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

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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 in 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.

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.

RNAs with more than 200 nt and limited protein-coding capability are defined as long non-coding RNAs (lncRNAs).[7] lncRNAs have been studied and play an increasingly important role in biological functions of multiple cancer cells,[8–10] including TNBC.[11,12] For instance, lncRNA GAS5 has been confirmed to be up-regulated in TNBC cells and to exert oncogenic influences on apoptosis of TNBC cells.[13] To date, lncRNAs have been studied in many important cancers and have been found to interact with many other cellular macromolecules.[14,15] lncRNA LUCAT1 has been identified as promoting tumorigenesis and metastasis of TNBC by sponging miR-5702.[16] In this study, we aimed to dig into the molecular functions of other lncRNAs in TNBC, which may provide us new insights into the study of cancer.

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.

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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-ethynyl-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 mRNAs 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 enriched in the same RNA-induced silencing complex (RISC) (Figure S1A). This finding showed that miR-16-5p could bind with DLG1-AS1 and JARID2. The following results of RNA pull-down assay demonstrated that more JARID2 was enriched in Bio-miR-16-5p after DLG1-AS1 knockdown (Figure S1B). Given these results, DLG1-AS1 competed with JARID2 as a sponge for miR-16-5p. Meanwhile, qRT-PCR and western blot were implemented to investigate the influence of DLG1-AS1 reduction or miR-16-5p overexpression on the RNA and protein levels of JARID2. We found that the expression of JARID2 was decreased after we transfected sh-DLG1-AS1#1/2 or miR-16-5p mimics into TNBC cells (Figure 3E). Inhibition efficiency of sh-JARID2#1/2 was then tested by qRT-PCR and western blot assays (Figure 3F). Subsequently, we verified whether JARID2 could affect the biological behavior of TNBC cells. We conducted EdU, transwell, and wound healing assays, and the results showed that JARID2 down-regulation restricted the proliferation, invasion, and migration of TNBC cells greatly (Figures 3G–3I). Moreover, we investigated the influence of PBX3 on the malignant behaviors of TNBC cells as shown in Figures S1C–S1E. And we found that PBX3 could act as an oncogene in TNBC cells too. In conclusion, JARID2 and PBX3 could act as an

