pMS2-GFP (Addgene). After 48 h, RIP experiments were performed using transfected cells, a GFP antibody (3 μg per reaction; ab290, Abcam), and the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) following the manufacturer's instructions.[22] The RNA fraction isolated by RIP was quantified using Real-time qPCR.

Nascent RNA Capture

To capture nascent RNAs, 0.2 mM ethylene uridine (EU) was incorporated into MCF-7 cells for 12 h. EU-labeled RNAs were biotinylated and captured using the Click-iT Nascent RNA Capture Kit (Invitrogen) following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software (La Jolla, CA, USA). Measurement data were expressed as mean ± SD. Differences between groups were analyzed using the *t*-test. P values <0.05 were considered to indicate significance. Figures are representative of three independent repeats.

Results

linc00261 Expression Is Reduced in Breast Cancer

To explore the function of linc00261 in breast cancer, linc00261 expression levels were determined by examining the extensive tumor transcriptome profiles in The Cancer Genome Atlas. Comparison with expression levels in paracarcinoma tissues revealed that linc00261 expression is significantly downregulated in breast cancer tissues (Figure 1A). To further validate these results, we determined linc00261 expression levels in 60 breast carcinoma tissue pairs. These

results show that linc00261 expression is lower in breast carcinoma tissue than in adjacent non-tumor tissues (Figure 1B). Furthermore, low linc00261 expression is observed in patients with advanced breast cancer (Figure 1C) and lymph node metastasis (Figure 1D).

linc00261 Suppresses the Proliferation and Migration of Breast Cancer Cells

We further evaluated whether linc00261 expression correlated with the malignant phenotype in breast carcinoma. Firstly, we examined linc00261 expression levels in various breast cancer cell lines. These results show that linc00261 is highly expressed in MCF7 cells and has low expression levels in MDA-MB-231 cells (Figure S1A). Then, we performed siRNA-mediated linc00261 knockdown in MCF7 cell lines (Figure S1B) and observed a dramatic elevation in their proliferative capacity (Figure 2A). Transwell migration assay results showed that linc00261 knockdown accelerates the migration of breast cancer cells (Figure 2B). Short hairpin RNA (shRNA) for linc00261 knockdown was constructed as another means to silence linc00261 expression (Figure S1C). Lentivirus-mediated shRNA linc00261 knockdown elevated the proliferative ability and migration capacity of cells (Figure 2C and D). Conversely, linc00261 over-expression inhibited breast cancer cell proliferation and migration (Figure 2E and F, S1D). Taken together, these data suggest that linc00261 knockdown increases proliferation and migration in breast cancer cells.

linc00261 Regulates the Epithelial–Mesenchymal Transition (EMT) in Breast Cancer Cells

Despite recent advances in diagnosis and treatment, as a result of metastasis and consequent recurrence after curative

