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A nuclear lncRNA Linc00839 as a Myc target to promote breast cancer chemoresistance via PI3K/AKT signaling pathway

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Abstract

Chemoresistance has become a leading cause of mortality in breast cancer patients and is one of the major obstacles for improving the clinical outcome. Long noncoding RNAs play important roles in breast cancer tumorigenesis and chemoresistance. However, the involvement and regulation of lncRNAs in breast cancer chemoresistance are not completely understood. Here, we reported that Linc00839 was localized in the nucleus and upregulated in chemoresistant breast cancer cells and tissues, and high level of Linc00839 was associated with a poor prognosis. Knockdown of Linc00839 significantly suppressed proliferation, invasion, and migration, sensitized cells to paclitaxel in vitro and inhibited transplant tumor development in vivo. Mechanistically, we found that Myc could directly bind to the promoter region of Linc00839 and activate its transcription. Furthermore, Linc00839 overexpression increased the expression of Myc and the RNA-binding protein Lin28B and activated the PI3K/AKT signaling pathway. We also discovered that Lin28B positively interacted with Linc00839 and was upregulated in breast cancer tissues. Taken together, for the first time, we showed that Linc00839 was activated by Myc and promoted proliferation and chemoresistance in breast cancer through binding with Lin28B. These findings provide new insight into the regulatory mechanism of Linc00839 and propose a Myc/Linc00839/Lin28B feedback loop that could be used as a novel therapeutic target for breast cancer.

KEYWORDS

breast cancer, chemosensitivity, Lin28B, Linc00839, Myc

Abbreviations: ASO, antisense Oligonucleotides; BC, breast cancer; GSEA, gene set enrichment analysis; lncRNAs, long noncoding RNAs; RBP, RNA-binding protein; RF, random forest; SVM, support vector machines.

1 | INTRODUCTION

BC is the most commonly diagnosed malignancy and has become the second leading cause of cancer-related mortality in females worldwide.[1] Patients with early stage and stage III diseases undergo mastectomy, and most also receive adjuvant chemotherapy.[2] Chemoresistance has become a major obstacle for BC therapy. Therefore, there is a clear need to understand the functional regulation of chemoresistance in BC to improve the overall survival rates.

Long noncoding RNAs (lncRNAs) have been identified as crucial components in BC development,[3] suggesting their potential abilities as diagnostic and therapeutic biomarkers.[4] Studies have shown that dysregulation of lncRNAs contributes to tumorigenesis,[5,6] metastasis,[7,8] and chemoresistance,[9] by modulating cell proliferation,[10] and apoptosis[11] through different signaling pathways. Different types of lncRNAs localize to the cytoplasm or nucleus, participating in transcriptional and posttranscriptional gene expression.[12] Microarray datasets analysis has successfully revealed a large number of altered lncRNAs, but only a small number has been indicated to be functional in detail. The oncogene Myc is a key transcription factor in human cancer, and it can regulate the transcription processes of protein-coding genes and lncRNAs, involved in cancer cell apoptosis and reprogramming.[13,14] In contrast, Myc can also act as a target by regulating downstream genes to promote cell cycle progression.[15] Interestingly, we also found that RNA-binding proteins (RBPs) could act as transcriptional regulators.[16] Several lncRNAs have been proven to modulate the activities and expression levels of RBPs.[17,18] Additionally, cancer-specific lncRNAs act as independently prognostic and diagnostic biomarkers.[19-21] Thus, the discovery of novel functions of lncRNAs undoubtedly calls for further research to characterize these functions.

In this study, we revealed a novel lncRNA, Linc00839, displayed a remarkable trend toward increasing expression levels in BC resistant cells and tissue samples, and for functional analysis in terms of proliferation and chemoresistance. High expression levels of Linc00839 were correlated with poor prognosis. Mechanistically, the upregulation of Linc00839 was transcriptionally activated by the crucial oncogene Myc, and it was surprisingly able to regulate Myc and Lin28B protein expression. Furthermore, we confirmed that Linc00839 promoted cell proliferation and chemoresistance by enhancing the activity of the PI3K/AKT pathway. Therefore, Linc00839 may be a potential biomarker and therapeutic target for BC patients.

2 | MATERIALS AND METHODS

2.1 | Gene Expression Omnibus data analysis

Microarray dataset GSE76540, GSE67916, GSE38376, and GSE125677 were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). TARNIC, a RNA sequence dataset, analyzed from TCGA and independent datasets, including expression level of newly annotate or named lncRNAs (https://ibl.mdanderson.org/tanric/design/basic/analysis.html) in breast invasive carcinoma(BRCA), which contained 837 BC tissue and 105 paired-normal tissue.

2.2 | Cell lines and reagents

BC cell lines MCF-7, BT549 and MDA-MB-231 (Chinese Academy of Sciences, China) were cultured in Minimum Essential Medium (MEM) and RPMI-1640 medium (Gibco) and supplemented with 100 U/mL penicillin/streptomycin and 10% fetal bovine serum (Gibco). MCF-7/ADR cells (Shanghai Antique Biotechnology Company) are cultured from parental MCF-7 cells in RPMI-1640 medium with increasing concentrations of doxorubicin. All the cells were cultured at 37°C with 5% CO_2. Doxorubicin (DOX; 10 mg) was obtained from Hisun Pharmaceutical Company. Paclitaxel (PTX, 6 mg/mL) was obtained from pharmacy of Fourth Affiliated Hospital, Jiangsu University.

2.3 | Patient samples

Primary BC tissues and adjacent normal tissues were collected from 32 patients with informed consent approved by Fourth Affiliated Hospital, Jiangsu University. The diagnoses of all the patients were pathologically confirmed, and the clinical tissue samples were collected after mastectomy, and no patient received any pre-operative chemotherapy and radiotherapy. The tissues were frozen and preserved at −80°C.

2.4 | RNA oligonucleotides, plasmid transfection, RT-PCR, western blot assay

Cell transfection was performed using Lipofectamine 2000 (Invitrogen) and Transfection Kit (RiboBio). RT-PCR and western blot were conducted according to previously described methods.[22] Details are also provided in Appendix S1.

2.5 | CCK8, EdU, apoptosis, and IHC analysis

CCK8, apoptosis, and immunohistochemical (IHC) analysis were conducted according to previously described methods.[22] Details are provided in Appendix S1.

2.6 | Fluorescence in situ hybridization (FISH) and immunofluorescence analysis

Cells were fixed with 4% formaldehyde and washed with PBS, and then, 0.5% Triton X-100 was added for 5min at 4°C. The fixed cells were incubated with 20 μM of the FISH probe (RiboBio) mix in 100 μL hybridization buffer at 37°C overnight. After hybridization, the slide was washed and mounted with DAPI. Cells were fixed again with 4%