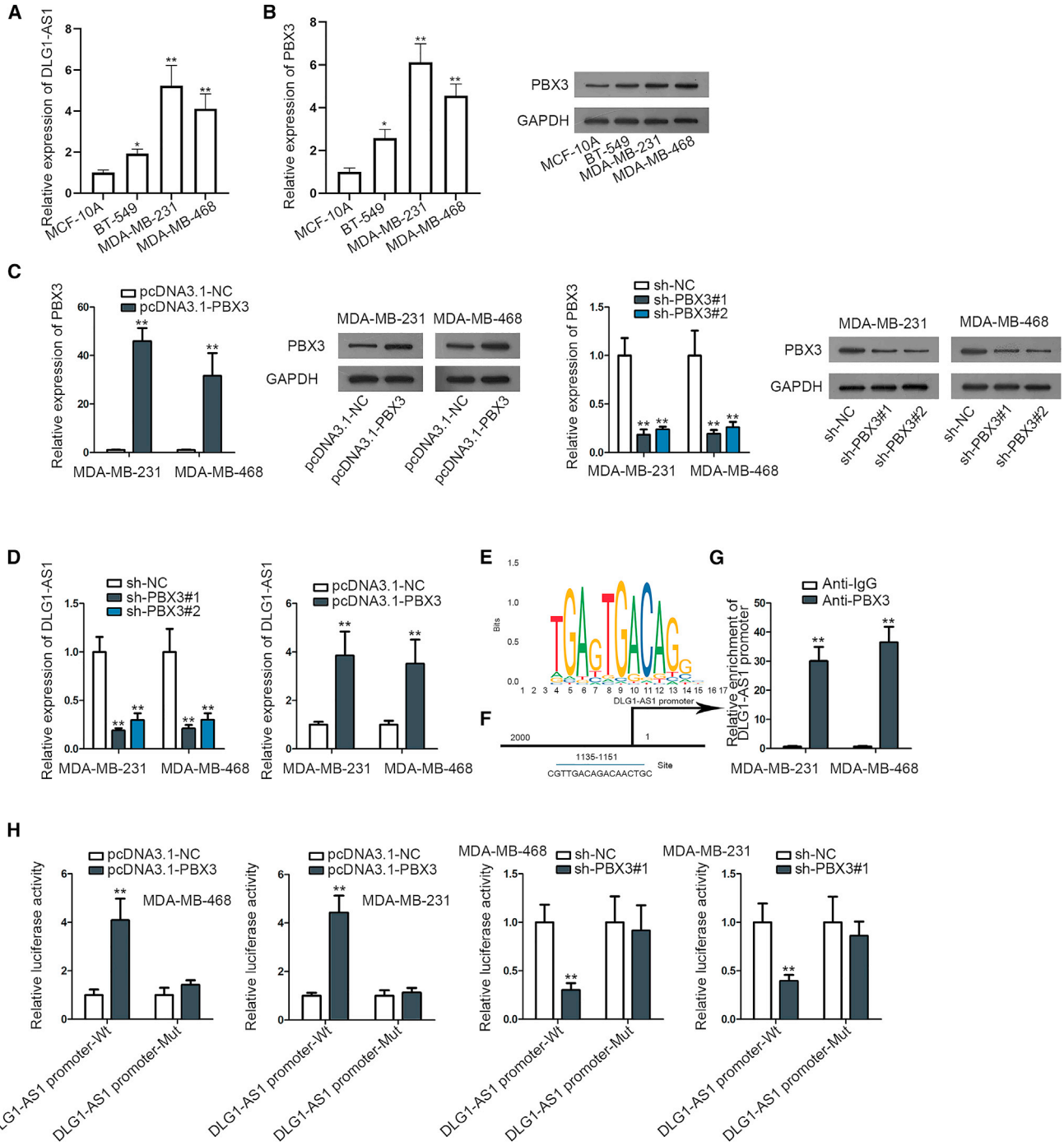

Figure 1. DLG1-AS1 and PBX3 are highly expressed in TNBC cells and PBX3 acts as the transcriptional activator of DLG1-AS1

(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.

