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Original Article

Long Non-coding RNA LOXL1-AS1 Drives Breast Cancer Invasion and Metastasis by Antagonizing miR-708-5p Expression and Activity

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LOXL1-AS1, a recently characterized long non-coding RNA (lncRNA), has been reported to modulate tumor progression in several types of cancer. However, the expression and role of LOXL1-AS1 in breast cancer remain unclear. In this study, we sought to identify novel lncRNA regulators engaged in breast cancer metastasis. To this end, we examined 42 cancer-related lncRNAs between MCF7 (with low metastatic potential) and MDA-MB-231 (with high metastatic potential) cells. These lncRNAs have been found to affect the invasiveness of several cancer types, but they are still undefined in breast cancer. Among the 42 candidates, LOXL1-AS1 is significantly increased in MDA-MB-231 cells relative to MCF7 cells. We also show that LOXL1-AS1 is upregulated in breast cancer tissues and cells compared to noncancerous counterparts. Increased LOXL1-AS1 expression is correlated with tumor stage and lymph node metastasis in breast cancer patients. Biologically, overexpression of LOXL1-AS1 enhances and knockdown of LOXL1-AS1 suppresses breast cancer cell migration and invasion. *In vivo* studies demonstrate that depletion of LOXL1-AS1 inhibits breast cancer metastasis. Mechanistically, LOXL1-AS1 sponges miR-708-5p to increase nuclear factor κB (NF-κB) activity. LOXL1-AS1 can also interact with EZH2 protein to enhance EZH2-mediated transcriptional repression of miR-708-5p. Rescue experiments indicate that co-expression of miR-708-5p attenuates LOXL1-AS1-induced invasiveness in breast cancer. In addition, there is a negative correlation between LOXL1-AS1 and miR-708-5p expression in breast cancer specimens. Overall, LOXL1-AS1 upregulation facilitates breast cancer invasion and metastasis by blocking miR-708-5p expression and activity. LOXL1-AS1 serves as a potential therapeutic target for breast cancer treatment.

INTRODUCTION

Breast cancer is the most prevalent and the second most lethal cancer among women.[1] Distant metastasis is the main cause of death in patients with breast cancer, which is associated with dismal prognosis.[2,3] Several strategies such as targeting cancer cell extravasation and stemness have been proposed to hamper metastatic progression of breast cancer.[4,5] Identification of key molecular regulators of breast cancer metastasis is important for development of effective therapies.

Long non-coding RNAs (lncRNAs) with a length of >200 nt have been found to regulate a wide range of biological processes and diseases.[6,7] Many studies have indicated that lncRNAs can regulate cancer development and metastasis.[8,9] For instance, the lncRNA UCA1 was reported to promote gastric cancer proliferation and migration and enhance immune escape.[7] Biochemically, lncRNAs have the ability to interact directly with RNA molecules or proteins to modulate gene expression at the transcriptional and post-transcriptional levels.[10] Wang et al.[11] have shown that LINC00339 sponges miR-377-3p to enhance triple-negative breast cancer growth. Similarly, the lncRNA TINCR was found to induce trastuzumab resistance in breast cancer by targeting miR-125b.[12] Therefore, lncRNAs are promising therapeutic targets for cancer management.

Recently, a new lncRNA, LOXL1-AS1, has been found to modulate the aggressive phenotype of glioblastoma, medulloblastoma, prostate cancer, and cholangiocarcinoma.[13–17] Long et al.[16] reported that LOXL1-AS1 downregulation inhibits cell proliferation and arrests cell cycle progression in prostate cancer. LOXL1-AS1 antagonizes miR-708-5p to enhance tumorigenesis and stemness in gastric cancer.[13] miR-708-5p functions as a tumor suppressor in multiple cancer types, including breast cancer.[18,19] However, the expression and role of LOXL1-AS1 in breast cancer remain elusive.

In the present study, we identified LOXL1-AS1 as a metastasis-related lncRNA in breast cancer. The mechanism by which LOXL1-AS1 regulates breast cancer cell invasion and metastasis was further explored. In addition, we evaluated the clinical significance of LOXL1-AS1 in breast cancer.