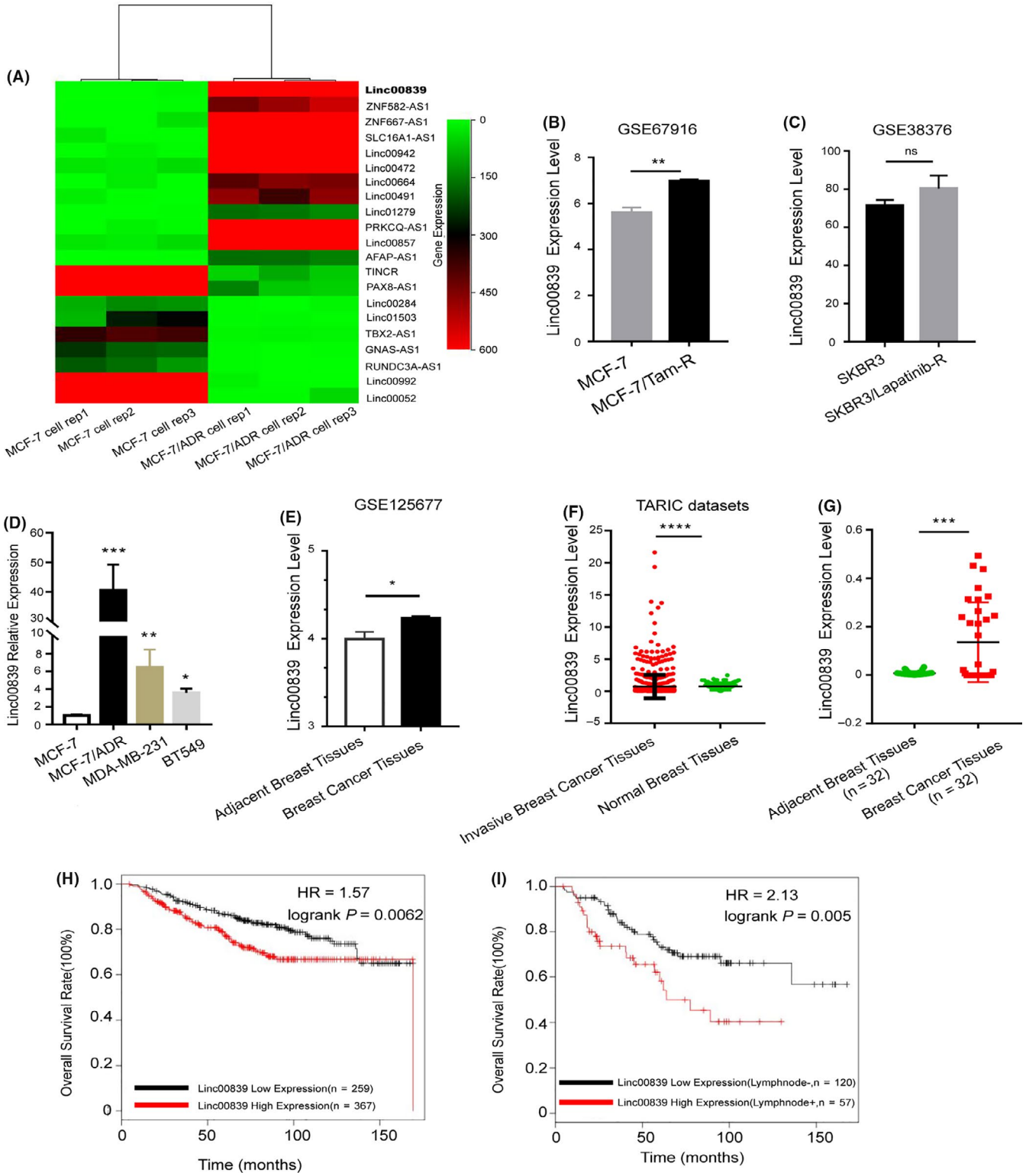


FIGURE 1 Identification of lncRNAs differentially expressed in breast cancer (BC). A, Heatmap of top dysregulated lncRNAs in resistant BC cells MCF-7/ADR, compared with sensitive BC cells MCF-7 with 3 biological replicates. The *P*-values < .05 and fold change |FC| > 2. B-E, The expression levels of Linc00839 in (B) GSE67916, (C) GSE38376, (D) different types of BC cells and (E) GSE125677. F, Validation of Linc00839 expression levels in TANRIC datasets including invasive breast cancer (n = 837) and normal tissues (n = 105). G, Validation of Linc00839 expression in 32 matched breast cancer tissues and adjacent cancer tissues collected from Fourth Affiliated Hospital. H-I, KM plotter analysis for the overall survival rate of Linc00839 differentially expressed level in BC tissues (H), and lymph node positive BC samples (I). Data were expressed as means ± SD in 3 independent experiments. **P* < .05, ***P* < .01, ****P* < .001

formaldehyde for further immunofluorescence staining. After blocking with 5% BSA, the cells were incubated with anti-Lin28B and anti-Myc and fluorescent secondary antibody (Cell Signaling Technology) for 48 h, and then incubated with DAPI (RiboBio). Images were visualized with an Olympus microscope.

2.7 | Xenograft Tumor Mice model

Female BALB/c nude mice (4-5 wk, 16-18 g) were purchased from Animal Laboratory Center of Nanjing University (Nanjing, China), maintained in a specific pathogen-free (SPF) animal laboratory in Jiangsu University, and was approved by the Laboratory Animal Management Committee of Jiangsu University. In total, 5×10^6 MDA-MB-231 cells were subcutaneously injected into the dorsal back. Tumor growth was monitored with mice weight and tumor volume, which was calculated using the formula: $V = ab^2/2$, where "*b*" is the minimal tumor diameter and "*a*" refers to the maximum tumor diameter. Mice were injected with ASO-Linc00839 or NC (50 μL/per) every 2 d. 30 d later, the mice were euthanized and the tissues were weighed, frozen in liquid nitrogen and stored at −80°C.

2.8 | RNA immunoprecipitation assay

Cells were lysed in complete RIP lysis buffer, and subsequently incubated with RIP buffer containing magnetic beads conjugated with anti-Lin28B- and anti-Myc-antibodies overnight at 4°C using the EZ-Magna RIP kit (Millipore). The beads were then washed with washing buffer, and the complexes were incubated with 0.1% SDS/0.5 mg/mL Proteinase K (30 min at 55°C) to remove proteins. RNA purified from the RNA-protein complex was amplified and detected by RT-PCR.