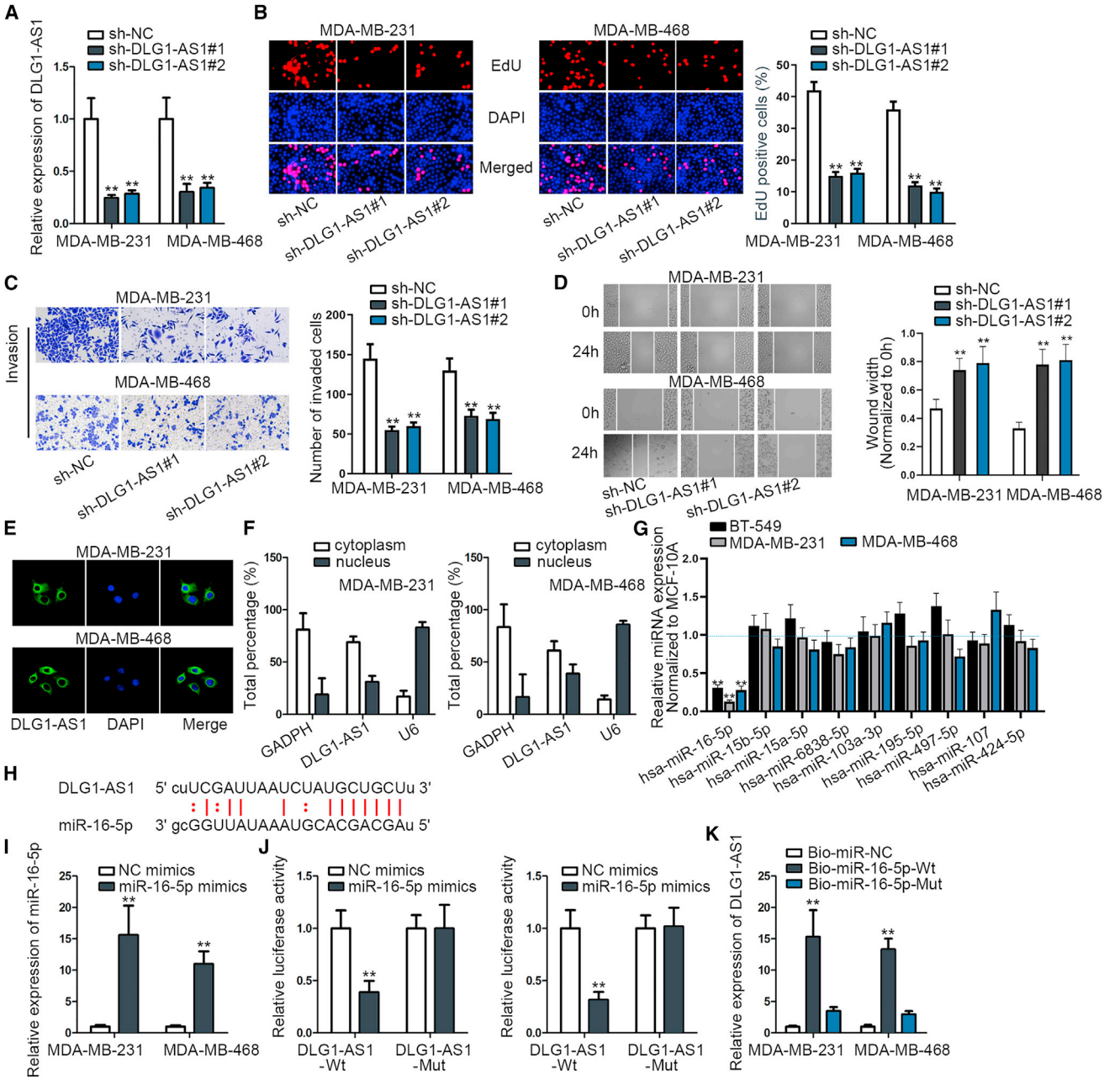

Figure 2. DLG1-AS1 acts as an oncogene in TNBC cells and as a sponge for miR-16-5p

(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.