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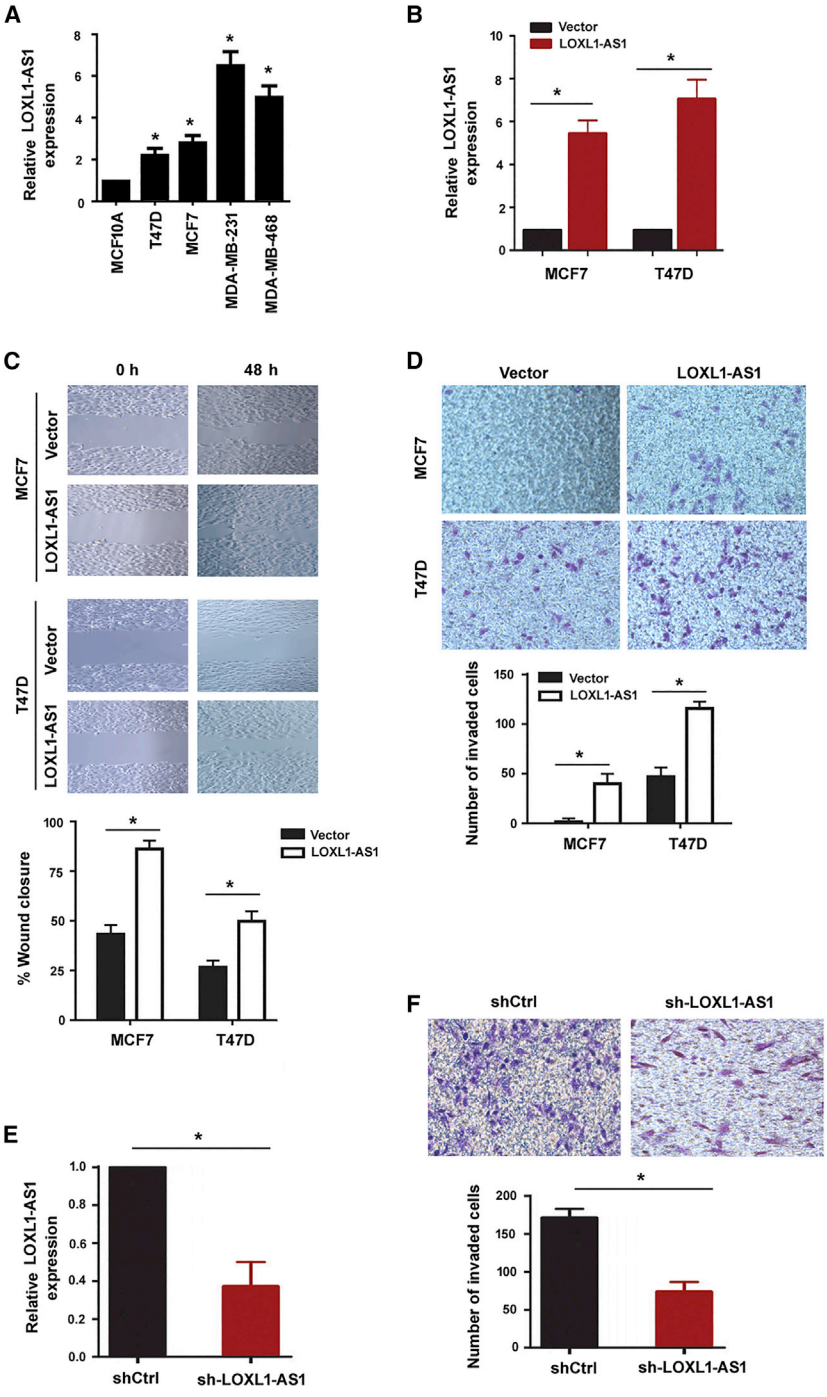

Figure 1. LOXL1-AS1 Promotes Breast Cancer Cell Migration and Invasion *In Vitro*

(A) qPCR analysis of LOXL1-AS1 levels in indicated cell lines. *p < 0.05 versus MCF10A. (B) qPCR analysis of LOXL1-AS1 levels in both MCF7 and T47D cells transfected with LOXL1-AS1-expressing plasmid or empty vector. (C) *In vitro* wound-healing assay demonstrates that overexpression of LOXL1-AS1 enhances the migration of MCF7 and T47D cells. (D) Transwell invasion assay shows the increased invasive potential in LOXL1-AS1-overexpressing cells. (E) Downregulation of LOXL1-AS1 in MDA-MB-231 cells was achieved by transfection with LOXL1-AS1-targeting shRNA (sh-LOXL1-AS1). (F) Transwell invasion assay shows the reduced invasive ability of MDA-MB-231 cells by LOXL1-AS1 depletion. *p < 0.05.

RESULTS

LOXL1-AS1 Promotes Breast Cancer Cell Migration and Invasion *in Vitro*

To identify novel lncRNA regulators of breast cancer metastasis, we analyzed 42 cancer-related lncRNAs between MCF7 (with low metastatic potential) and MDA-MB-231 (with high metastatic potential) cells using quantitative PCR (qPCR) analysis. These lncRNAs were selected since they can affect the invasiveness of several cancer types but are less characterized in breast cancer (Table S1). Among them, only LOXL1-AS1 expression remarkably differed between MCF7 and MDA-MB-231 cells (Figure 1A). We also examined the expression of LOXL1-AS1 in other breast cancer cell lines. Compared to MCF10A non-malignant epithelial cells, breast cancer cell lines displayed an upregulation of LOXL1-AS1, especially in highly metastatic cell lines MDA-MB-231 and MDA-MB-468 (Figure 1A). To determine the function of LOXL1-AS1 in breast cancer, we performed gain-of-function studies in MCF7 and T47D cells (Figure 1B). Notably, overexpression of LOXL1-AS1 led to a significant increase in MCF7 cell migration (Figure 1C) and invasion (Figure 1D). Similar results were noted in T47D cells (Figures 1C and 1D). In contrast, the proliferative ability of MCF7 and T47D cells remained unchanged after ectopic expression of LOXL1-AS1 (Figure S1). To verify whether LOXL1-AS1 is required for the invasive phenotype of breast cancer, we knocked down its expression in MDA-MB-231 cells (Figure 1E). As shown in Figure 1F, depletion of LOXL1-AS1 significantly diminished the invasion of MDA-MB-231 cells. These results suggest that LOXL1-AS1 contributes to the invasive phenotype of breast cancer.

