PBX3-activated DLG1-AS1 can promote the proliferation, invasion, and migration of TNBC cells by sponging miR-16-5p

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INTRODUCTION

As one of the most malignant cancers in women, breast cancer has caused much death worldwide.1-3 Triple-negative breast cancer (TNBC) cases account for nearly 13%-18% of breast cancer cases and have a worse prognosis than other subtypes of breast cancer.4 Recently, TNBC has been intensely investigated, because it may lead to crucial clinical problems. TNBC has an aggressive nature. Patients with TNBC are relatively young, but they are usually diagnosed at the advanced stage, or even worse, they are more likely to have tumor metastasis, causing death.5,6 Therefore, we need to dig into the molecular mechanisms of TNBC and try to find a new and effective therapeutic target.

Discs large MAGUK scaffold protein 1 antisense RNA 1 (DLG1-AS1) has been studied in a previous study, where it was found that it can act as an oncogene in cervical cancer (CC). More specifically, DLG1-AS1 can promote the proliferation of CC cells.17 Moreover, DLG1-AS1 has been shown to facilitate TNBC cell proliferation by down-regulating miR-203.18 Nevertheless, the role of DLG1-AS1 in TNBC cell migration and invasion had not been well explored. Therefore, this study focused on functions of DLG1-AS1 in TNBC cells. Further, Pre-B-cell leukemia homeobox 3 (PBX3) has been reported to have high expression in CC cells, which is associated with the poor overall survival of CC patients, and it promotes the growth of CC cells.19 PBX3 has also been revealed to be remarkably up-regulated in letrozole-resistant breast cancer (BC) cells and tissues and is linked to relatively unfavorable progression-free survival of BC patients.20 In this study, we attempted to test our speculation about the influence of DLG1-AS1 and PBX3 on TNBC cell growth. The potential mechanism of DLG1-AS1 was also investigated in this study.

DLG1-AS1 and PBX3 have been identified as acting as an oncogene in cervical cancer. However, they have not been well explored in triple-negative breast cancer (TNBC). As TNBC is one of the malignancies causing increasing death throughout the world, this study aimed to probe into the regulatory relationship between DLG1-AS1 and PBX3 in TNBC cells. In this study, real-time quantitative PCR (qRT-PCR) and western blot experiments were conducted to investigate the RNA and protein levels of genes of interest on TNBC cells. Functional experiments were implemented, such as 5-ethynyl-2'-deoxyuridine (EdU), transwell, and wound healing assays, to assess the changes in TNBC cell phenotype. Chromatin immunoprecipitation, luciferase reporter, RNA binding protein immunoprecipitation, and RNA pull-down assays were conducted to investigate the binding relationships among subject genes. The results show that DLG1-AS1 and PBX3 displayed high expression in TNBC cells, and PBX3 worked as the transcriptional activator of DLG1-AS1. Also, DLG1-AS1 served as an oncogene in TNBC cells and as a sponge for miR-16-5p to up-regulate JARID2. Meanwhile, JARID2 and PBX3 exerted oncogenic effects on TNBC cell growth. In conclusion, PBX3-activated DLG1-AS1 can promote the proliferation, invasion, and migration of TNBC cells by sponging miR-16-5p and elevating JARID2 expression.
RESULTS