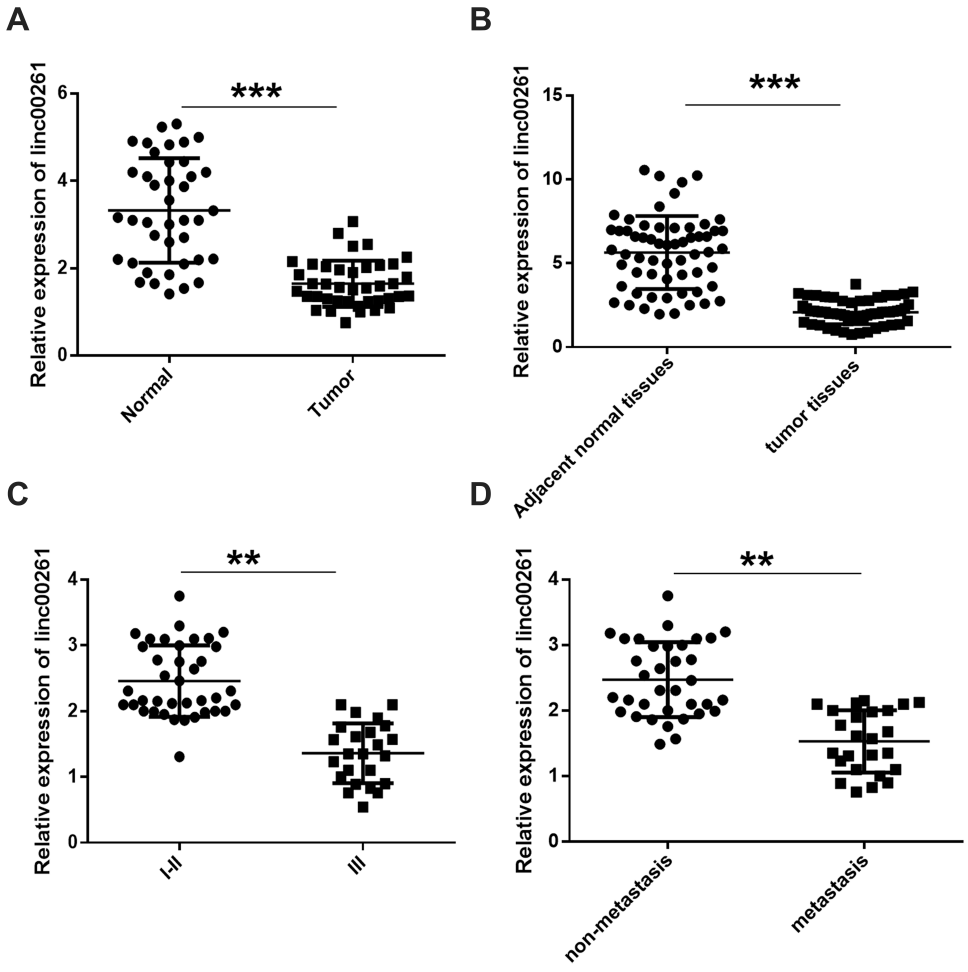

Figure 1 Linc00261 expression is significantly down-expressed in breast cancer.

Notes: (**A, B**) Linc00261 expression was significantly downregulated in breast cancer tissues, the data was obtained from TCGA (**A**) and clinical specimens (**B**). (**C**) Low linc00261 expression was observed in patients with advanced breast cancer. (**D**) linc00261 expression was significantly downregulated in metastatic breast carcinoma tissue. Data are reported as mean ± SD. **P < 0.01; ***P < 0.001.