2.9 | Dual-luciferase reporter assay

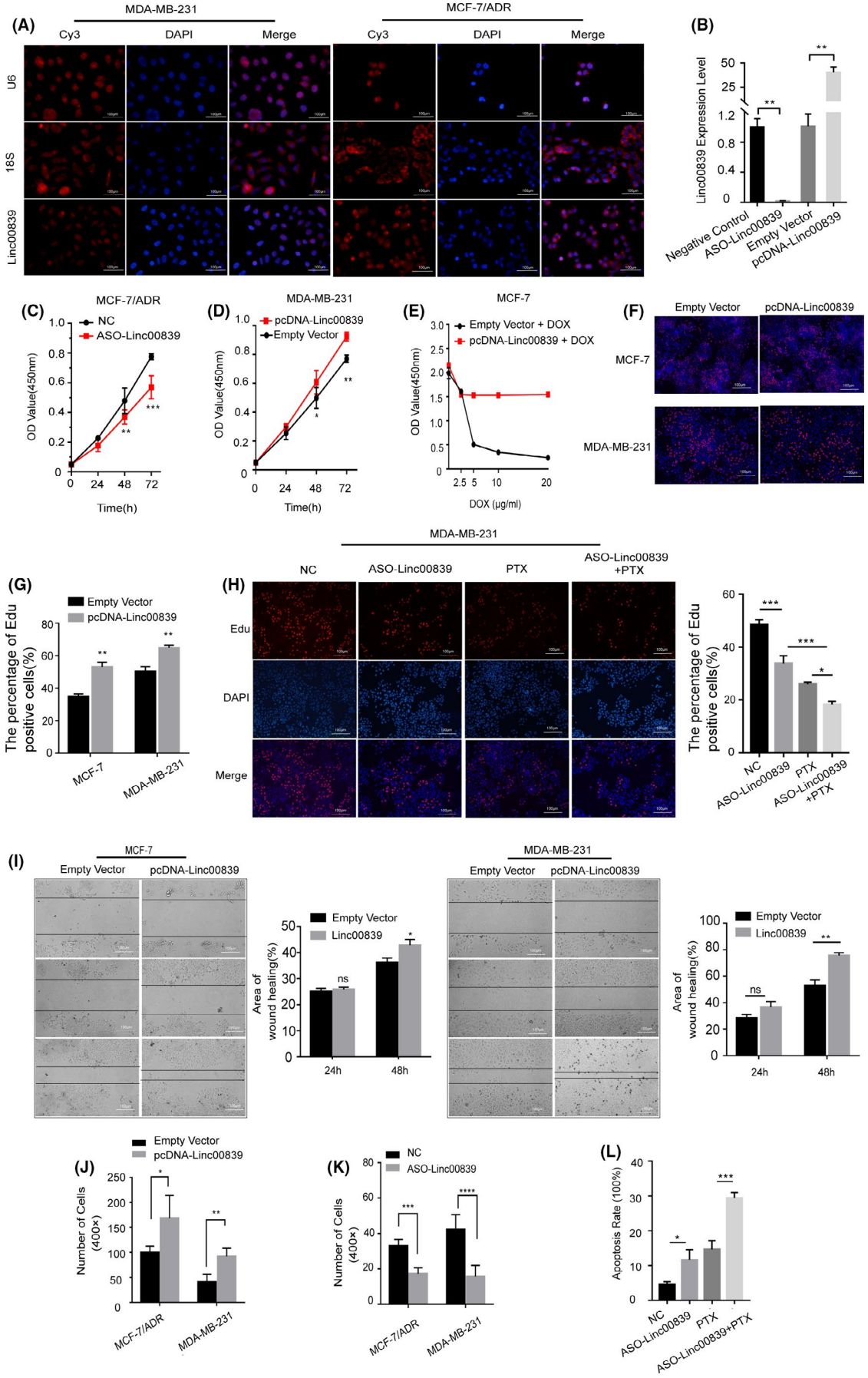
The wide type and the mutation of the promoter segment sequences of Linc00839 were synthesized and inserted into a pGL3-basic vector (Genecreate), respectively. HEK-293T cells were, respectively, plated in 24-well plates at 5×10^3 cells per well 8 h before transfection. The cells were co-transfected with a mixture 2 μg of pGL3-basic-Linc00839 promoter, Renilla, and pcDNA3.1-Myc or control (Dual Luciferase Reporter Gene Assay Kit, Biovision). 48 h later, the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega).

TABLE 1 Associations between Linc00839 expression and clinicopathological features (n = 32)

		Linc00839 expression		*P*-value
	n = 32	High	Low	
Age				
>45	21	11	10	.542
≤45	11	7	4	
Lymph node metastasis				
Yes	18	13	5	.039*
No	14	5	9	
ER				
Positive	15	12	3	.011*
Negative	17	6	11	
PR				
Positive	19	8	11	.051
Negative	13	10	3	
HER2				
Positive	14	6	8	.178
Negative	18	12	6	
TNM stage				
T1/T2	21	15	6	.017*
T3/T4	11	3	8	
Ki-67				
≤10%	18	7	11	.025*
>10%	14	11	3	

Note: P-value was acquired by Pearson chi-square test.
*$P < .05$.

FIGURE 2 Linc00839 knockdown suppressed cell proliferation and increased sensitivity to paclitaxel in vitro. A, MCF-7/ADR and (B) MDA-MB-231 cells were labeled with a lncRNA Linc00839 probe using FISH analysis. The h-U6 and h-18S FISH probe served as markers for nuclear and cytoplasmic gene localization, respectively. Red: Cy3 label showing FISH probe labeled Linc00839, U6, and 18S; Blue: DAPI staining showing nucleus. The h-18S is almost in the cytoplasm, and h-U6 is almost in the nucleus. B, Linc00839-overexpressing MCF-7 cell lines were established by the transfection of pcDNA3.1-Linc00839. MCF-7/ADR cells were infected with ASO, targeting Linc00839 nucleotides to knock down endogenous expression. LincC00839 expression levels were detected by RT-PCR. CCK8 assays were performed to determine the proliferation of MCF-7/ADR (C) and MDA-MB-231 (D) cells after transfection of ASO-Linc00839 or pcDNA-Linc00839 vector. E, CCK8 assay was performed to determine the proliferation of MCF-7 cells after transfection of Linc00839-overexpressed and different concentration (2.5, 5, 10, 20 μg/mL) of doxorubicin for 24 h. F, G, EdU staining after overexpression of Linc00839 in MDA-MB-231 and MCF-7 cells. Original magnification, ×100. Scale bars, 100 μm. **$P < .01$. H, EdU analysis after knockdown of Linc00839 in MDA-MB-231 cells with or without PTX. Original magnification, ×100. Scale bars, 100 μm. I, Wound healing assay in MCF-7 and MDA-MB-231 cells transfected with pcDNA-Linc00839 for 24 and 48 h. Original magnification, ×100. Scale bars, 100 μm. The scratched area of wound healing was measured and calculated as a percentage of the area at 0h. *$P < .05$, **$P < .01$. J, K, Transwell assay for MCF-7/ADR and MDA-MB-231 cells transfected with pcDNA-Linc00839 and ASO-Linc00839, respectively, compared with the control. L, Flow cytometry was performed to assess MDA-MB-231 cells apoptosis in response to transfection with ASO-Linc00839 with or without PTX


(A)
MDA-MB-231
MCF-7/ADR
Cy3
DAPI
Merge
U6
18S
Linc00839
(B)
Linc00839 Expression Level
Negative Control
ASO-Linc00839
Empty Vector
pcDNA-Linc00839
(C)
MCF-7/ADR
NC
ASO-Linc00839
OD Value(450nm)
Time(h)
(D)
MDA-MB-231
pcDNA-Linc00839
Empty Vector
(E)
MCF-7
Empty Vector + DOX
pcDNA-Linc00839 + DOX
DOX (μg/ml)
(F)
Empty Vector
pcDNA-Linc00839
MCF-7
MDA-MB-231
(G)
Empty Vector
pcDNA-Linc00839
The percentage of Edu positive cells(%)
(H)
MDA-MB-231
NC
ASO-Linc00839
PTX
ASO-Linc00839 +PTX
Edu
DAPI
Merge
(I)
MCF-7
MDA-MB-231
Linc00839
Area of wound healing(%)
24h
48h
ns
(J)
Number of Cells (400×)
MCF-7/ADR
(K)
(L)
Apoptosis Rate (100%)
ASO-Linc00839+PTX

2.10 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 software. Student's *t* test was used for comparison between 2 groups. One-way ANOVA followed by Bonferroni correction was used for multiple comparisons. Pearson χ^2 test or Fisher exact test was used to estimate the correlation between the expression and clinicopathologic features. Overall survival was estimated using the Kaplan-Meier method (the log-rank test). A probability value of .05 or less was considered as significance.

Migration and invasion assays and chromatin immunoprecipitation (ChIP) assay are available in Supporting Information (Appendix S1).