PBX3 can act as the transcriptional activator of DLG1-AS1

At first, we detected the expression of DLG1-AS1 and PBX3 in normal human breast epithelial cells and TNBC cells. The data of qRT-PCR and western blot assays demonstrated that DLG1-AS1 and PBX3 were highly expressed in TNBC cells, especially in MDA-MB-231 and MDA-MB-468 cells (Figures 1A and 1B). Meanwhile, according to the prediction of UCSC (http://genome.ucsc.edu/), we found that PBX3 could act as a transcriptional activator of DLG1-AS1, which indicated that DLG1-AS1 and PBX3 may interact with each other in TNBC cells. Therefore, we tested the overexpression or knockdown efficiency of pcDNA3.1-PBX3 or sh-PBX3#1/2 via qRT-PCR and western blot assays. And the results showed PBX3 could be successfully up-regulated or down-regulated by pcDNA3.1-PBX3 or sh-PBX3#1/2 (Figure 1C). Next, we used qRT-PCR to investigate the effect of reduced or overexpressed PBX3 on the expression of DLG1-AS1 in TNBC cells. The results showed that inhibited PBX3 decreased the expression of DLG1-AS1, and overexpressed PBX3 increased the expression of DLG1-AS1 (Figure 1D). PBX3 has been identified as a transcription factor that regulates gene expression in cancers.21 Hence, we presumed PBX3 might modulate DLG1-AS1 as a transcriptional factor. We then utilized the JASPAR website (http://jaspar.genereg.net/) and found that there was a binding site between PBX3 and DLG1-AS1. We present the DNA motif of PBX3 as well as the binding site between DLG1-AS1 and PBX3 in Figures 1E and 1F. Chromatin immunoprecipitation (ChIP) assay was implemented to prove the binding relationship of DLG1-AS1 and PBX3. We found that they could bind to each other since the DLG1-AS1 promoter could be remarkably enriched in anti-PBX3 (Figure 1G). Moreover, the luciferase assay was performed to verify the function of the predicted binding site. And it was revealed that the binding site could exert its function, as the luciferase activity of DLG1-AS1-Wt rather than DLG1-AS1-Mut was greatly affected by PBX3 augmentation or knockdown (Figure 1H). Hence, we could conclude that DLG1-AS1 and PBX3 were highly expressed in TNBC cells and PBX3 acted as the transcriptional activator of DLG1-AS1.

DLG1-AS1 could act as an oncogene and a sponge for miR-16-5p in TNBC cells

In this section, we studied the role that DLG1-AS1 played in TNBC cells. First, we tested the interference efficiency of sh-DLG1-AS1#1/2 shown in Figure 2A. And then, we conducted functional assays to reveal the possible influence DLG1-AS1 on TNBC cells. According to the results of a 5-ethyl-2'-deoxyuridine (EdU) assay, we find that the growth of MDA-MB-231 and MDA-MB-468 was highly impeded after transfection with sh-DLG1-AS1#1/2 (Figure 2B). Also, we performed transwell and wound healing assays to investigate the invasion and migration of TNBC cells. And the results showed that DLG1-AS1 knockdown inhibited the invasion and migration of TNBC cells greatly (Figures 2C and 2D). Afterward, the location of DLG1-AS1 in cells was tested by fluorescence in situ hybridization (FISH) and subcellular fraction assays. As shown in Figures 2E and 2F, DLG1-AS1 was mainly distributed in the cell cytoplasm. Cytoplasmic lncRNA has been shown to function as a competing endogenous RNA (ceRNA) to regulate the miRNA/mRNA axis in cancer cells.22 So, we searched on the starBase database (http://starbase.sysu.edu.cn/) with no specific conditions chosen, and nine potential miRNAs likely binding with DLG1-AS1 were screened out. Based on the qRT-PCR data, we found that, among these candidate miRNAs, only miR-16-5p was expressed less in TNBC cells compared with normal cells (Figure 2G). And there is evidence proving that miR-16-5p can act as a tumor suppressor.23 So miR-16-5p was finally chosen. Next, we present the binding site between DLG1-AS1 and miR-16-5p (Figure 2H). We confirmed the high overexpression efficiency of miR-16-5p mimics via qRT-PCR assay (Figure 2I). For testing the binding relationship between DLG1-AS1 and miR-16-5p, we implemented luciferase reporter assays and RNA pull-down assays. Results showed that the luciferase activity of DLG1-AS1-Wt was noticeably decreased in BC cells transfected with miR-16-5p mimics, which indicated that miR-16-5p could bind to DLG1-AS1 (Figure 2J). And RNA pull-down assays gave the same conclusion that DLG1-AS1 could be overtly enriched in Bio-miR-16-5p-Wt (Figure 2K). In conclusion, DLG1-AS1 acted as an oncogene in TNBC cells and as a sponge for miR-16-5p.