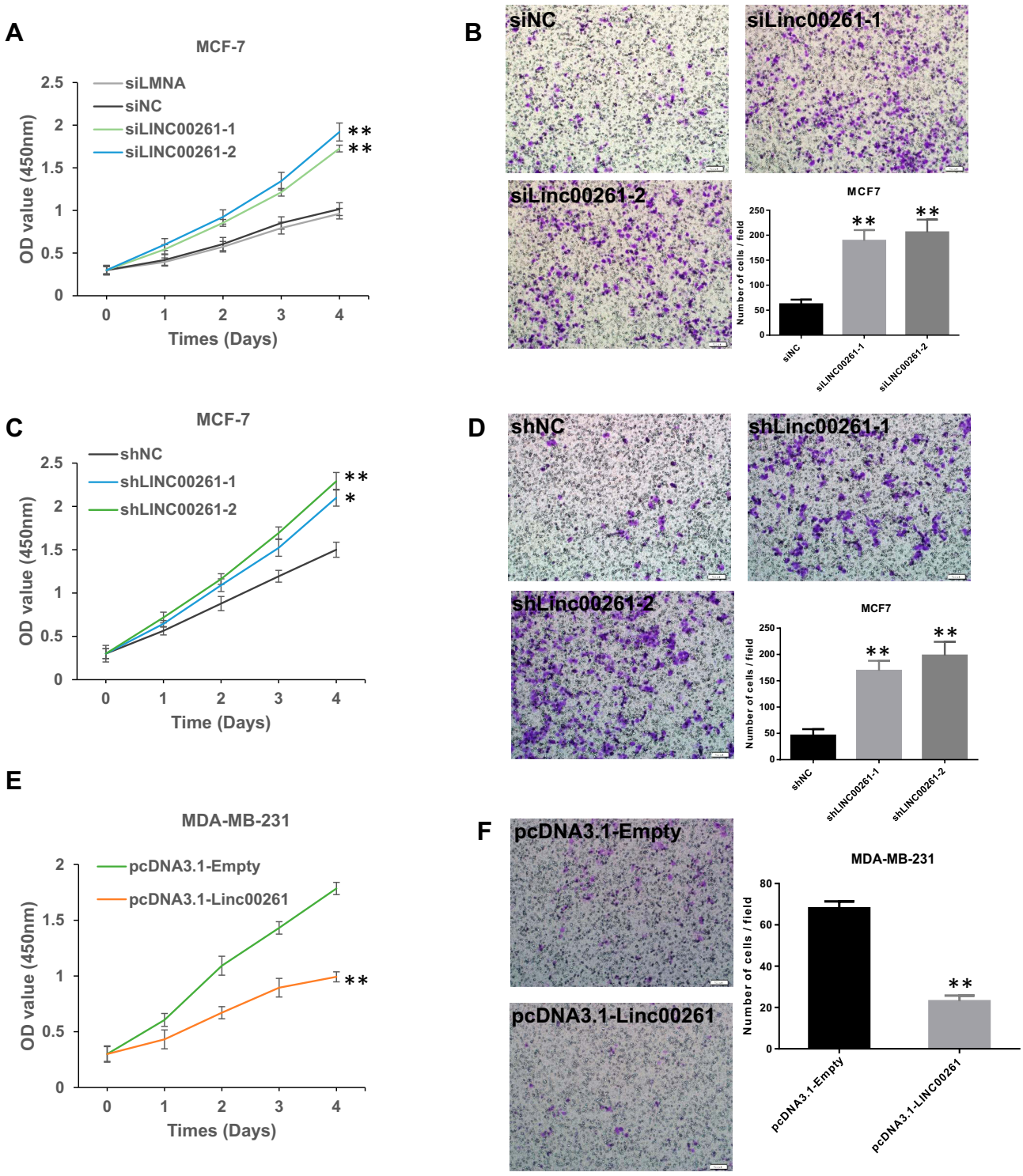

Figure 2 Linc00261 suppresses proliferation in breast cancer cell.

Notes: (**A**) MTT assay results show that linc00261 inhibition promotes the proliferative capacity of breast cancer cells. (**B**) Transwell assay results show that linc00261 inhibition promotes the capacity of breast cancer cells to migrate. (**C**) MTT assay results show that shLinc00261 promotes breast cancer cell proliferation. (**D**) Transwell assays show that shLinc00261 promotes breast cancer cell migration. (**E**) MTT assay results show that Linc00261 inhibits breast cancer cell proliferation. (**F**) Transwell assays show that Linc00261 inhibits breast cancer cell migration. Data are reported as mean ± SD. *P < 0.05; **P < 0.01.

therapies, breast carcinoma remains a highly lethal disease. In gastric cancer, linc00261 suppresses growth and metastasis by repressing the EMT.[19] The hallmarks of the EMT are decreased E-cadherin expression and increased N-cadherin expression. Therefore, we examined whether linc00261 affects EMT in breast cancer cells. Our results show that

E-cadherin expression is decreased and N-cadherin expression increased following linc00261 knockdown (Figure 3A). Consistent with this, E-cadherin expression increased and N-cadherin expression decreased when linc00261 is overexpressed in breast cancer cells (Figure 3A). These results show that linc00261 inhibits the EMT in breast cancer. We further explored how linc00261 regulated the EMT during breast cancer pathogenesis. *NME1* is a metastasis suppressor gene implicated as an important EMT regulator. Interestingly, linc00261 knockdown significantly inhibit *NME1* mRNA expression (Figure 3B). Furthermore, linc00261 overexpression can inhibit the expression of the important EMT regulators *snail* and *twist* (Figure 3B).