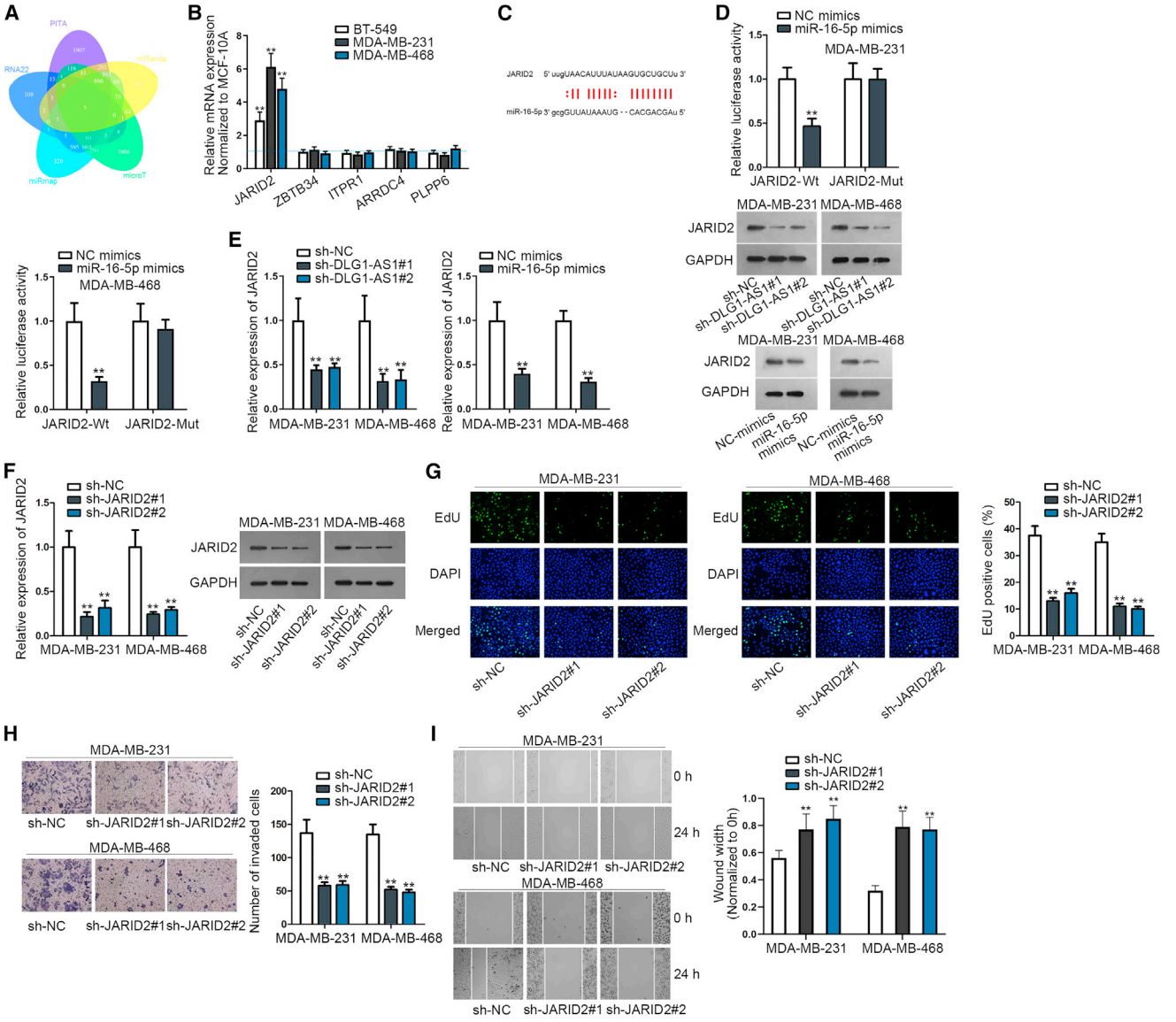

Figure 3. JARID2 acts as an oncogene in TNBC cells and its expression is regulated by DLG1-AS1 and miR-16-5p

(A) A Venn diagram was drawn to display the candidate mRNAs of miR-16-5p according to starBase. (B) The expression of mRNAs in TNBC cells (BT-549, MDA-MB-231, and MDA-MB-468) was investigated by qRT-PCR to find the cells with highly expressed mRNA. (C) The binding site between miR-16-5p and JARID2 is presented according to starBase. (D) The binding relationship between miR-16-5p and JARID2 was proven by luciferase reporter assays. (E) qRT-PCR and western blot were implemented to investigate the influence of reduced DLG1-AS1 and overexpressed miR-16-5p on the expression and protein level of JARID2. (F) The interference efficiency of JARID2 was investigated by qRT-PCR and western blot assays in MDA-MB-231 and MDA-MB-468 cells. (G–I) The proliferation, invasion, and migration situation of MDA-MB-231 and MDA-MB-468 cells was tested to investigate the influence of JARID2 depletion on TNBC cells by EdU (G), transwell (H), and wound healing (I) assays. $^{**}p < 0.01$. 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