JARID2 and PBX3 can act as an oncogene in TNBC cells

At first, we used a Venn diagram to present the five miRNAs we predicted from starBase (Figure 3A). Then, the expression of mRNAs in TNBC cells (BT-549, MDA-MB-231, and MDA-MB-468) was investigated. And we found that among the five mRNAs, JARID2 was the only one to be highly expressed in TNBC cells (Figure 3B). Also, the binding site between miR-16-5p and JARID2 is presented (Figure 3C). Then, we used luciferase reporter assays to prove their binding relationship, and the results showed that they could bind with each other (Figure 3D). Further, we conducted RNA-binding-protein immunoprecipitation ( RIP) assay and found that DLG1-AS1, miR-16-5p, and JARID2 were chosen. Next, we present the binding site between DLG1-AS1 and JARID2. The following results of RNA pull-down assay demonstrated that more JARID2 was enriched in Bio-miR-16-5p-Wt (Figure 3E). In conclusion, DLG1-AS1 could act as an oncogene in TNBC cells and as a sponge for miR-16-5p.
Figure 1. DLG1-AS1 and PBX3 are highly expressed in TNBC cells and PBX3 acts as the transcriptional activator of DLG1-AS1

(A and B) The expression levels of DLG1-AS1 (A) as well as PBX3 (B) were examined by qRT-PCR, and the protein level of PBX3 was detected through western blot assay in normal cells (MCF-10A) and TNBC cells (BT-549, MDA-MB-231, and MDA-MB-468). (C) The efficiency of PBX3 overexpression and knockdown was tested by qRT-PCR and western blot assays in MDA-MB-231 and MDA-MB-468 cells. (D) qRT-PCR was used to investigate the effect on the expression of DLG1-AS1 caused by inhibited and overexpressed PBX3. (E and F) The DNA motif of PBX3 (E) and the binding site between the DLG1-AS1 promoter and PBX3 (F) are presented with the data recorded from JASPAR. (G) ChIP assay was used to prove that PBX3 can bind to DLG1-AS1. (H) The activity of the binding site of PBX3 was examined by luciferase reporter assays. *p < 0.05, **p < 0.01. The data were measured using mean ± SD.
Figure 2. DLG1-AS1 acts as an oncogene in TNBC cells and as a sponge for miR-16-5p

(A) The interference efficiency of DLG1-AS1 was tested by qRT-PCR. (B) EdU experiments were implemented to test the influence of inhibited DLG1-AS1 (sh-DLG1-AS1#1 and sh-DLG1-AS1#2) on the proliferation of TNBC cells (MDA-MB-231 and MDA-MB-468). (C) Transwell assays were conducted to study the invasion situation of MDA-MB-231 and MDA-MB-468 cells affected by sh-DLG1-AS1#1 or sh-DLG1-AS1#2. (D) The migration situation of MDA-MB-231 and MDA-MB-468 cells transfected with sh-DLG1-AS1#1 or sh-DLG1-AS1#2 was evaluated by wound healing experiments. (E and F) The location of DLG1-AS1 in MDA-MB-231 and MDA-MB-468 cells was tested by FISH (E) and subcellular fraction assays (F). (G) Potential miRNAs were dug out by testing their expression in TNBC cells (BT-549, MDA-MB-231, and MDA-MB-468) through qRT-PCR experiments. (H) The binding site of DLG1-AS1 and miR-16-5p is presented according to starBase. (I) The overexpression efficiency of miR-16-5p was tested by qRT-PCR. (J and K) The binding relationship of DLG1-AS1 and miR-16-5p was examined by luciferase reporter assays (J) and RNA pull-down assays (K). **p < 0.01. The data were measured using mean ± SD.
oncogene in TNBC cells and the expression of JARID2 could be affected by DLG1-AS1 and miR-16-5p.

The DLG1-AS1/JARID2 axis modulates the malignant processes of TNBC cells

Finally, we conducted rescue experiments to test the biological behaviors of TNBC cells affected by DLG1-AS1 and JARID2. We first detected the overexpression efficiency of JARID2 via qRT-PCR and western blot assays (Figure 4A). And we used EdU, transwell, and wound healing assays to investigate the TNBC cell proliferation, invasion, and migration separately. As the results showed, decreased proliferation, invasion, and migration of MDA-MB-231 and MDA-MB-468 caused by sh-DLG1-AS1#1 were regained by pcDNA3.1-JARID2. This meant the interaction between DLG1-AS1 and JARID2...
could change the biological behavior of TNBC cells greatly (Figures 4B–4D). In summary, PBX3-activated DLG1-AS1 can promote the proliferation, invasion, and migration of TNBC cells by sponging miR-16-5p and up-regulating JARID2.