linc00261 Regulates EMT by Enhancing *NME1* mRNA Stability

To elucidate the mechanisms underlying the effect of linc00261 on the EMT, we asked whether linc00216 binds with an EMT marker using immunoprecipitation (IP). MS2-RIP experiment results reveal that linc00261 interacts with *NME1* mRNA, but not with *snail, twist, E-cadherin*, or *N-cadherin* mRNA (Figure 4A). We used in vitro transcribed biotin-labelled *NME1* to further validate that *NME1* pulls down endogenous linc00261 mRNA (Figure 4B). These results indicate that linc00261 may regulate *NME1* mRNA stability. We transiently expressed linc00261 in breast cancer cells treated with a-amanitin to block the synthesis of RNA and measured the *NME1* mRNA loss (Figure 4C). linc00261 increases the half-life of *NME1* mRNA (Figure 4C) and *NME1* mRNA half-life decreases after linc00261 knockdown (Figure S2A). These results suggest that linc00261 is required for the regulation of *NME1* mRNA stability. Furthermore, newly synthesized nascent *NME1* mRNA levels were measured in shLinc00261 cells and control cells. We found that linc00261 does not influence *NME1* gene transcription synthesis (Figure S2B). Assessment of *NME1* expression in 60 breast carcinoma tissues revealed a positive correlation with linc00261 expression ($R2 = 0.6393$, $p < 0.001$) (Figure S2C). Importantly, *NME1* overexpression completely suppressed the proliferation ability and EMT phenotype caused by linc00261 silencing (Figure 4D and E, S1E). Additionally, silencing *NME1* disrupted linc00261 overexpression-mediated tumor suppression (Figure S1F,S3A and B). These data show that