LOXL1-AS1 Depletion Blocks Breast Cancer Metastasis *In Vivo*

Next, we determined the effect of LOXL1-AS1 depletion on breast cancer metastasis. To this end, we injected control,

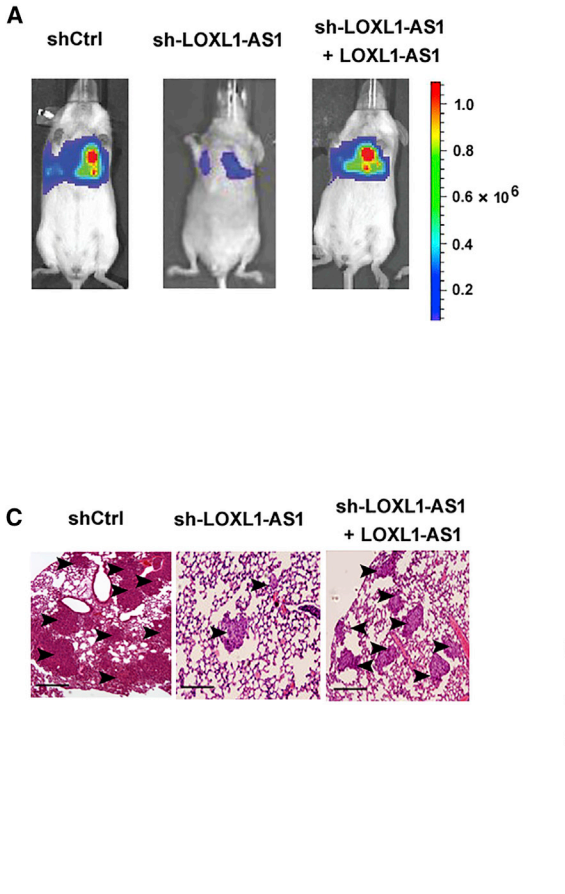

Figure 2. LOXL1-AS1 Depletion Blocks Breast Cancer Metastasis *In Vivo*

(A) Representative bioluminescence images of animals showing lung metastasis after injection of MDA-MB-231 cells transfected with indicated constructs through the tail vein. (B) Quantitation of lung metastasis as assessed by bioluminescence imaging (n = 4). (C) Representative H&E of lungs showing metastatic nodules. Scale bars, 50 μm. (D) Determination of the number of metastatic lesions in the lung. *$p < 0.05$.

LOXL1-AS1-depleted, and LOXL1-AS1-restored MDA-MB-231 cells via the tail vein. Bioluminescent imaging of tumor-harboring animals showed that depletion of LOXL1-AS1 in MDA-MB-231 cells markedly suppressed lung metastasis in mice, which was rescued by overexpression of LOXL1-AS1 (Figures 2A and 2B). H&E staining of lung sections confirmed the reduced lung metastasis in the LOXL1-AS1-depleted group (Figures 2C and 2D). Taken together, these results support the importance of LOXL1-AS1 in breast cancer metastasis.

LOXL1-AS1 Sponges miR-708-5p in Breast Cancer Cells

Given the functional association between lncRNAs and microRNAs (miRNAs),[20] we attempted to identify the key miRNAs that mediate the pro-metastatic activity of LOXL1-AS1. We predicted potential miRNA partners of LOXL1-AS1 using starBase v3.0 (http://starbase.sysu.edu.cn/panCancer.php). As a consequence, we obtained 33 candidate miRNAs (Table S2). Among them, both miR-708-5p and miR-101-3p were suppressed in LOXL1-AS1-overexpressing MCF7 cells relative to control cells (Figure 3A). To check whether both miRNAs can directly target LOXL1-AS1, we performed luciferase reporter assays. It was found that the expression of the LOXL1-AS1 luciferase reporter was inhibited by overexpression of miR-708-5p but not miR-101-3p (Figures 3B and 3C). Moreover, miR-708-5p-mediated repression of the LOXL1-AS1 luciferase reporter was blocked by disruption of the putative binding site for miR-708-5p (Figure 3D). These observations suggest that miR-708-5p may play a major role in mediating the oncogenic activity of LOXL1-AS1.

To confirm the direct interaction between LOXL1-AS1 and miR-708-5p, an RNA immunoprecipitation (RIP) assay was conducted to pull down endogenous Ago2 complexes in MCF7 cells. We found that both LOXL1-AS1 and miR-708-5p were enriched in Ago2 immunoprecipitates relative to control immunoglobulin G (IgG) immunoprecipitates (Figure 3E). Taken together, these observations suggest a crosstalk between LOXL1-AS1 and miR-708-5p in breast cancer.

LOXL1-AS1 Interacts with EZH2 to Inhibit the Expression of miR-708-5p

It has been previously demonstrated that the polycomb repressor complex 2 (PRC2), which contains SUZ12 and EZH2 subunits, is responsible for the transcriptional repression of miR-708-5p in breast cancer.[21] Next, we asked whether LOXL1-AS1 interacts with the PRC2 subunits. To address this, we performed LOXL1-AS1 pull-down assays. Western blot analysis of the pull-down proteins revealed that EZH2 protein associated with LOXL1-AS1 in MCF7 and MDA-MB-231 cells (Figure 4A). Overexpression of LOXL1-AS1 led to the stabilization of EZH2 (Figure 4B) and enhancement of EZH2 expression (Figure 4C) in MCF7 cells. In addition, we examined the impact of LOXL1-AS1/EZH2 interaction on the PRC2-induced histone H3 on lysine 27 (H3K27) trimethylation (H3K27me3). Interestingly, we found that LOXL1-AS1 overexpression resulted in increased levels of H3K27me3 on the promoter region of miR-708-5p (Figure 4D). Taken together, we suggest that LOXL1-AS1-mediated repression of miR-708-5p involves the interaction with EZH2 and enhancement of PRC2-induced H3K27me3.