DISCUSSION
Being one of the most serious subtypes of BC, TNBC has a poor prognosis and a higher death rate.1,24,25 Recent years, liposomal doxorubicin (produced by Changzhou Kinyond Pharmaceutical Manufacturing Co. LTD) has been used for chemotherapy of TNBC patients.26,27 Whereas other subtypes of cancers have found effective therapy, TNBC still needs more effective therapeutic methods.24,28 Recently, lncRNAs have been identified as being closely connected to the progression and poor prognosis of various cancers, including TNBC.12,29,30 For instance, the lncRNA AWPPH has been shown to propel proliferation and chemosensitivity of TNBC by interacting with miRNA-21.29 To further understand TNBC and improve the poor prognosis of it, we conducted this research to expand the knowledge of underlying molecular mechanisms in TNBC and provide evidence for developing therapeutic targets of TNBC.

In the sphere of genes, lncRNAs have attracted increasing attention from researchers, and they have made great efforts to explore their intricate mechanisms on diverse cancer cells.31 As one of the lncRNAs, DLG1-AS1 has been verified to act as an oncogene in...
CC, which functionally promotes the proliferation of CC cells. However, the knowledge of DLG1-AS1 remains insufficient. In the present study, EdU experiments, transwell assays, and wound healing experiments were implemented to test the influence of inhibited DLG1-AS1 (sh-DLG1-AS1#1 and sh-DLG1-AS1#2) on the proliferation of TNBC cells (MDA-MB-231 and MDA-MB-468). The results showed that DLG1-AS1 can work as an oncogene to prompt the proliferation, migration, and invasion of TNBC cells.

PBX3 has also been reported to be highly expressed in CC cells, and it can reduce overall survival of patients and promote the growth of CC cells. Moreover, PBX3 has been demonstrated to play a promoting role in BC development. Also, in our study, we conducted a line of functional experiments and investigated the influence of PBX3 on TNBC cell phenotype. Based on our collected data, PBX3 was highly expressed in TNBC cells. Meanwhile, it was revealed to function as an oncogene in TNBC cells as well. Moreover, we studied PBX3 playing the role of transcriptional activator of DLG1-AS1. ChIP assay and luciferase reporter assays proved that PBX3 is the transcriptional activator of DLG1-AS1.

As a kind of ncRNA, miRNA has a length of only 20–25 nt. However, it can bind to complementary bases to search and down-regulate target genes, which can restrain their translation. miR-16-5p can perform its function as a tumor suppressor. In our study, we investigated whether miR-16-5p can bind to DLG1-AS1 and JARID2 by luciferase reporter, RIP, and RNA pull-down assays. Through qRT-PCR, we found that DLG1-AS1 positively regulated the expression of JARID2, whereas miR-16-5p negatively regulated JARID2 expression. Further, JARID2 was confirmed to play an oncogene role to facilitate TNBC cell growth. Given all the above findings, we conclude that DLG1-AS1 sponged miR-16-5p to lift the expression of JARID2. Hence, a DLG1-AS1-miR-16-5p-JARID2 pathway was validated. In conclusion, PBX3-activated DLG1-AS1 can promote the proliferation, invasion, and migration of TNBC cells by acting as a sponge for miR-16-5p.

As for the limitations, in vivo experiments and clinical samples were not involved in this study. In the future, we intend to perform in vivo experiments and explore the expression of relevant genes in TNBC patient samples. We hope that the findings of the current research can provide novel insight for understanding the correlation between DLG1-AS1 and TNBC and support the development of an effective treatment for TNBC.

**MATERIALS AND METHODS**

**Cell lines**

BC cell lines (BT-549, MDA-MB-231, MDA-MB-468) were acquired from American Type Culture Collection (Manassas, VA, USA), together with the human mammary epithelial cells (MCF-10A) as normal control. Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Rockville, MD, USA) was supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics. The media were maintained in an incubator with 5% CO₂ at 37°C.

**Real-time quantitative polymerase chain reaction**

The TRizol kit from Invitrogen (Carlsbad, VA, USA) was used for total cellular RNA extraction from MCF-10A, BT-549, MDA-MB-231, and MDA-MB-468 cells. RNA samples were reverse transcribed into cDNA for qPCR with the help of a SYBR Premix Ex Taq II kit (Takara, Tokyo, Japan). Gene expression was monitored by an ABI Prism 7900HT system (Applied Biosystems, Foster City, CA, USA), calculated by 2⁻^ΔΔC_{T}, with GAPDH or U6 as internal reference. Primer sequences are presented in Table 1.