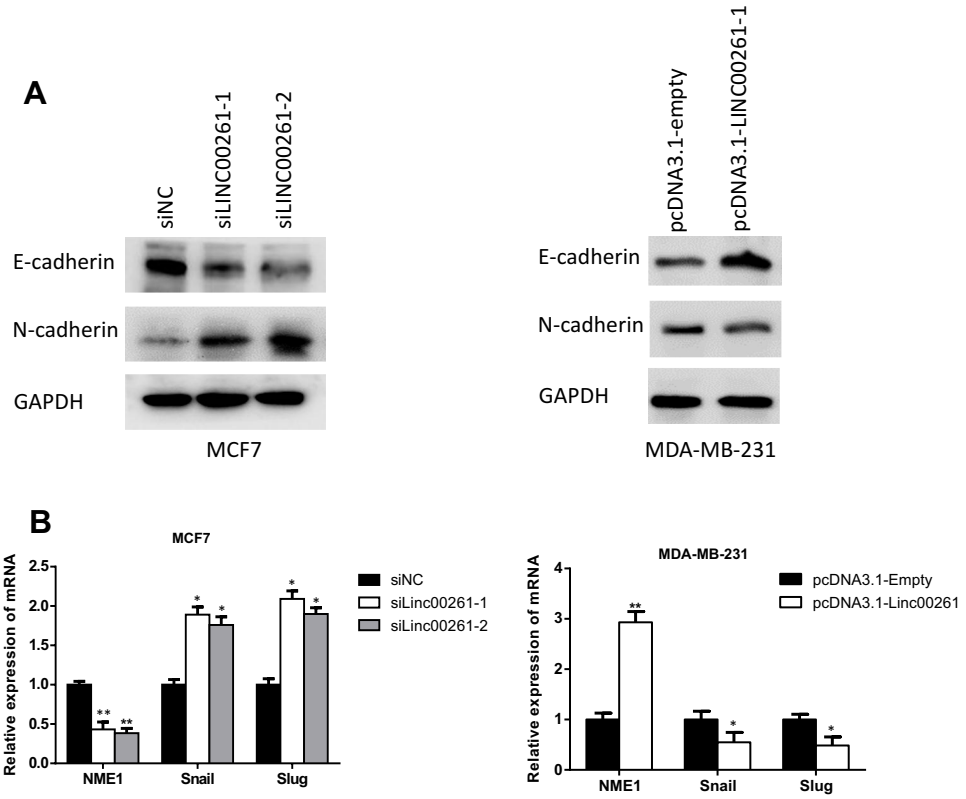

Figure 3 linc00261 regulates the epithelial–mesenchymal transition (EMT) in breast cancer cells.
Notes: (**A**) Western blot of E-cadherin and N-cadherin. EMT marker expression in experimental cells. (**B**) NEM1, Slug, and Snail expression in breast cancer cells transduced with siRNA-Linc00261 was determined qRT-PCR. Data are reported as mean ± SD. *P < 0.05; **P < 0.01.