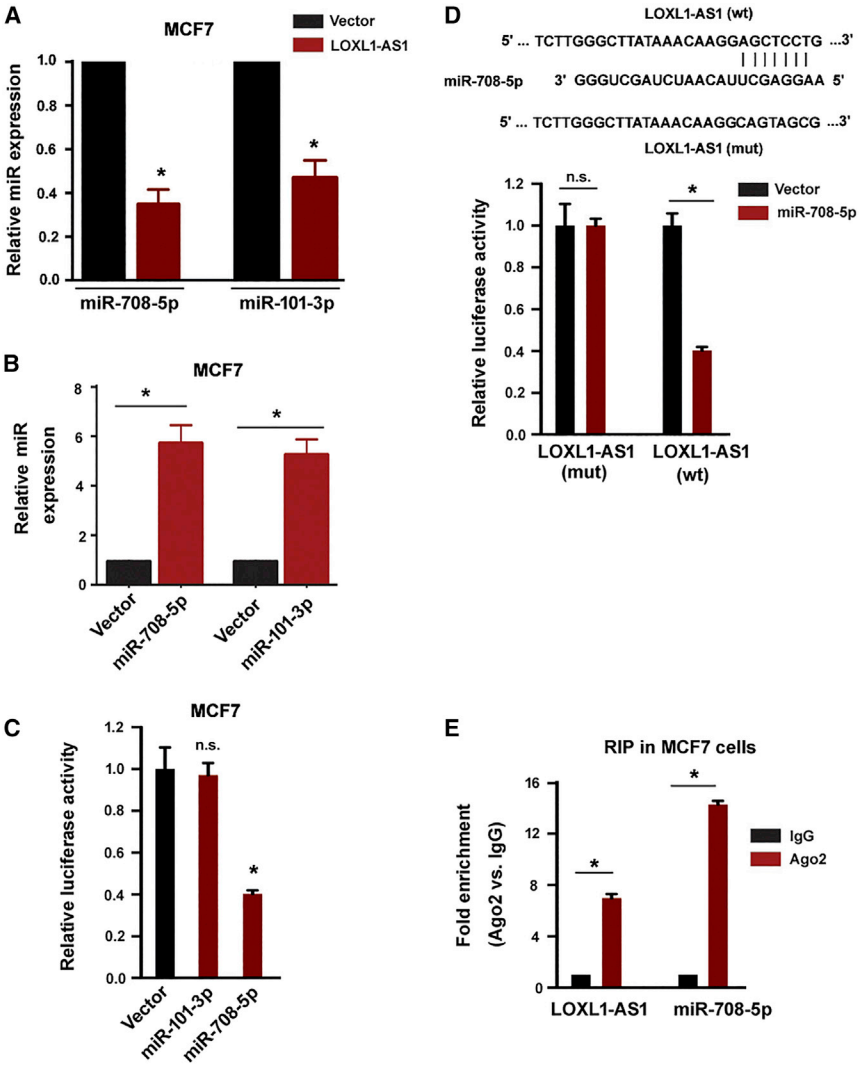

Figure 3. LOXL1-AS1 Sponges miR-708-5p in Breast Cancer Cells

(A) Effect of overexpression of LOXL1-AS1 on miR-708-5p and miR-101-3p expression. (B) qPCR analysis confirms the upregulation of miR-101-3p or miR-708-5p in MCF7 cells transfected with indicated constructs. (C) Overexpression of miR-101-3p does not affect the activity of the LOXL1-AS1 luciferase reporter. In contrast, overexpression of miR-708-5p leads to a repression of the LOXL1-AS1 luciferase reporter. (D) Luciferase reporter assay shows that overexpression of miR-708-5p suppresses the luciferase reporter harboring wild-type (WT) but not mutant (mut) LOXL1-AS1. (E) RIP assay confirms that both LOXL1-AS1 and miR-708-5p are detected in the Ago2 immunoprecipitates from MCF7 cells. *$p < 0.05$. n.s., not significant.

miR-708-5p Is Implicated in the Oncogenic Activity of LOXL1-AS1

Next, we asked whether LOXL1-AS1-dependent malignant behaviors are linked to downregulation of miR-708-5p. To this end, breast cancer cells were co-transfected with LOXL1-AS1- and miR-708-5p-expressing plasmids. Co-transfection with the miR-708-5p-expressing plasmid restored the expression of miR-708-5p in LOXL1-AS1-overexpressing MCF7 and T47D cells (Figure 5A). LOXL1-AS1-induced cell migration and invasion was dramatically attenuated by co-expression of miR-708-5p (Figures 5B and 5C). A previous study has indicated that miR-708-5p-dependent anticancer effects on breast cancer involve repression of IKKB and inactivation of nuclear factor κB (NF-κB) signaling.[18] Interestingly, we found that LOXL1-AS1 overexpression increased NF-κB activity and promoted p65 nuclear translocation and IKKB mRNA expression in MCF7 and T47D breast cancer cells, which was reversed by restoration of miR-708-5p (Figures 5D–5F). In addition, silencing of LOXL1-AS1 led to a reduction in IKKB mRNA expression and NF-κB-dependent transcriptional activity (Figure S2). Taken together, these data suggest that LOXL1-AS1 promotes breast cancer invasiveness through the miR-708-5p/NF-κB pathway.

LOXL1-AS1 Upregulation Is Associated with Aggressive Phenotype of Breast Cancer

To determine the clinical significance of LOXL1-AS1 in breast cancer, we examined the expression of LOXL1-AS1 in 85 pairs of breast cancer and adjacent normal tissues by qPCR analysis. The results showed that LOXL1-AS1 expression levels were significantly higher in breast cancer samples than those in corresponding normal tissues ($p < 0.001$; Figure 6A). Further analysis revealed that LOXL1-AS1 expression was correlated with tumor stage ($p = 0.0013$) and lymph node metastasis ($p = 0.0001$; Table 1). In addition, we found a negative correlation between LOXL1-AS1 and miR-708-5p expression in breast cancer specimens ($r = -0.303$, $p = 0.0048$; Figure 6B).