**Cell transfection**

BC cells were pre-seeded into six-well plates (3.0 × 10⁵ cells/well) for transfection with various plasmids for 48 h in line with the instructions for Lipofectamine 2000 (Invitrogen). For overexpression, the pcDNA3.1-PBX3, pcDNA3.1-JARID2 and corresponding control (pcDNA3.1-NC), miR-16-5p mimics, and control (NC mimics) were procured from Genechem (Shanghai, China). For silencing, short hairpin RNAs (shRNAs) against DLG1-AS1 (sh-DLG1-AS1#1/2), PBX3 (sh-PBX3#1/2), and JARID2 (sh-JARID2#1/2), as well as the negative control (sh-NC), were produced by Genechem (Shanghai, China). Sequences for gene knockdown are listed in Table 1.

**Chromatin immunoprecipitation**

ChIP assay was conducted as per the protocol of the EZ ChIP chromatin immunoprecipitation kit (Millipore, Bedford, MA, USA). The cross-linked chromatin DNA was sonicated and then immunoprecipitated with anti-PBX3 antibody (ab109173, Abcam, Cambridge, MA, USA). Normal IgG (MABE-253, Sigma-Aldrich, St. Louis, MO, USA) served as the negative control. After precipitates were purified, the enrichment of relevant genes was quantified by qRT-PCR.

**Dual-luciferase reporter assays**

The DLG1-AS1 promoter containing wild-type (WT) or mutant (Mut) PBX3 binding sites was obtained and cloned into pGL3-basic vector (Promega, Madison, WI, USA), followed by co-transfection with pcDNA3.1-PBX3 or pcDNA3.1-NC, sh-PBX3, or sh-NC into MDA-MB-231 and MDA-MB-468 cells. Muta-Direct enzyme (SDM-15, SBS Genetech, Beijing, China) and designed primers were used for PCR amplification to induce mutagenesis at the target site (binding sites of DLG1-AS1/PBX3, miR-16-5p/DLG1-AS1, and miR-16-5p/JARID2). After PCR, Mutazyme enzyme (SDM-15, SBS Genetech) was utilized to digest non-mutated parental plasmid DNA templates, leaving the mutated plasmid DNA. The mutated products were analyzed by a sequencing technique. The DLG1-AS1 full-length or JARID2 3’ UTR sequence covering WT or Mut miR-16-5p binding sites was inserted into the pmirGLO reporter vector (Promega) to construct DLG1-AS1-WT/Mut and JARID2 3’ UTR-Wt/Mut, then miR-16-5p mimics or NC-mimics were co-transfected into the indicated TNBC cells. After 48 h transfection, luciferase activity was detected by a dual-luciferase reporter assay system (Promega). Sequences inserted into pGL3 and pmirGLO vectors are displayed in Table 1.
Table 1. All sequences used in the current study

| Gene name | Primer sequences |
|-----------|------------------|
| DLG1-AS1 | F:AGACACCCCTACAACCTCAGCTGTTTGG  |
|           | R:AAAGGAACCAGCTGTTTGG |
| PBX3     | F:GACATGGGGTGCAATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| miR-107  | F:CCGAAGAGCTGTTTGG  |
|           | R:GCTCCTGATGCTGAGACC |
| miR-424-5p | F:CCGAAGAGCTGTTTGG |
|           | R:GCTCCTGATGCTGAGACC |
| miR-16-5p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| miR-15b-5p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| miR-15a-5p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| miR-6838-5p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| miR-103a-3p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| miR-195-5p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| miR-497-5p | F:CCGAAGAGCTGTTTGG |
|           | R:CTCAACTGGTGTCGTGGA |
| JARID2   | F:GGACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| ZBTB34   | F:GACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| ITPR1    | F:GACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| ARDC4    | F:GACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| PLP6     | F:GACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| U6       | F:GACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |
| GAPDH    | F:GACATCGGCGACATCCTCC  |
|           | R:GGTCCCTGATGCTGAGACC |