3 | RESULTS

3.1 | Identification of dysregulated lncRNAs in resistant BC cells and tissue samples

We observed that 196 lncRNAs were differentially expressed in the BC cell dataset GSE76540, which included normal BC MCF-7 and resistant cancer MCF-7/ADR cells (Table S1). Of the 196 identified lncRNAs in the heatmap, the top 10 differentially expressed lncRNAs were identified (Figure 1A). The sequence alignments were performed by using the NCBI network. Interestingly, sequence analysis of Linc00839, which was one of the most obviously DE-lncRNAs, revealed a poly-A tail, suggesting that it could be considered full length (Table S2). To further explore the common expression pattern of Linc00839 in resistant cancer cell lines, we confirmed that the expression levels of Linc00839 were upregulated in tamoxifen (TAM)-resistant cells compared with TAM-sensitive cells in the GSE67916 dataset (Figure 1B), as well as higher in lapatinib-resistant (SKBR3-R) cell lines compared with parental SKBR3 cell lines in the GSE38376 dataset, but the difference showed no significance unfortunately (Figure 1C). Furthermore, the expression of Linc00839 was much higher in MCF-7/ADR cells than in MCF-7, MDA-MB-231, and BT-549 cells (Figure 1D). Together, these data support that the expression of Linc00839 may have certain effects on chemosensitivity. In addition, we confirmed that BC tissue samples showed higher expression levels of Linc00839 than normal breast tissue in GSE125677 (Figure 1E) or invasive BC tissue (Figure 1F). Experimentally, we investigated the clinical significance of Linc00839 in BC tissue samples collected from BC patients. Consistent with TCGA datasets, Linc00839 expression levels were upregulated in BC tissues compared with adjacent breast tissues (Figure 1G), and high expression of Linc00839 was significantly correlated with lymph

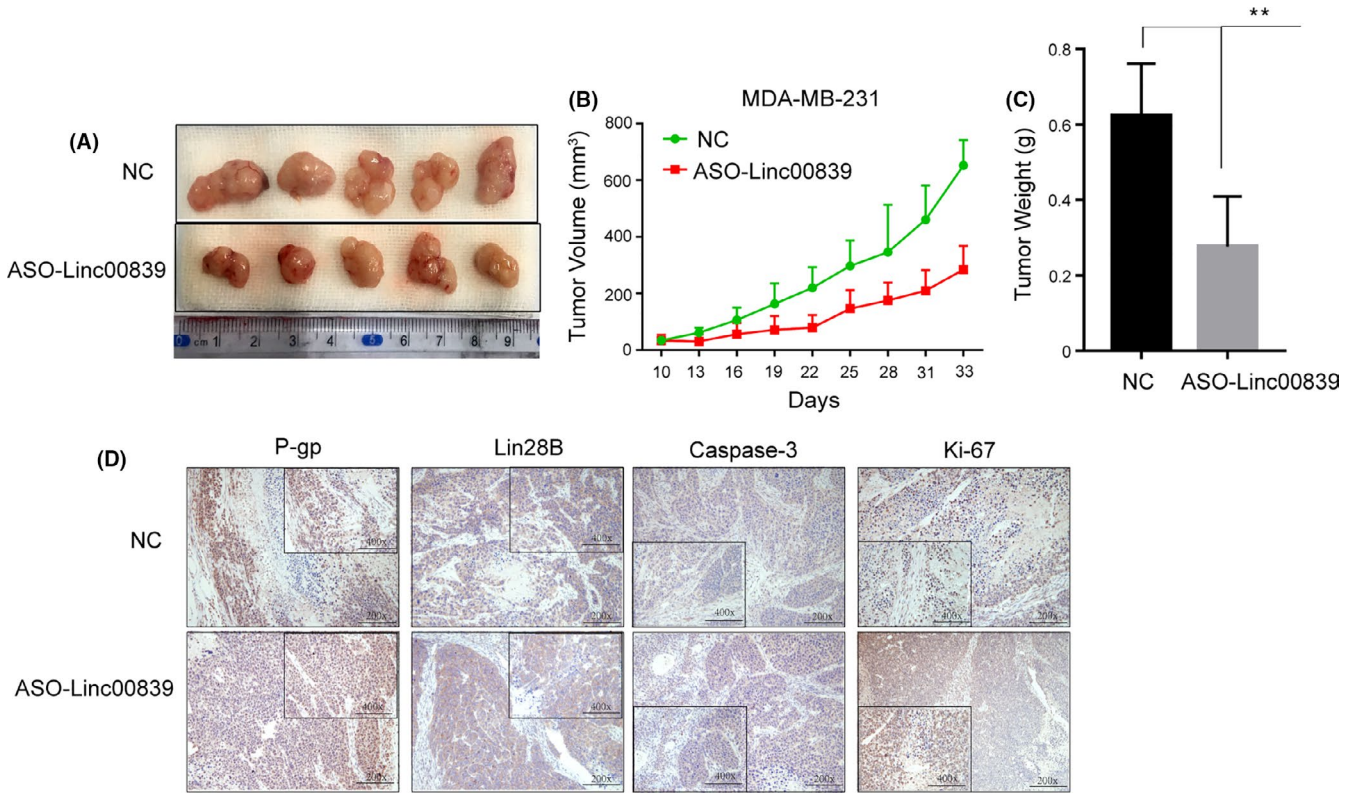


FIGURE 3 Linc00839 knockdown inhibited tumor proliferation in vivo. A, MDA-MB-231 cells were injected into nude mice (n = 5). The mice were treated with negative control or ASO-Linc00839 for 7 times every 2 d. (B) Tumor volume was measured every 3 d (mm). The mice were killed 21 d after injection, and (C) the tumors were excised and weighed. D, The images of IHC for P-gp, Lin28B, caspase-3, and Ki-67 in xenografts were shown. The results are shown as means ± SD in 3 independent experiments. $^*P < .05$, $^{**}P < .01$