DISCUSSION

lncRNAs are frequently dysregulated in cancers and engaged in cancer progression.[6,8,9] Zhang et al.[22] reported that MIR22HG is significantly downregulated in hepatocellular carcinoma, and its overexpression suppresses the proliferation and metastasis of hepatocellular carcinoma (HCC) cells both *in vitro* and *in vivo*. Zhu et al.[23] reported that lncRNA HAS2-AS1 mediates hypoxia-induced invasiveness in oral squamous cell carcinoma. In this study, we examined a set of recently characterized lncRNAs in breast cancer cells with different metastatic potential. Among the 42 candidate lncRNAs, LOXL1-AS1 was found to be expressed at a higher level in MDA-MB-231 cells than in MCF-7 cells. Moreover, LOXL1-AS1 is generally overexpressed in multiple breast cancer cell lines compared to MCF10A non-malignant epithelial cells. Clinically, breast cancer

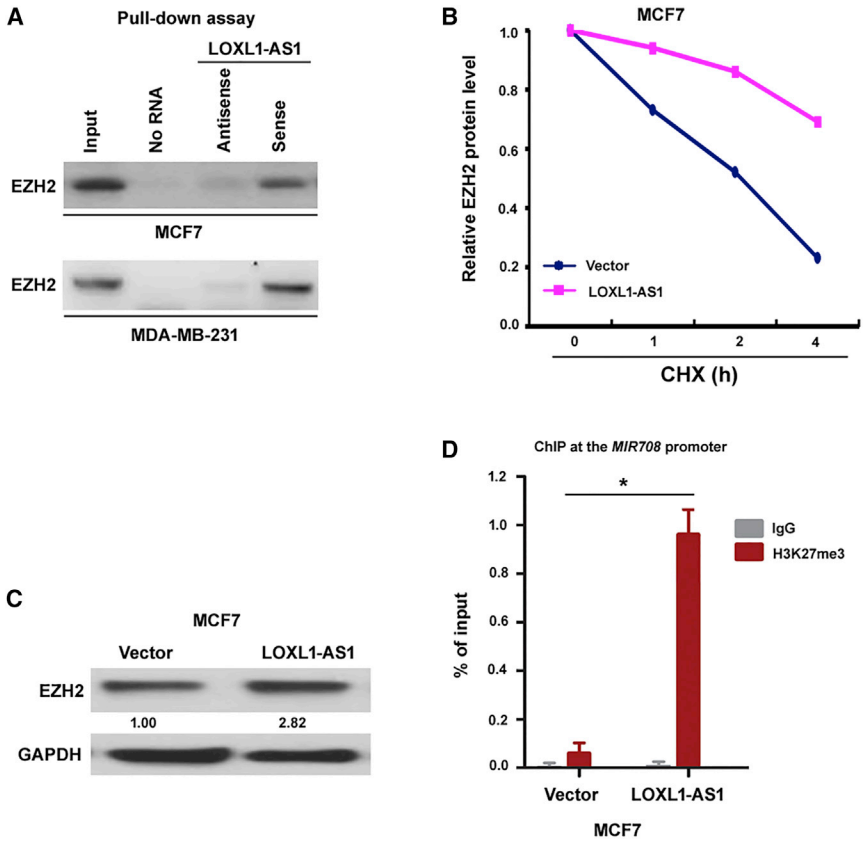

Figure 4. LOXL1-AS1 Interacts with EZH2 to Inhibit the Expression of miR-708-5p

(A) RNA pull-down assay reveals that EZH2 protein associates with LOXL1-AS1 in MCF7 and MDA-MB-231 cells. (B) Overexpression of LOXL1-AS1 slows the turnover of EZH2 protein. CHX was used to block *de novo* protein synthesis. (C) Western blot analysis demonstrates that overexpression of LOXL1-AS1 increases EZH2 protein levels. (D) Overexpression of LOXL1-AS1 promotes the enrichment of H3K27me3 on the promoter region of miR-708-5p. *$p < 0.05$.

tissues show overexpression of LOXL1-AS1 relative to adjacent noncancerous breast tissues. The upregulation of LOXL1-AS1 is significantly associated with advanced tumor stage and lymph node metastasis. Therefore, we suggest LOXL1-AS1 as a potential driver of breast cancer metastasis. The clinical significance of LOXL1-AS1 was further validated in a larger cohort of breast cancer patients.

Several studies have indicated that LOXL1-AS1 contributes to tumor growth and progression in glioblastoma, medulloblastoma, prostate cancer, and cholangiocarcinoma.[13–17] In agreement with these reports, we show that overexpression of LOXL1-AS1 remarkably increases the invasive property of breast cancer cells. However, LOXL1-AS1 overexpression has no effect on the proliferation of breast cancer cells. These data suggest that LOXL1-AS1 is a pro-invasive gene in breast cancer. To explore the potential significance of LOXL1-AS1 as a therapeutic target, we knocked down endogenous expression of LOXL1-AS1 in MDA-MB-231 cells. Of note, depletion of LOXL1-AS1 leads to impairment of MDA-MB-231 cell invasion *in vitro*. *In vivo* studies confirm that that LOXL1-AS1-depleted MDA-MB-231 cells show a reduced metastatic potential compared to control cells. Moreover, overexpression of LOXL1-AS1 restores the metastatic ability of LOXL1-AS1-depleted MDA-MB-231 cells. Overall, these observations suggest LOXL1-AS1 as a promising therapeutic target for breast cancer.