Knockdown vectors Sequences

sh-NC (for DLG1-AS1)
5'-CCGGGTCTTTGTGAAATGCTTCTGTCCTGAGACAGGAGAGCTGTTTGG  
sh-DLG1-AS1#1 5'-CCGGGTCTTTGTGAAATGCTTCTGTCCTGAGACAGGAGAGCTGTTTGG  
sh-DLG1-AS1#2 5'-CCGGGTCTTTGTGAAATGCTTCTGTCCTGAGACAGGAGAGCTGTTTGG  
sh-NC (for PBX3) 5'-CCGGGTCTTTGTGAAATGCTTCTGTCCTGAGACAGGAGAGCTGTTTGG  
sh-PBX3#1 5'-CCGGGTCTTTGTGAAATGCTTCTGTCCTGAGACAGGAGAGCTGTTTGG  
sh-PBX3#2 5'-CCGGGTCTTTGTGAAATGCTTCTGTCCTGAGACAGGAGAGCTGTTTGG  

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EdU staining
EdU assay was carried out with Cell-Light EdU Apollo567 in vitro imaging kit (RiboBio, Guangzhou, China), according to the supplier’s guidelines. TNBC cells in 96-well plates (8.0 × 10^5 cells/well) were treated with 25 μM EdU solution for 4 h. Then, 4% paraformaldehyde was used for 30 min fixation. After that, 0.5% Triton X-100 was used for 10 min. After DAPI (Beyotime, Guangzhou, China) was added, images of proliferative cells were taken by fluorescence microscope.

Transwell assay
The transfected TNBC cells were harvested and put into the top chamber of transwell inserts with 8 μm pore filters (Corning, Corning, NY, USA) and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in 24-well plates. After 48 h, the fixed invasive cells were treated with crystal violet staining and counted by microscope at 200X magnification.

Wound healing assay
The confluent BC cells in six-well culture plate (3.0 × 10^5 cells/well) were scratched vertically by the sterile 10 μL micropipette tip. The exfoliated cells were removed with phosphate-buffered saline (PBS). Serum-free medium was added for 4 h. Later, cell migratory ability was analyzed by inverted microscope. The width of the wound was recorded and imaged.

FISH
The PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA) was used in a FISH assay to determine the distribution of DLG1-AS1 in MDA-MB-231 and MDA-MB-468 cells. The DLG1-AS1 FISH probe was designed by RiboBio. The fixed cells were incubated with 40 nM DLG1-AS1 FISH probe in hybridization buffer and then treated with DAPI solution. An Olympus fluorescence microscope (Olympus, Tokyo, Japan) was utilized to capture images.

Nucleus-cytoplasam isolation
BC cells (5.0 × 10^6) were resuspended in cell fraction buffer and centrifuged for 15 min. The supernatant was collected as the cytoplasmic fraction. The pellet was cultured in cell disruption buffer on ice and centrifuged to collect the nuclear fraction. After the RNAs were extracted, the GADPH, DLG1-AS1, or U6 expression was determined by qRT-PCR.

RNA binding protein immunoprecipitation
In the beginning, BC cells were lysed with RIP lysis buffer. Subsequently, cell lysates were incubated with anti-immunoglobulin G (IgG), anti-Ago2 (TS-10X10ML-U, Millipore), and magnetic beads. After the incubation, enriched RNAs in the precipitated protein-RNA complexes were purified and examined by qRT-PCR analysis.

RNA pull-down assay
The RNA sample was purified and labeled with biotin using a Pierce RNA 3’ End Desthiobiotinylation Kit (Thermo Fisher Scientific, Waltham, MA). Lysates of MDA-MB-231 and MDA-MB-468 cells were cultivated with the biotinylated miR-16-5p (Bio-miR-16-5p-Wt/Mut) and Bio-miR-NC. The Bio-miR-16-5p-Mut was synthesized by mutating the sites of miR-16-5p complementary to DLG1-AS1. Magnetic beads were added for 1 h incubation at room temperature, prior to qRT-PCR analysis. Biotin-labeled sequences are presented in Table 1.