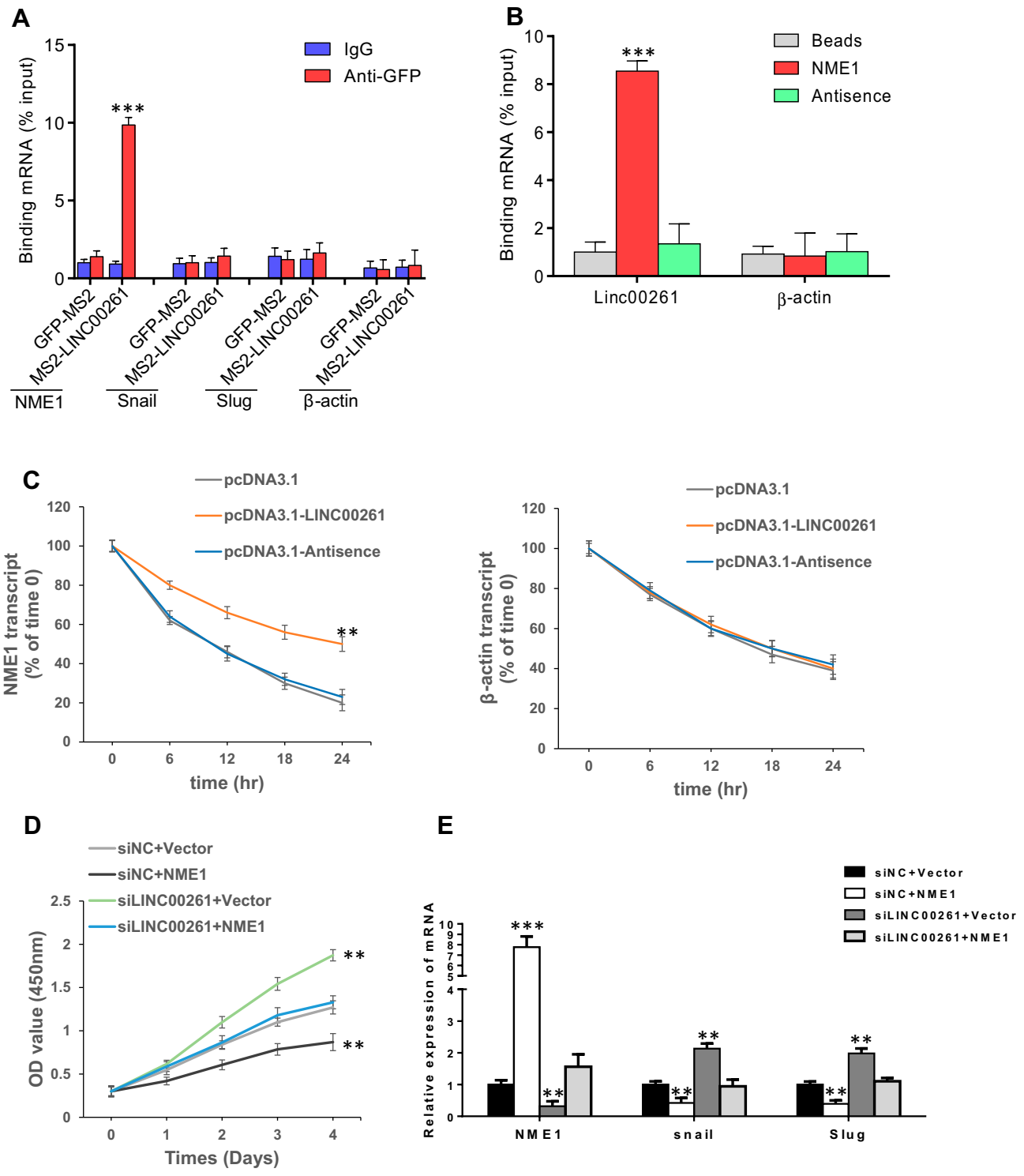

Figure 4 linc00261 regulates the epithelial–mesenchymal transition (EMT) by enhancing *NME1* mRNA stability.

Notes: (**A**) We used MS2-RIP followed by qRT-PCR to detect mRNAs endogenously associated with linc00261. (**B**) Cell lysates were incubated with biotin-labeled *NME1* mRNA. After pull-down, mRNA was extracted and assessed using qRT-PCR. (**C**) NME1 and β-actin mRNA stability over time was measured by qRT-PCR. Expression levels at time 0, after RNA synthesis was blocked with a-amanitin (50 mM), were determined in breast cancer cells and normalized to 18S rRNA expression levels (a product of RNA polymerase I that is unchanged by a-amanitin) by over-expressing linc00261. (**D**) NME1 reduces MCF-7 cell proliferation capability after linc00261 knockdown in MTT assays. (**E**) NME1 reduces the linc00261 knockdown-induced EMT phenotype. Data are reported as mean ± SD. **P < 0.01; ***P < 0.001.

linc00261 suppresses the EMT process by enhancing *NME1* mRNA stability, preventing breast carcinoma tumorigenesis.

Discussion

In this study, we identified a tumor suppressor lncRNA, linc00261. linc00261 is located on chromosome 20, spanning sites 22,560,552 to 22,578,642.[23] LINC00261 has been identified as a tumor suppressor in various cancers, and a novel prognostic marker,[24,25] for example, in patients with lung cancer.[26–28] Here, for the first time, we report that linc00261 expression is significantly decreased in breast cancer tissues and cells. Furthermore, we show that LINC00261 inhibits breast cancer by competitively binding to *NME1* mRNA, a critical step in mediating the progression of breast cancer. Our functional studies show that linc00261 significantly drives, and is necessary for, *NME1* mRNA stability. We also identified a mechanistic link between linc00261 and breast carcinoma tumorigenesis.

NME1 is a well-known tumor suppressor that regulates p53 function to prevent cancer progression and metastasis in some malignancies.[29–32] NME1 is involved in cell-adhesion-related signaling pathways, and may play an important role in growth control, cellular proliferation, signal transduction, embryonic development, and differentiation.[33–36] lncRNAs are known to be involved in a variety of biological functions, such as regulating local chromatin structure, gene expression, mRNA levels, and protein binding for posttranscriptional modification.[9,37-39] However, the mechanism through which lncRNA regulates NME1 is unknown. In our study, we found that linc00261 interacts with *NME1* mRNA and stabilizes NEM1 expression. We also showed that linc00261 has no effect on *NME1* transcript synthesis, and only inhibits the *NME1* mRNA degradation pathway. Therefore, the interaction between *NME1* mRNA and linc00261 may result in a positive regulatory effect on breast carcinoma tumorigenesis.

In summary, our results show that linc00261 can inhibit the progression of breast cancer cells by enhancing *NME1* mRNA stability. Here, we have shown a mechanistic link between the linc00261-NME1 axis and tumorigenesis, and demonstrated that linc00261 may be an attractive therapeutic target for breast carcinoma treatment.

Abbreviations

Linc00261, long intergenic noncoding RNA-00261; NME1, NME/NM23 Nucleoside Diphosphate Kinase 1; EMT, epithelial–mesenchymal transition.

Ethics and Consent Statement

Informed consent was obtained from all individual participants included in the study. All procedures involving humans were performed in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Disclosure

The authors report no conflicts of interest in this work.

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