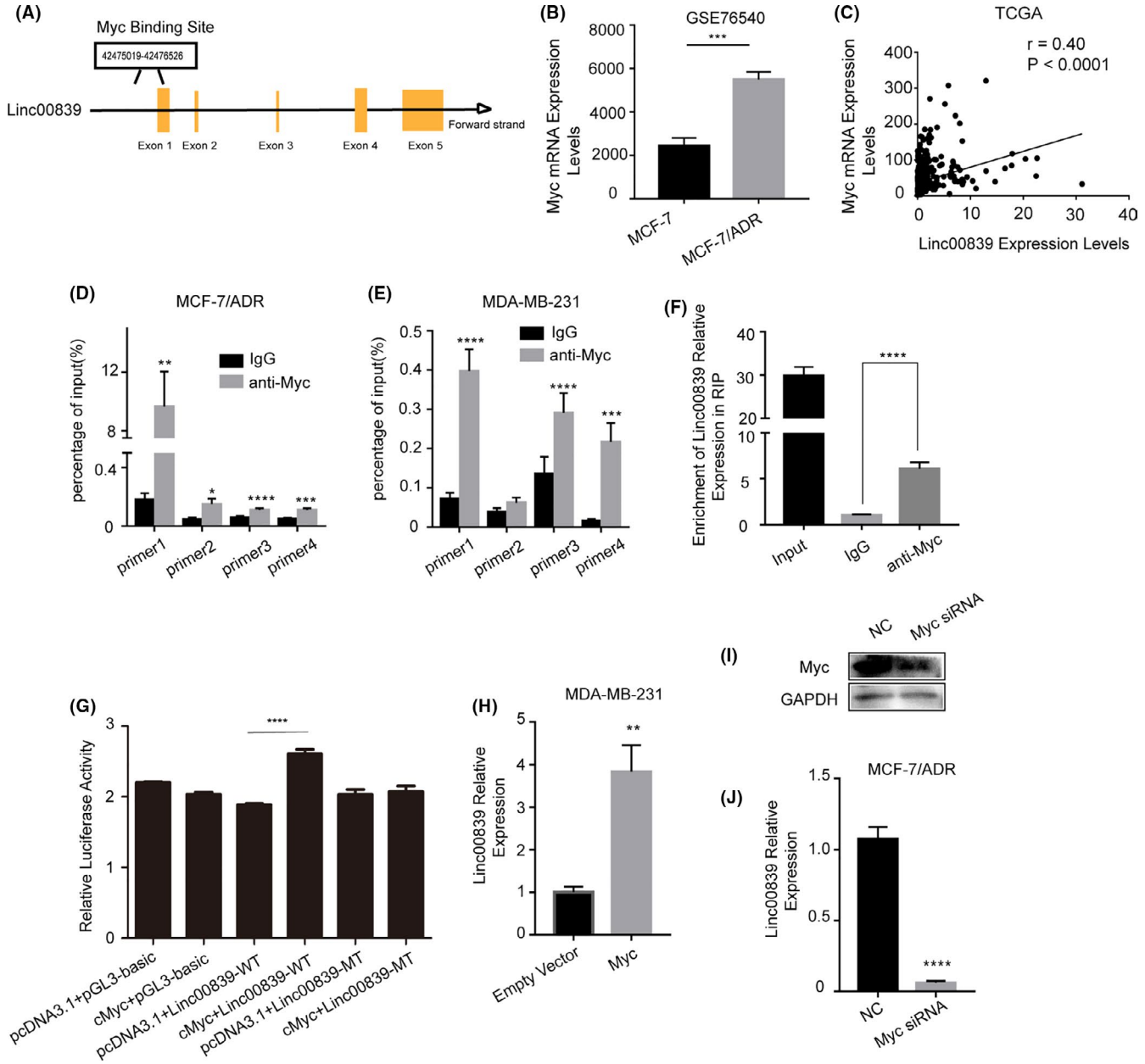


FIGURE 4 Myc activates Linc00839 transcription and regulates its expression level. A, Potential Myc transcription factor-binding sites with Linc00839 were analyzed by ChIPBase datasets. B, The expression level of Myc mRNA was determined in GSE76540 dataset. C, Correlation analysis between Linc00839 and Myc mRNA level in 1212 BC patients in the TCGA database. D, The binding of Myc to the promoter region of the Linc00839 was determined by a ChIP assay in MCF-7/ADR cells and MDA-MB-231 cells (E). The normal rabbit IgG was used as a negative control. F, RIP analysis of Linc00839 enriched by Myc antibody in MCF-7/ADR cells. IgG antibody served as negative controls. G, Dual luciferase assays on MDA-MB-231 cells co-transfected with firefly luciferase constructs containing the Linc00839 promoters and vectors or Myc plasmid. H, Expression level of Linc00839 was determined using RT-PCR in MDA-MB-231 cells treated with Myc plasmid vector. I, Western blot was used to evaluate the myc protein level after Myc siRNA treatment. J, The expression level of Linc00839 in MCF-7/ADR after Myc siRNA treatment. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

node metastasis, ER, TNM stage and Ki67 levels (Table 1). For the overall survival analysis, high expression of Linc00839 was related to a lower overall survival rate (Figure 1H), especially in the patients with who were lymph node positive (Figure 1I). Taken together, Linc00839 expression correlated with chemoresistance and was considered an independent prognostic factor associated with poor outcome in BC.

3.2 | Linc00839 regulates proliferation and chemoresistance in vitro

The FISH assay was performed to analyze the subcellular distribution of Linc00839, and showed that Linc00839 was predominately localized in the nucleus of MCF-7/ADR and MDA-MB-231 cells (Figure 2A), suggesting that Linc00839 mainly functioned through transcriptional regulation.

Respectively, it was validated that the expression of Linc00839 was decreased in MCF-7/ADR cells using ASOs, while the expression was increased after transfection with pcDNA-Linc00839 (Figure 2B). Linc00839 knockdown reduced cell proliferation in a time-dependent manner in MCF-7/ADR cells (Figure 2C), while ov-Linc00839 caused an induction of cells proliferation both in MDA-MB-231 and MCF-7 (Figure 2D). In addition, Linc00839 overexpression increased cell survival after cotreatment with DOX (Figure 2E). Edu assay also suggested that overexpression of Linc00839 increased proliferation in MCF-7 and MDA-MB-231 cells (Figure 2F,G). We also found that Linc00839 downregulation could increase MDA-MB-231 cell sensitivity to PTX, and the cell proliferation was significantly lower in the ASO cotreated group than in the ASO or PTX alone groups (Figure 2H). Furthermore, Linc00839 overexpression promoted cell migration and invasion (Figure 2I,J and Figure S1C), while Linc00839 knockdown significantly inhibited the migration and invasion of MDA-MB-231 and MCF-7/ADR cells (Figures S1A-C and 2K). Next, Linc00839 knockdown increased apoptosis rate of MDA-MB-231 cells induced by PTX (Figures 2L and S1D). Accordingly, all these investigations indicate that Linc00839 can promote cell proliferation and mediate chemoresistance in vitro.

3.3 | Knockdown of Linc00839 inhibits transplantation tumor development

To further investigate whether decreasing Linc00839 would inhibit tumor development in vivo, we used a xenograft mouse model. As expected, the ASO-Linc00839 group showed significantly reduced tumor volume and tumor weight compared with those in the controls (Figure 3A-C). Consistently, Ki67, P-gp, and Lin28B expression was lower, while caspase-3 expression was higher in tumor tissues derived from the ASO-Linc00839 group than in those derived from the control group (Figure 3D). These findings suggest that Linc00839 has an important role in BC tumor development in vivo.

3.4 | Myc activates Linc00839 transcription and promotes its expression level

The potential transcription factors of Linc00839 were predicted by the ChIPBase database (http://rna.sysu.edu.cn/chipbase/), and we determined that the Myc-binding site was located at chr10:42475019-42476526 downstream of Linc00839 (Figure 4A). In addition, the Myc mRNA expression level was higher in chemoresistant MCF-7/ADR cells than in MCF-7 cells from the GSE76540 dataset (Figure 4B). In detail, we analyzed the data from the TCGA database, the results showed that the Myc mRNA expression level was positively correlated with Linc00839 in BC tissues (Figure 4C). To further verify the predicted information from ChIPBase, we performed a ChIP assay and found that the amount of immunoprecipitated DNA from the Linc00839 promoter increased, indicating that Myc directly binds to regions of the Linc00839 promoter (Figure 4D,E). Meanwhile, we performed a RIP assay to verify the interaction between Linc00839 and Myc protein in MCF-7/ADR cells, and an enriched Linc00839 was identified by anti-Myc antibody in contrast to anti-IgG (Figure 4F). To determine whether Linc00839 is a direct transcriptional target of Myc, luciferase reporter assay was performed and Myc expression increased the luciferase activity of the wild-type Linc00839 reporter vector, but not that of the mutant reporter vector (Figure 4G). Importantly, we found that Myc overexpression caused positive upregulation of Linc00839 in MDA-MB-231 cells (Figure 4H), while the Myc protein level was decreased by Myc siRNA (Figure 4I), and the expression of Linc00839 was markedly downregulated (Figure 4J) in MCF-7/ADR cells. Our results indicated that there exists transcriptional regulation between Myc and Linc00839. Taken together, the results suggested that Myc was a directly transcriptional factor of Linc00839.