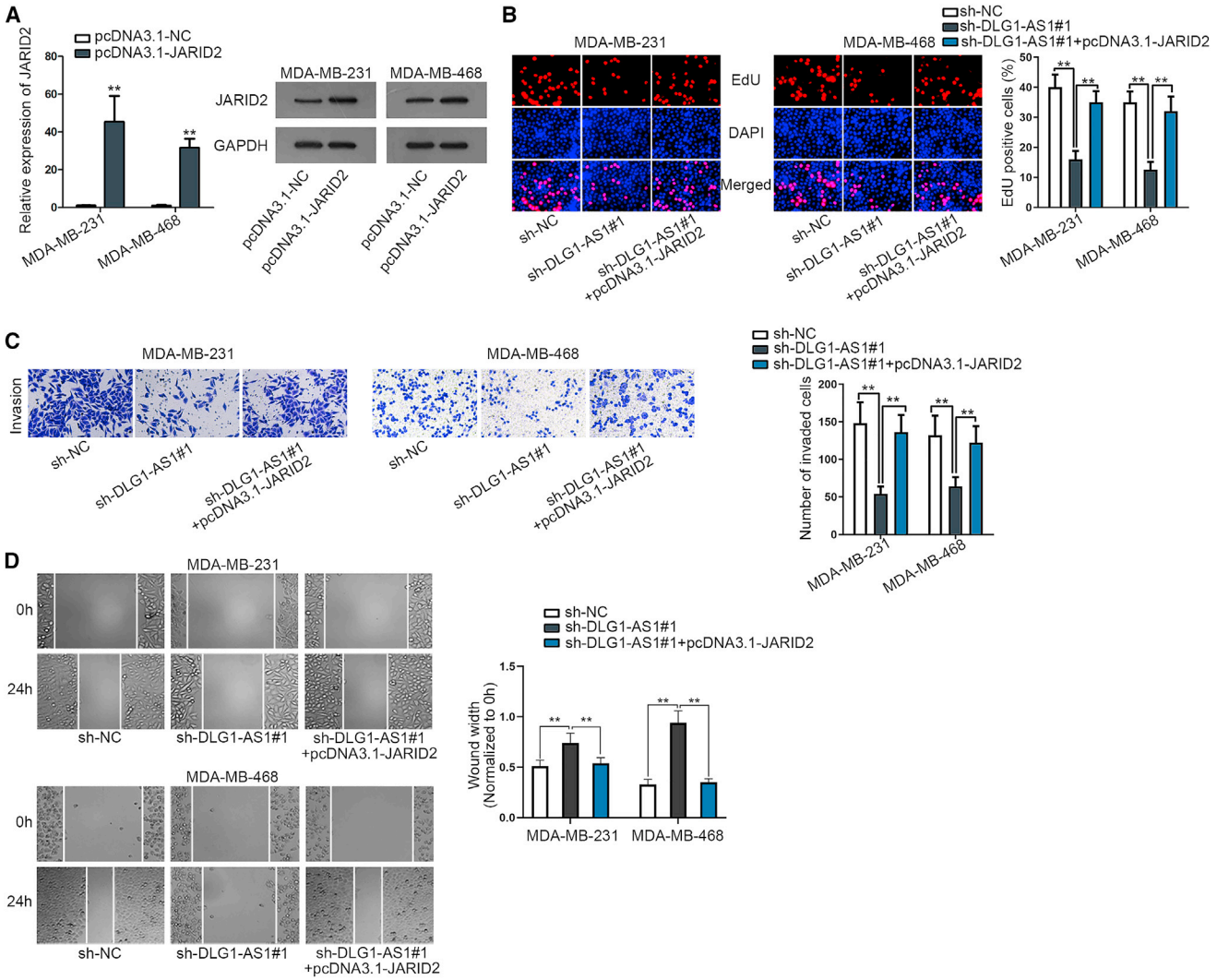

Figure 4. The malignant behaviors of TNBC cells are affected through the DLG1-AS1/JARID2 axis

(A) The overexpression efficiency of JARID2 was tested by qRT-PCR and western blot assays in MDA-MB-231 and MDA-MB-468 cells. (B–D) EdU (B), transwell (C), and wound healing (D) assays were implemented in different groups (sh-NC, sh-DLG1-AS1#1, and sh-DLG1-AS1#1+pcDNA3.1-JARID2) to explore the effect of DLG1-AS1 and JARID2 on TNBC cell proliferation, invasion, and migration. **p < 0.01. The data were measured using mean ± SD.