The interaction with miRNAs has been proposed as an important mechanism for LOXL1-AS1-mediated aggressive phenotype. LOXL1-AS1 has shown the ability to sponge miR-541-3p in prostate cancer and miR-324-3p in cholangiocarcinoma.[16,17] However, LOXL1-AS1 interaction with these miRs was not detected in breast cancer (data not shown). When this manuscript was under review, Sun et al.[13] reported the sponging of miR-708-5p by LOXL1-AS1 in gastric cancer. Consistently, we observe that LOXL1-AS1 interacts with miR-708-5p in breast cancer cells. Specially, miR-708-5p overexpression suppresses the LOXL1-AS1 luciferase reporter. A RIP assay demonstrated that both LOXL1-AS1 and miR-708-5p were enriched in Ago2 immunoprecipitates from MCF7 cells. In contrast, another candidate miR-101-3p does not repress the LOXL1-AS1 luciferase reporter activity. These results indicate the specific interaction between LOXL1-AS1 and miR-708-5p in breast cancer.

Our data also show that LOXL1-AS1 overexpression leads to an inhibition of miR-708-5p expression in breast cancer cells. It has been reported that the PRC2 complex participates in the transcriptional repression of miR-708-5p.[21] Intriguingly, we find that LOXL1-AS1 can bind to and stabilize EZH2 protein, an important subunit of the PRC2 complex. EZH2 belongs to the SET domain family of lysine methyltransferases, which methylates (H3K27), consequently resulting in transcriptional repression of genes.[24,25] Multiple lncRNAs have been identified to directly interact with EZH2 in different cellular contexts.[26–28] For instance, lncRNA TUG1 can regulate miR-144-3p in osteosarcoma and miR-194-5p in bladder cancer via binding to EZH2 protein.[27,29] Consistent with the increased expression of EZH2, the H3K27me3 on the promoter region of miR-708-5p was enhanced in LOXL1-AS1-overexpressing cells. Clinically, there is a significant negative correlation between LOXL1-AS1 and miR-708-5p expression in breast cancer specimens. Therefore, our data suggest that LOXL1-AS1 antagonizes both the expression and activity of miR-708-5p in breast cancer cells.

Previous studies have reported that miR-708-5p expression is reduced and exerts anti-metastatic activity in breast cancer.[21,30] Ramchandani