Western blot
Transfected cells were placed in an ice bath for 30 min and then centrifuged for 15 min. The processed protein samples were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After being washed in PBS, samples on PVDF were sealed with 5% skimmed milk powder for 2 h at room temperature. The primary antibodies against PBX3, JARID2, and GAPDH and horseradish peroxidase (HRP)-labeled IgG secondary antibody were all procured from Abcam. Immunoactive blots were analyzed by ECL Prime Detection reagent (GE Healthcare, Chicago, IL, USA).

Statistical analyses
Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was employed for statistical analysis. Student’s t test and analysis of variance (ANOVA) were applied for difference comparison between groups, with the significance level of p < 0.05. All assays were run in triplicate and the representative results were exhibited as mean ± standard deviation (SD).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.12.023.

ACKNOWLEDGMENTS
We appreciate the experimenters. No funding.

AUTHOR CONTRIBUTIONS
H.Z. conceived and designed the research. X.S. and X.Q. performed the experiments. G.G. and W.X. analyzed the data. Y.G., Z.G., and...
Z.W. interpreted the results of experiments and prepared the figures. All authors have read and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
1. Foulkes, W.D., Smith, I.E., and Reis-Filho, J.S. (2010). Triple-negative breast cancer. New Engl J. Med. 363, 1938–1948.
2. Anastasiadi, Z., Lianos, G.D., Ignatiadou, E., Harissis, H.Y., and Mitsis, M. (2017). Breast cancer in young women: an overview. Updates Surg. 69, 313–317.
3. Merino Bonilla, J.A., Torres Tabanera, M., and Ros Mendoza, L.H. (2017). Breast cancer in the 21st century: from early detection to new therapies. Radiologia 59, 368–379.
4. Siegel, R.L., Miller, K.D., and Jemal, A. (2015). Cancer statistics. CA Cancer J. Clin. 65, 5–29.
5. Podo, F., Buydens, L.M., Degani, H., Hilhorst, R., Klipp, E., Gribbestad, I.S., Van Huffel, S., van Laarhoven, H.W., Luts, J., Monleon, D., et al. (2010). Triple-negative breast cancer: present challenges and new perspectives. Mol. Oncol. 4, 209–229.
6. Carey, L., Winer, E., Viale, G., Cameron, D., and Gianni, L. (2010). Triple-negative breast cancer: disease entity or title of convenience? Nat. Rev. Clin. Oncol. 7, 683–692.
7. St Laurent, G., Wahlestedt, C., and Kapranov, P. (2015). The Landscape of long non-coding RNA classification. Trends Genetics 31, 239–251.
8. Geisler, S., and Coller, J. (2013). RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat. Rev. Mol. Cell Biol. 14, 699–712.
9. Kondo, Y., Shinjo, K., and Katsushima, K. (2017). Long non-coding RNAs as an epigenetic regulator in human cancers. Cancer Sci. 108, 1927–1933.
10. Dey, B.K., Mueller, A.C., and Dutta, A. (2014). Long non-coding RNAs as emerging regulators of differentiation, development, and disease. Transcription 5, e44014.
11. Wang, L., Liu, D., Wu, X., Zeng, Y., Li, L., Hou, Y., Li, W., and Liu, Z. (2018). Long non-coding RNA (LncRNA) RMST in triple-negative breast cancer (TNBC): expression analysis and biological roles research. J. Cell. Physiol. 233, 6603–6612.
12. Li, P., Zhou, B., Lv, Y., and Qian, Q. (2019). LncRNA HEIH regulates cell proliferation and apoptosis through miR-4485/503C1 axis in triple-negative breast cancer. Hum. Cell 32, 522–528.
13. Li, J., Li, L., Yuan, H., Huang, X.W., Xiang, T., and Dai, S. (2019). Up-regulated lncRNA GAS5 promotes chemosensitivity and apoptosis of triple-negative breast cancer cells. Cell Cycle 18, 1965–1975.
14. Huarte, M. (2015). The emerging role of lncRNAs in cancer. Nat. Med. 21, 1253–1261.