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.[9] 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.[10] Moreover, PBX3 has been demonstrated to play a promoting role in BC development.[32] 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.[33,34] miR-16-5p can perform its function as a tumor suppressor.[23] 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 modulated 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% CO_2 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 $2^{-\Delta\Delta CT}$, 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 (3.0×10^5 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 Genepharma (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 augmentation 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:AGACACCCCTCAACCTCACT R:AAGGGACCGTCAGAGTTTGG
PBX3	F:GACATCGGCGACATCCTCC R:GCTCCTCTGATGCTGAGACC
miR-107	F:CCGAGagcagcattgtacagggctatca R:GCTCCTCTGATGCTGAGACC
miR-424-5p	F:CCGAGcagcagcaattcatgttttgaa R:GCTCCTCTGATGCTGAGACC
miR-16-5p	F:CCGAGtagcagcacgtaaatattggcg R:CTCAACTGGTGTCGTGGA
miR-15b-5p	F:CCGAGtagcagcacatcatggtttaca R:CTCAACTGGTGTCGTGGA
miR-15a-5p	F:CCGAGtagcagcacataatggtttgtg R:CTCAACTGGTGTCGTGGA
miR-6838-5p	F:CCGAGaagcagcagtggcaagactcct R:CTCAACTGGTGTCGTGGA
miR-103a-3p	F:CCGAGagcagcattgtacagggctatgaag R:CTCAACTGGTGTCGTGGA
miR-195-5p	F:CCGAGtagcag cacagaaata ttggc R:CTCAACTGGTGTCGTGGA
miR-497-5p	F:CCGAGcagcagcacactgtggtttgt R:CTCAACTGGTGTCGTGGA
JARID2	F:TGACTCTTCTGCTCGCACTG R:GAGCCAAAGACGAACGAAGC
ZBTB34	F:AGTCGCATCTCTTTGGCACA R:CGATTTAGCAAAGCCGCTCC
ITPR1	F:CTGCCACCAGTTCAAAAGCC R:CTGAGGGCTGAAACTCCAGG
ARRDC4	F:TCGATGTCAACACACCAGCA R:CTCCCAGAAGCGATGTGGTT
PLPP6	F:GTCACTCTCTCGGTGGACAA R:GGGGTGAGAGCCAGCAATAG
U6	F:TCCCTTCGGGGACATCCG R:AATTTTGGACCATTTCTCGATTTGT
GAPDH	F:GACAGTCAGCCGCATCTTCT R:GCGCCCAATACGACCAAATC
Knockdown vectors	Sequences
sh-NC (for DLG1-AS1)	5'-CCGGTGTTAACTTTCTCAGCTTCGTC TCGAGACGAAGCTGAGAAAGTTAACAT TTTTG-3'
sh-DLG1-AS1#1	5'-CACCGTGTTAACTCCTTTGTTCTCAGCTC GAGCTGAGAACAAAGGAGTTAACA-3'
sh-DLG1-AS1#2	5'-CACCGTCAGATTCCTTTAAAAGCGTGCT CGAGCACGCTTTTAAAGGAATCTGA-3'
sh-NC (for PBX3)	5'-CCGGAGTTATCTGAAGACTGAACTC CTCGAGGAGTTCAGTCTTCAGATAACT TTTTTG-3'
sh-PBX3#1	5'-CACCATAGAGTTATCTGAAGAACCT CTCGAGAGGTTCTTCAGATAACTCTA-3'
sh-PBX3#2	5'-CACCGTAAGAATCCCCATTCAGACTCCT CGAGGAGTCTGAATGGGGATTCTTA-3'

(*Continued*)