3.5 | Linc00839 regulates Lin28B and Myc expression

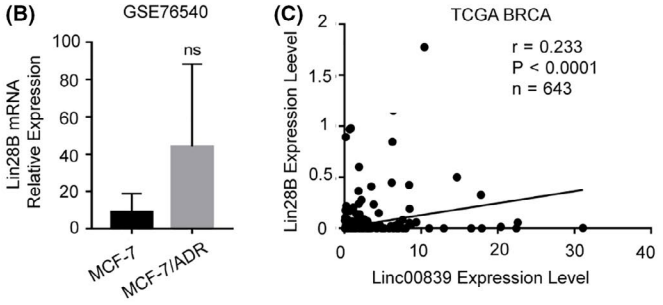
Studies have shown that aberrantly expressed lncRNAs exert pleiotropic effects on mRNAs and proteins by altering their expression or stability, as well as through exerting effects on protein structure or interaction with transcriptional regulators.[17,18,23] Using the Starbase database (http://starbase.sysu.edu.cn/starbase2/index.php),[24] we found that Linc00839 potentially targeted Lin28B, and the target location was chr10:42972740-42972763. Studies have shown that Lin28B mainly localizes to the nucleus and acts as an oncogene in human cancer.[25] Based on the sequence analysis, the RPISeq (http://pridb.gdcb.

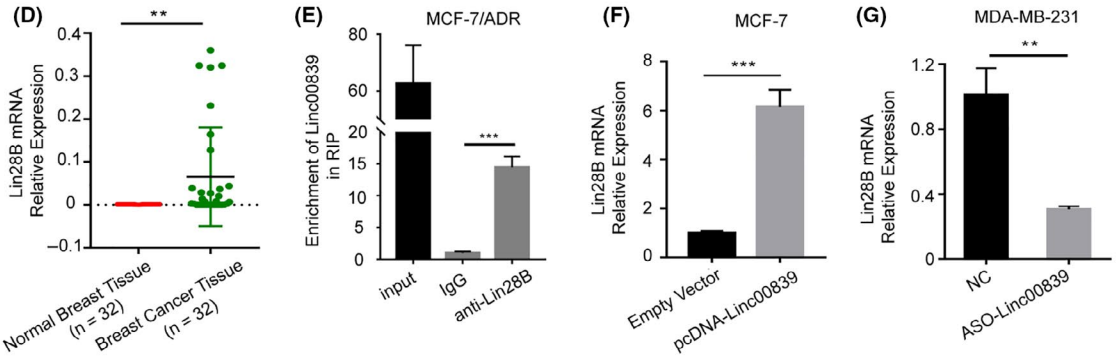
FIGURE 5 Linc00839 regulates Lin28B and Myc mRNA and protein expression. A, The prediction of the interaction probabilities of Linc00839 with Myc and Lin28B by RPISeq. Predictions with probabilities >0.5 were considered "positive," indicating that the corresponding RNA and protein are likely to interact with each other. B, Lin28B expression level in the dataset GSE76540. C, Correlation analysis between Linc00839 and Lin28B expression level in TCGA dataset, which contains 643 invasive breast cancer (BC) patients. D, Lin28B expression levels in 32 BC tissues and adjacent breast tissues. These tissue samples were selected from Fourth Affiliated Hospital of Jiangsu University. E, RIP analysis was performed in MCF-7/ADR cells, the co-precipitated RNA Linc00839 was subjected to qRT-PCR analysis. The fold enrichment of Linc00839 after Lin28B-pull down is relative to IgG control. F, The Lin28B mRNA expression level after pcDNA-Linc00839 transfection in MCF-7 cells and (G) ASO-Linc00839 in MDA-MB-231 cells. H, The expression level of Lin28B protein was detected after Linc00839 knockdown by immunofluorescence staining. I, The protein levels of Lin28B and Myc were detected after Linc00839 knockdown using western blot assay. J, Lysates from MCF-7/ADR cells were incubated with anti-Myc or anti-Lin28B. The protein levels of Myc and Lin28B were validated by western blot analysis with the indicated antibodies. K, FISH analysis and IF analysis were performed to analyze the colocalization of Linc00839 and Myc, and the expression level of Myc after ov- or aso-Linc00839. Results are shown as the mean ± SD based on 3 independent experiments. $^{*}P < .05$, $^{**}P < .01$

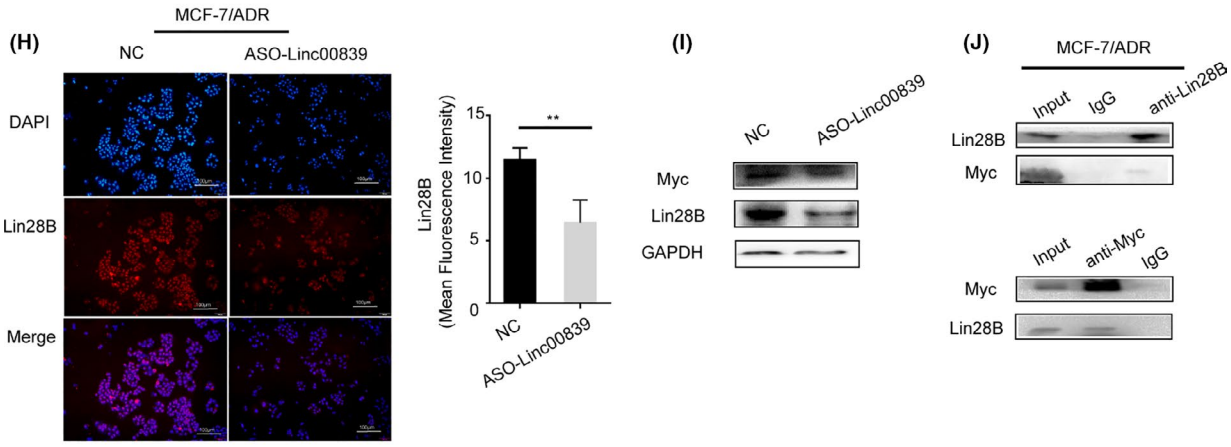
(A)