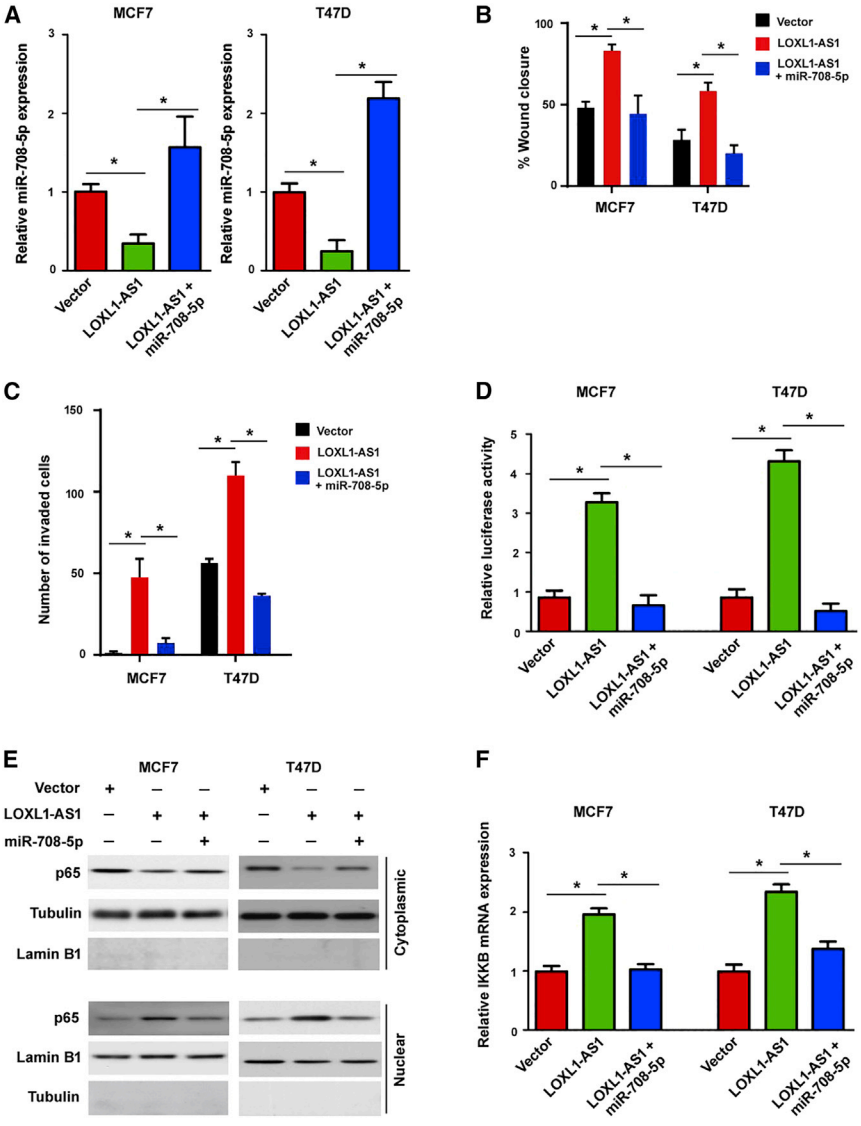

Figure 5. miR-708-5p Is Implicated in the Oncogenic Activity of LOXL1-AS1

(A) qPCR analysis shows that overexpression of LOXL1-AS1 reduces the expression of miR-708-5p, which is reversed by co-expression of miR-708-5p. (B) *In vitro* wound-healing assay confirms that miR-708-5p overexpression rescues LOXL1-AS1-induced migration in both MCF7 and T47D cells. (C) Transwell invasion assay shows that LOXL1-AS1-mediated invasiveness is reversed by overexpression of miR-708-5p. (D) NF-κB luciferase reporter assay shows that miR-708-5p attenuates LOXL1-AS1-dependent increase in NF-κB activity. (E) Western blot analysis of p65 proteins in nuclear and cytoplasmic extracts from MCF7 and T47D cells transfected with indicated constructs. Tubulin and lamin B1 was used as the loading control for cytoplasmic and nuclear fractions, respectively. (F) qPCR analysis of IKKB mRNA in MCF7 and T47D cells transfected with indicated constructs. *$p < 0.05$.

et al.[30] reported that nanoparticle delivery of miR-708 mimetic effectively suppresses lung metastasis in an orthotopic model of triple-negative breast cancer. Senthil Kumar et al.[18] demonstrated that miR-708-5p hampers breast cancer tumorigenesis and metastasis via inactivation of NF-κB signaling. To validate the functional interplay between LOXL1-AS1 and miR-708-5p in breast cancer invasion, we performed rescue experiments by co-overexpressing miR-708-5p and LOXL1-AS1. As a result, LOXL1-AS1-induced cell migration and invasion are reversed by enforced expression of miR-708-5p. At the molecular level, LOXL1-AS1-mediated induction of NF-κB activity and IKKB expression is blocked by overexpression of miR-708-5p. Constitutive activation of NF-κB signaling has been reported to contribute to breast cancer invasion and metastasis.[31,32] Targeting NF-κB signaling attenuates aggressive properties of breast cancer.[32] Taken together, we propose that LOXL1-AS1-dependent breast cancer aggressiveness involves the suppression of miR-708-5p and induction of NF-κB signaling (Figure 6C).

Conclusion

In summary, we identify LOXL1-AS1 as a pro-metastatic gene in breast cancer. Upregulation of LOXL1-AS1 promotes the invasive property of breast cancer through sponging miR-708-5p to induce NF-κB activation. LOXL1-AS1 is capable of binding to EZH2 and enhancing EZH2-mediated transrepression of miR-708-5p. Our findings suggest LOXL1-AS1 as a novel therapeutic target for the treatment of breast cancer.

MATERIALS AND METHODS

Cell Culture

A panel of breast cancer cell lines (i.e., T47D, MCF7, MDA-MB-231, and MDA-MB-468 cells), as well as MCF10A nonmalignant breast epithelial cells, were purchased from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA).

Human Tissue Specimens

We collected a total of 85 primary tumor specimens and adjacent noncancerous tissues from breast cancer patients who underwent resection surgery between 2010 and 2011. These patients did not receive any anticancer treatment before surgery. The study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China). Each patient gave written informed consent for research purposes. The resected specimens were plunged immediately into liquid nitrogen and stored at −80°C until use.

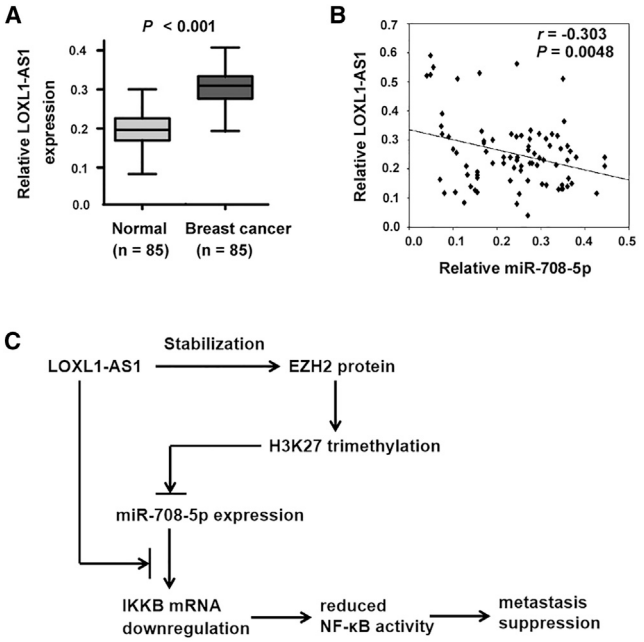

Figure 6. LOXL1-AS1 Upregulation Is Associated with Aggressive Features of Breast Cancer

(A) qPCR analysis demonstrates that LOXL1-AS1 expression levels are significantly higher in breast cancer samples than those in corresponding normal tissues. (B) There is a negative correlation between LOXL1-AS1 and miR-708-5p expression in breast cancer specimens, as assessed by Pearson correlation analysis. (C) A schematic model showing that LOXL1-AS1 promotes breast cancer metastasis by enhancing EZH2-mediated transrepression of miR-708-5p and antagonizing miR-708-5p-indced IKKB downregulation and reduction of NF-κB activity.

qPCR Analysis

Total RNA from tissues and cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were reverse transcribed into cDNA using the RNeasy mini kit (QIAGEN, Hilden, Germany). A qPCR assay was performed using the SYBR Green RT-PCR kit (Takara, Dalian, China). The PCR primers are as follows: *LOXL1-AS1*, forward, 5′-AGTCCACAA ATCCTAGGTGTA-3′, reverse, 5′-CTCGTTTCCGATCCAGCCA GG-3′; *IKKB*, forward, 5′-TGGAGCTGGTTACAGACGGAAG-3′, reverse, 5′-CAGCAGGAACCACCATGTGAGAG-3′; *GAPDH*, forward, 5′-ACCACAGTCCATGCCATCAC-3′, reverse, 5′-TCCACC CTGTTGCTGTA-3′.