Table 1. *Continued*

Knockdown vectors	Sequences
sh-NC (for JARID2)	5'-CCGGATCTTCTTGAATGATCTATGT CTCGAGACATAGATCATTCAAGAAGA TTTTTTG-3'
sh-JARID2#1	5'-CACCATCGTATTTCTTCTGAATGATC TCGAGATCATTCAGAAGAAATACGA-3'
sh-JARID2#2	5'-CACCATTAATCAGCCGGAAAAAGCACTC GAGTGCTTTTTCCGGCTGATTAA-3'
Vector name	Sequences
pGL3-DLG1-AS1-Wt	ttaaaatgtggacagttgaccatctccatgataggtagtttaggcactga attaatgctttttaatggaagagccaccctaagattcctggagtgtggta taatggagtactaaatcagcataggactaataactgcctcccgtgtaa actggtctatccggagagtggtatagcctctcatacagtgttcaaaggc agatttaactcactgcaggtccacagcaaccgataagataaactcgtt ggttatggtctcccactttgcaaaggagagcacagaggaagaggaa actgagataacttggtttaggaccccacagctggctggagctgggctg aacattagttcattgtttCttcgattaatctatgctgctt tttctctactt ggctccttttctaaattaaacaaaacaaaacaaa
pGL3-DLG1-AS1-Mut	ttaaaatgtggacagttgaccatctccatgataggtagtttaggcactga attaatgctttttaatggaagagccaccctaagattcctggagtgtggta taatggagtactaaatcagcataggactaataactgcctcccgtgtaa actggtctatccggagagtggtatagcctctcatacagtgttcaaagg cagatttaactcactgcaggtccacagcaaccgataagataaactcgt tggttatggtctcccactttgcaaaggagagcacagaggaagaggaa actgagataacttggtttaggaccccacagctggctggagctgggctg aacattagttcattgtttcttggtataaacgaacgacgat tttctctactt ggctccttttctaaattaaacaaaacaaaacaaa
pmirGLO-DLG1-AS1-Wt	uuaaaauguggacaguugaccaucuccaugauagguaguu aggcacugaauuaaugcuuuuuaauggaagagccacccua agauuccuggaguguggguauaauggaguacuaaaucagca uaggacuaauaacugccucccguguaaacuggucuauccg gagaguggguauagccucucuacauaguguucaaaggcagau uuaacucacugcagguccacagcaaccgauaagauaaacuc guuggguuauggucucccacuuugcaaaggagagcacagag gaagaggaaacugagauaacuugguuuaggaccccacagc uggcuggagcugggcugaacauuaguucauuguuuCuuc gauuaaucuaugcugcuu uuucucuacuuggcuccuuu ucuaaauuaaacaaaacaaaacaaa
pmirGLO-DLG1-AS1-Mut	uuaaaauguggacaguugaccaucuccaugauagguaguuag gcacugaauuaaugcuuuuuaauggaagagccacccuaagau uccuggaguguggguauaauggaguacuaaaucagcauaggac uaauaacugccucccguguaaacuggucuauccggagagugg uauagccucucauacaguguucaaaggcagauuuaacucacu gcagguccacagcaaccgauaagauaaacucguuggguuaugg ucucccacuuugcaaaggagagcacagaggaagaggaaacuga gauaacuuggguuuaggaccccacagcuggcuggagcugggc ugaacauuaguucauuguuucuuggguauaaacgaacgac gau uuucucuacuuggcuccuuuucuaaauuaaacaaaa caaaacaaa
pmirGLO-JARID2-Wt	auuuuuucucuucauguuguaacaaaaaggaaaaaagaaaaa aaaaucccauccccuuuuguacauauguccuguaaauuguuuua aauacuugagccuuuuucucgguggggggguggggaggggg ugagaagacaagaugaagaaaagccuuacauuucaguuucuu caucgguuggauuggaugcuuacagggUUUUUCUuguaaca uuuauaaugugcugcuuacaucacugaacaacaacaaaaaaaua auaauggaguagcuguugcccuucuccggguuguguguacag uauguguggaauaaaaaaggggaaacuguuuucacaagcugu ucuuuguuucauaauuggauucaucaauccguagcuaccc auauugcacugagcuugccaguggugacugccaggaacguc cuaugaucc

(*Continued on next page*)

Table 1. *Continued*

Vector name	Sequences
pmirGLO-JARID2-Mut	auuuuuucucuucauguuguaacaaaaaggaaaaaagaaaaa aaaaucccaucccuuuuguacauaugccuguaaaauuguuuu aaauacuugagccuuuuucucggugggggugggggagggg ggugagaagacaagaugaagaaaagccuuacauuucaguuu cuucaucgguuggauuggaugcuuacaggguuuuucUugu uucuaaauuaacacgacgauacaucacugaacaacaacaaaa aaauaauaauggaguagcuguugcccuucucggguugugu guacaguauguguggaauaaaaaagggaaacuguuuucac aagcuguucuuuguuucauaauuggauucaucaaucccgua gcuacccauauugcacugagcuugccaguggugacugccag gaacguccuaugaucc

Biotin probes	Sequences
Bio-miR-NC	Empty
Bio-miR-16-5p-Wt	biotin-uagcagcacguaaauauuggcg
Bio-miR-16-5p-Mut	biotin-uucgucgucguuaauuaucgcg

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 (8.0×10^3 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 200× 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-cytoplasm 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.

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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.

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