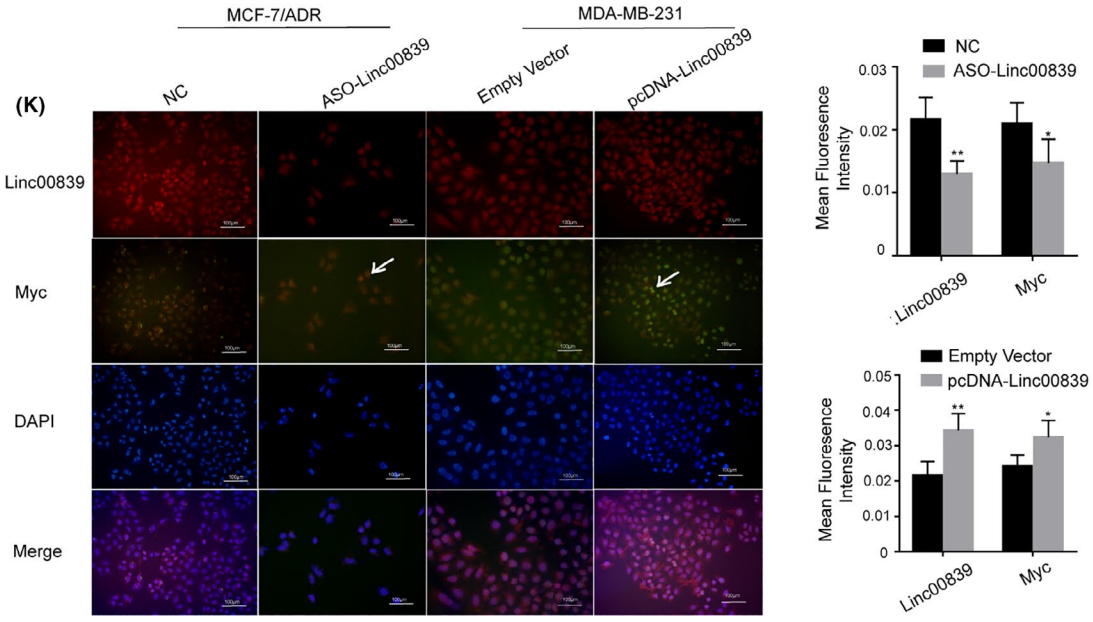
LncRNA-Protein Interaction Prediction

Protein	RF Classifier	SVM Classifier
Lin28B	0.75	0.6
Myc	0.7	0.71









iastate.edu/RPISeq/) was performed to evaluate the scores between Linc00839, Lin28B, and Myc. Both of the scores for the RF Classifier and SVM Classifier are, respectively, more than 0.5 (Figure 5A), indicating that Linc00839 is likely to bind with Lin28B and Myc protein. Furthermore, the expression level of Lin28B was higher in MCF-7/ADR cells than in MCF-7 cells (Figure 5B). Although there was no significant difference between the 2 groups, there was a positive correlation between Lin28B mRNA level and Linc00839 level in BC tissue samples from the TCGA (Figure 5C). Furthermore, Lin28B expression was much higher in BC tissues than in adjacent normal tissues (Figure 5D). The RIP assay showed that the enrichment of Linc00839 in the anti-Lin28B group compared with the anti-IgG group (Figure 5E). Importantly, Linc00839 overexpression induced upregulation of Lin28B mRNA (Figure 5F), while Linc00839 downregulation caused decreased expression of Lin28B mRNA and protein (Figure 5G-I).

Lin28B is a validated target of c-Myc transcriptional regulation and enhances c-Myc expression in BC patients.[26,27] Consistently, the levels of c-Myc and Lin28B were significantly enriched in the anti-Lin28B group and anti-c-Myc group, respectively (Figure 5J). Additionally, we performed FISH combined with immunofluorescence analysis, and the results revealed colocalization of c-Myc and Linc00839. The expression level of c-Myc was significantly decreased after Linc00839 knockdown, while the level of c-Myc in the nucleus of MDA-MB-231 cells was brightly enriched after Linc00839 overexpression (Figure 5K). Therefore, we concluded that Linc00839 induced Lin28B expression and nuclear Myc expression in BC cells.

3.6 | Linc00839 activates the PI3K/AKT signaling pathways

Gene set enrichment analysis (GESA) showed that upregulation of Linc00839 in TCGA datasets might be related to different signaling pathways in cancer, the apoptosis pathway and the Jak-STAT3 signaling pathway (Figure 6A). Recent studies have proven that the MAPK/AKT signaling pathway functions as a crucial regulator in cancer cell apoptosis and chemoresistant processes.[28-30] Therefore, we assessed the activation status of the MAPK/AKT pathways regulated by Linc00839. Interestingly, upon Linc00839 downregulation, the expression levels of p-AKT, p-STAT3, p-P38, and Bcl-2 were apparently decreased, while the expression levels of p-ERK1/2, cleaved caspase-3 and Bax were correspondingly increased (Figure 6B). In contrast, Linc00839 overexpression enhanced the expression of p-AKT, p-P38, p-STAT3, and Bcl-2, while p-ERK1/2, cleaved caspase-3 and Bax expression were decreased (Figure 6B). These results indicated that Linc00839 might promote apoptotic progression through the activation of PI3K/AKT signaling pathways.

Ly294002, a PI3K/AKT signaling pathway inhibitor, was applied to validate the gain/loss of function Linc00839-mediated migration, invasion, and proliferation of BC cell lines. Furthermore, inhibition of the PI3K/AKT signaling pathway in Linc00839-overexpressing cells not only reversed the Linc00839-enhanced cell invasion (Figure 6C), but also decreased the cell proliferation induced by Linc00839 (Figure 6D,E). To investigate whether Linc00839-activated PI3K/AKT signaling pathway contributes to BC chemoresistance, under the effect of different concentration of doxorubicin, cell viability was increased after overexpression of Linc00839, and then showed a significantly decrease after the addition of Ly294002. The results showed that overexpression of Linc00839 inducing resistance to doxorubicin could be reversed by Ly294002 (Figure 6F). Additionally, Ly294002 decreased the phosphorylation of AKT and the Linc00839-induced decrease in caspase-3 expression, but reversed Linc00839-induced increase in Bcl-2 expression (Figure 6G). Together, these results suggested that Linc00839 promoted BC cell migration, invasion, and chemoresistance through the PI3K/AKT signaling pathways.

4 | DISCUSSION

Dysregulation of lncRNAs, revealed by re-annotation and analysis of microarray data, has been successfully reported in different types of cancer, and plays a crucial role in tumor development, invasion, and metastasis.[9,31-33] In our study, we screened a novel lncRNA, Linc00839, using microarray datasets, and we explored its potential functions in the regulation of proliferation, invasion, and chemoresistance in BC for the first time. According to our clinical characteristics analysis, we confirmed that high level of Linc00839 was significantly correlated with lymph node metastasis, ER, TNM stage, and Ki67 levels, and that a high expression level mediated a poor prognosis, especially in patients with lymph node metastasis, suggesting that it might serve as an oncogenic and prognostic biomarker. Furthermore, we found that Linc00839 expression was especially increased in chemoresistant cells. Linc00839 overexpression markedly promoted cell proliferation, invasion, and chemoresistance in vitro, while knockdown of Linc00839 inhibited transplanted tumor development in vivo. FISH analysis showed that Linc00839 was primarily located in the nucleus. These results have called for us to explore the underlying mechanism by which Linc00839 modulates BC progression.

To explore the potential mechanism of Linc00839, we identified that Myc could directly interact with the promoter and activate the transcription of Linc00839. Transcriptional dysregulation of Myc is among the most frequent events in aggressive tumor cells,[34] endowing cells with physiological changes that have the potential to feedback on RNA production,[35] regulating cell proliferation, migration, apoptosis, and activating vital signaling pathways.[33,36,37] Studies have shown that lncRNAs may function as vital components of the Myc transcriptional network,[37] adding crucial additional layers to the regulation of Myc and its downstream effects.[38] MycLos (Myc-regulated lncRNAs) functions in cell proliferation and the cell cycle by regulating Myc target genes, such as CDKN1A and CDKN2B.[13] Subcellular localization analysis indicated that Linc00839 may participate in regulating