15. Evans, J.R., Feng, F.Y., and Chinnaiyan, A.M. (2016). The bright side of dark matter: lncRNAs in cancer. J. Clin. Invest. 126, 2775–2782.
16. Mou, E., and Wang, H. (2019). LncRNA LUCAT1 facilitates tumorigenesis and metastasis of triple-negative breast cancer through modulating miR-5702. Biosci. Rep. 39, BSR20190489.
17. Rui, X., Xu, Y., Huang, Y., Ji, L., and Jiang, X. (2018). LncRNA DLG1-AS1 promotes cell proliferation by competitively binding with miR-107 and up-regulating ZHX1 expression in cervical cancer. Cell Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. 49, 1792–1803.
18. Li, S. (2020). LncRNA DLG1-AS1 promotes cancer cell proliferation in triple negative breast cancer by downregulating miR-203. J. Breast Cancer 23, 343–354.
19. Li, H., Sun, G., Liu, C., Wang, J., Jing, R., Wang, J., Zhao, X., Xu, X., and Yang, Y. (2017). PX3 is associated with proliferation and poor prognosis in patients with cervical cancer. Oncotargets Ther. 10, 5685–5694.
20. Pang, Z.Y., Wei, Y.T., Shang, M.Y., Li, S., Li, Y., Jin, Q.X., Liao, Z.X., Cui, M.K., Liu, X.Y., and Zhang, Q. (2021). Leptin-elicited PX3 confers letrozole resistance in breast cancer. Endocrine-related Cancer. https://doi.org/10.1530/ERC-20-0328.
21. Morgan, R., and Pandhia, H.S. (2020). PX3 in cancer. Cancers 12, 431.
22. Yang, Y., Tai, W., Li, N., Li, T., Liu, Y., Wu, W., Li, Z., Pu, L., Zhao, X., Zhang, T., et al. (2020). LncRNA ZFAS1 promotes lung fibroblast-to-myofibroblast transition and ferroptosis via functioning as a ceRNA through miR-150-5p/SLC38A1 axis. Aging 12, 9085–9102.
23. Ruan, L., and Qian, X. (2019). MiR-16–5p inhibits breast cancer by reducing AKT3 to restrain NF-kappaB pathway. Biosci. Rep. 39, BSR20190489.
24. Park, J.H., Ahn, J.H., and Kim, S.B. (2018). How shall we treat early triple-negative breast cancer (TNBC): from the current standard to upcoming immuno-molecular strategies. ESMO Open 3, e000357.
25. Camorani, S., Fedele, M., Zannetti, A., and Cerchia, L. (2018). TNBC challenge: oligonucleotide aptamers for new imaging and therapy modalities. Pharmaceuticals 11, 123.
26. Franco, Y.L., Vaidya, T.R., and Ar-t-Oudhia, S. (2018). Anticancer and cardio-protective effects of liposomal doxorubicin in the treatment of breast cancer. Breast Cancer (Dove Med. Press) 10, 131–141.
27. Füredi, A., Szebenyi, K., Tóth, S., Cserpes, M., Hámori, L., Nagy, V., et al. (2017). Pegylated liposomal formulation of doxorubicin overcome drug resistance in a genetically engineered mouse model of breast cancer. J. Control. Release. 261, 287–296.
28. Cancer Genome Atlas Network (2012). Comprehensive portraits of human breast tumours. Nature 490, 61–70.
29. Liu, A.N., Qu, H.J., Gong, W.J., Xiang, J.Y., Yang, M.M., and Zhang, W. (2019). LncRNA AWPPH and miRNA-21 regulates cancer cell proliferation and chemosensitivity in triple-negative breast cancer by interacting with each other. J. Cell. Biochem. 120, 14860–14866.
30. Song, X., Liu, Z., and Yu, Z. (2019). LncRNA NEF is downregulated in triple negative breast cancer and correlated with poor prognosis. Acta Biochim. Biophys. Sin. 51, 386–392.
31. Schmitt, A.M., and Chang, H.Y. (2016). Long noncoding RNAs in cancer pathways. Cancer Cell 29, 452–463.
32. Zhao, J., Meng, R., Yao, Q., Wang, H., Niu, J., Cui, Y.U., Chen, S., and Bai, Y. (2019). Long non-coding RNA HEIH promotes breast cancer development via negative modulation of microRNA-200b. Die Pharmazie 74, 471–476.
33. Liu, B., Li, J., and Cairns, M.J. (2014). Identifying miRNAs, targets and functions. Pharmaceuticals 7, 123.
34. Chhabra, R. (2015). miRNA and methylation: a multifaceted liaison. Chembiochem Eur. J. Chem. Biol. 16, 195–203.