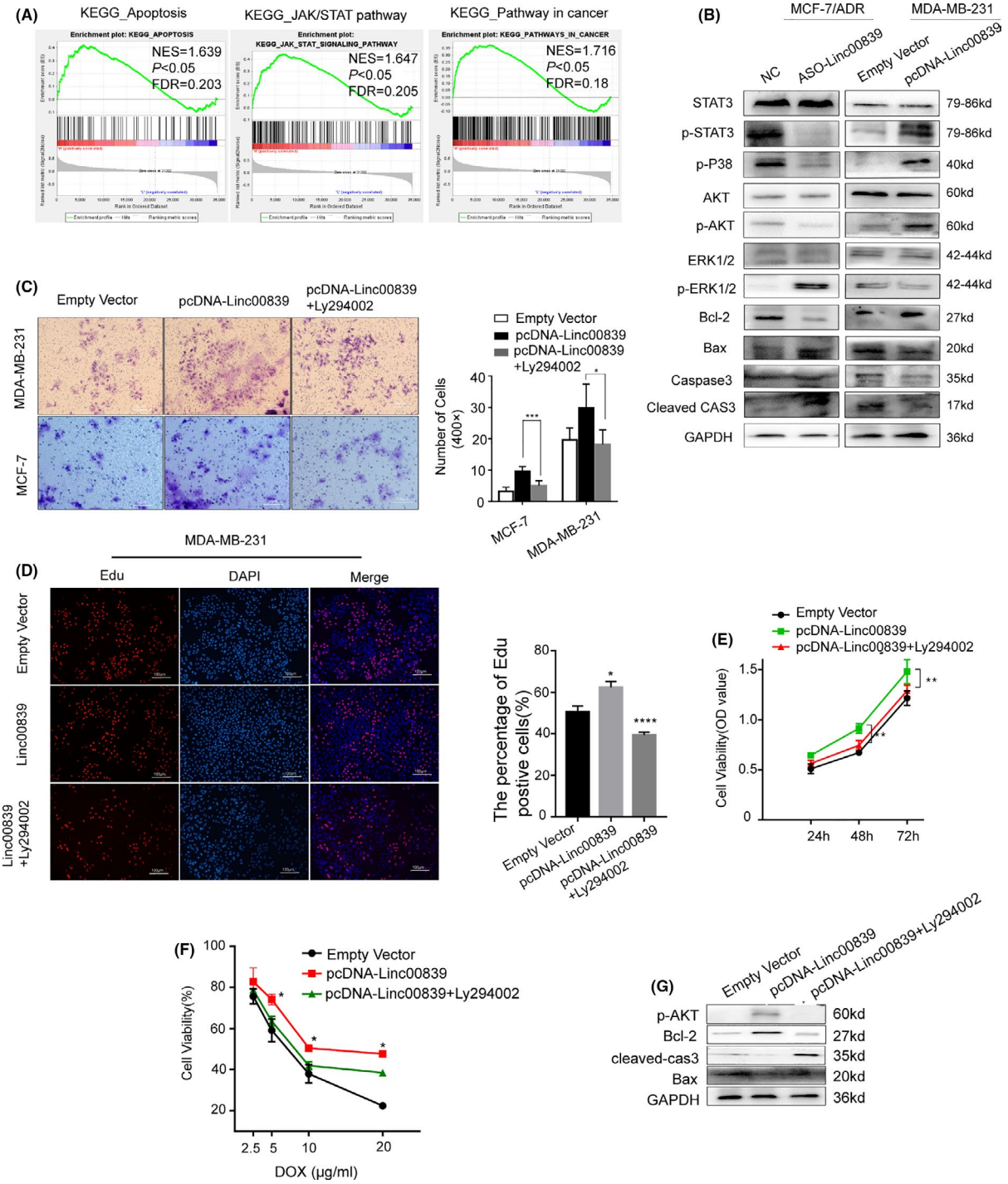


FIGURE 6 Linc00839 promotes cell invasion and proliferation via PI3K/AKT Signaling Pathways. A, From 1212 invasive breast cancer patients of the TCGA database, GSEA analysis showed the enrichment of key signaling pathways that dysregulated Linc00839 was involved in. B, Western blot analysis was performed to evaluate the phosphorylation of STAT3, P38, AKT and ERK1/2, and Bcl-2, caspase-3 antibodies after transfection of aso-Linc00839 and Linc00839 plasmid after 48 h in MDA-MB-231 and MCF-7/ADR cells. C, MDA-MB-231 and MCF-7 cell were transfected with pcDNA-Linc00839 plasmid, and treated with Ly294002 (10 μm) for 48 h for transwell assay. D, MDA-MB-231 cells were transfected with pcDNA-Linc00839 plasmid, and treated with Ly294002 (10 μm) for 24 h for Edu assay. E, MDA-MB-231 cells were transfected with pcDNA-Linc00839 plasmid, and then treated with Ly294002 (10 μm) for 24 h, 48 h and 72 h for CCK8 assay. (F) MCF-7 cells were transfected with pcDNA-Linc00839 plasmid, and then treated with a series dose (2.5, 5, 10, 20 μg/mL) of doxorubicin with or without Ly294002 (10 μm) for 48 h. G, MDA-MB-231 cells were transfected with pcDNA-Linc00839 plasmid, and then treated with Ly294002 (10 μm) for 48 h. Western blot assay was performed using p-AKT, Bcl-2, caspase-3, and Bax antibodies. Data are presented as mean ± SD for 3 independent experiments. $^{*}P < .05$, $^{**}P < .01$

nuclear transcription and posttranscriptional gene expression. Surprisingly, using bioinformatics tools, RPISeq, RIP, and luciferase reporter assays, we confirmed that Myc transcriptionally activated Linc00839 expression and modulated Myc protein expression levels. Therefore, the regulatory effect of Myc on lncRNAs opens up new insight into the lncRNA effect on tumorigenesis. For instance, lncRNA-MIF is directly induced by c-Myc, subsequently leading to c-Myc degradation and tumor inhibition.[39] Low expression levels of lncRNA MYMLR have been identified and experimentally shown to maintain Myc transcriptional activity and cell cycle progression.[40]

Previous studies have reported that RBPs could interact with lncRNAs, influencing the expression of mRNA at the posttranscriptional level.[19,20] LncRNA BCAR4 recruits the PNUTS to acetylated H3K18, resulting in the activation of GL12 target genes.[21] The RBP Lin28B exhibits upregulated expression in various cancer cells, and is involved in carcinogenesis and metastasis.[17,41] Here, we demonstrated that Lin28B expression was increased in BC tissues and shared positive relationships with Linc00839. Using RIP and co-IP assays, we confirmed that Lin28B can directly bind to Linc00839, and that the Lin28B expression level was also regulated by Linc00839 overexpression or knockdown. Studies have shown that pancreatic carcinoma with high expression of Lin28B are strongly enriched for the expression of Myc targets.[42] Importantly, Lin28B enhances AKT phosphorylation and nuclear translocation of Myc.[43] Our results suggest that Lin28B acts as an oncogene due to its effect on BC cells by targeting Myc through the PI3K/AKT pathway. Therefore, our study demonstrates another point: Myc transcriptionally activates Linc00839, which simultaneously binds to Lin28B, leading to positive regulation of Myc and Lin28B protein.

Cell apoptosis and Jak/STAT3 signaling pathways are significantly enriched in response to Linc00839 alteration in BC patients. Indeed, many lncRNAs have been identified to function in these pathways, such as TSLNC8, suppresses metastasis by inactivating the IL-6/STAT3 signaling pathway.[44] HOXD-AS1 inhibits colorectal cell growth and metastasis through inactivation of the MAPK/AKT signaling pathway.[8] Furthermore, we proved that Linc00839 overexpression could activate the phosphorylation of Akt, STAT3, and P38, but the expression level of p-ERK1/2 was inactivated. There must be another underlying mechanism in regulation of the MAPK signaling pathway. Here, our study confirmed that Linc00839 regulated the PI3K/AKT pathway, and that the inhibition of PI3K/AKT signaling was required for BC cell proliferation, apoptosis, and invasion by Linc00839. Based on our work, it is reasonable to conclude that Linc00839 promotes BC proliferation and chemoresistance via Lin28B-mediated upregulation of Myc and activation of the PI3K/AKT pathway.

In conclusion, we first characterized that upregulation of Linc00839 was specifically mediated by an oncogene, Myc. We confirmed a feedback loop involving Myc-activated Linc00839 and Lin28B that modulates Myc expression and activity of the PI3K/AKT pathway, thus regulating BC cell proliferation, invasion, and chemoresistance. Although more clinical samples and further investigations of the exact binding site of Linc00839 with Myc and Lin28B are required. Based on these findings, the proposed a Myc/Linc00839/Lin28B feedback loop provides a better understanding of BC proliferation and chemoresistance, and may be a potential target for the BC therapy.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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