For measurement of miR-708-5p and miR-101-3p, reverse transcription was performed using the TaqMan microRNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR reactions were conducted using the MystiCq microRNA qPCR assay primer (Sigma-Aldrich). RNU48 served as an internal control. The relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method.[33]

Plasmids

A fragment expressing LOXL1-AS1 was cloned to the pcDNA3.1(+) vector. The miR-708-5p and miR-101-3p expression plasmids were generated by cloning their precursor sequences to pSuper vector. The short hairpin RNA (shRNAs) targeting LOXL1-AS1 or nonspecific control shRNAs were inserted to the pLKO.1 vector. For generation of the LOXL1-AS1 luciferase reporter, the fragment encoding LOXL1-AS1 was cloned downstream of the pGL3 firefly luciferase coding sequence. To disrupt the potential miR-708-5p target site in *LOXL1-AS1*, site-directed mutagenesis was performed using a site-directed mutagenesis kit (New England Biolabs, Ipswich, MA, USA). An NF-κB luciferase reporter was purchased from Promega (Madison, WI, USA).

Cell Transfection

Cell transfection was performed using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's instructions. For generation of stable cell lines, transfected cells were selected using G418 (600 μg/mL) or puromycin (2 μg/mL) for 1–2 weeks.

Wound-Healing and Transwell Invasion Assays

Indicated cells were plated in six-well plates and allowed to grow to confluence. An artificial gap was created in the cell monolayer with a pipette tip. The cells were washed to remove cellular debris and cultured in complete medium containing 10% FBS for an additional 48 h. The wound area was photographed at 0 and 48 h after wounding. A Transwell invasion assay was performed using 24-well Transwell chambers (8.0 μm in pore size; Corning, Cambridge, MA, USA), which were coated with Matrigel (BD Biosciences, Bedford, MA, USA). Cells (5 × 104) were previously starved for 24 h and then plated in the top chamber in serum-free medium. The lower chamber was filled with DMEM supplemented with 10% FBS. After a 48-h incubation, the cells on the upper compartment were removed. The invaded cells on the bottom side of the inserts were stained with 0.2% crystal violet solution (Sigma-Aldrich) for 15 min and counted under an inverted microscope.

Animal Studies

Six-week-old female BALB/c nude mice were used in this experiment. Luciferase-labeled MDA-MB-231 cells (4 × 106 cells/mouse) transfected with the indicated constructs were injected into the mice via the tail vein. After 30 days, bioluminescence imaging was performed using the IVIS Spectrum *in vivo* imaging system (Caliper Life Sciences, PerkinElmer, Hopkinton, MA, USA). The animals were then sacrificed after bioluminescence imaging. Lungs were fixed, paraffin embedded, and sectioned. Hematoxylin and eosin (H&E) staining was performed on sections to analyze micrometastases. The procedure was carried out in accordance with the ethics guidelines and approved by the Institutional Animal Care and Use Committee of China Medical University.

Luciferase Reporter Assay

MCF7 cells were plated on a 24-well plate and co-transfected with *Renilla* luciferase-expressing pRL-TK vector, LOXL1-AS1 reporter vector, as well as miR-708-5p-expressing vector or empty vector. After a 24-h culture, cells were collected and tested for luciferase activities

Table 1. Association of LOXL1-AS1 Expression with Clinicopathological Parameters of Breast Cancer Patients

Parameter	n	LOXL1-AS1		p
		Low Expression (n = 34)	High Expression (n = 51)	
Age (years)				0.7036
≤45	27	10	17	
>45	58	24	34	
Tumor size (cm)				0.3176
≤2	33	11	22	
>2	52	23	29	
Estrogen receptor				0.8440
Negative	24	10	14	
Positive	61	24	37	
Progesterone receptor				0.4618
Negative	31	14	17	
Positive	54	20	34	
HER2				0.6017
Negative	65	27	38	
Positive	20	7	13	
TNM stage				0.0013
I	9	7	2	
II	43	21	22	
III	33	6	27	
Lymph node metastasis				0.0001
Negative	36	23	13	
Positive	49	11	38	

TNM, tumor/node/metastasis.

using the Dual-Luciferase reporter assay kit (Promega). Firefly luciferase activity was normalized on *Renilla* luciferase activity.

Similarly, NF-κB luciferase reporters together with LOXL1-AS1- and miR-708-5p-expressing plasmids were transfected to both MCF7 and T47D cells. Twenty-four hours after transfection, the cells were lysed and luciferase activities were measured.

RIP Assay

Ago2 immunoprecipitation was performed as described previously.[34] Briefly, MCF7 cells were lysed and incubated with anti-Ago2 antibody (Sigma-Aldrich) or mouse IgG for 4 h, which was followed by incubation with precleared protein A/G-Sepharose beads for 1 h. The immunoprecipitated RNA was extracted using TRIzol reagent, and qPCR was performed to analyze both LOXL1-AS1 and miR-708-5p levels.

RNA Pull-Down Assay

The pull-down assay was conducted as previously described.[35] Briefly, biotin-labeled LOXL1-AS1 (sense or antisense) was synthesized with a biotin RNA labeling mix and T7 RNA polymerase (Roche Diagnostics, Indianapolis, IN, USA). MCF7 cells were lysed and incubated with the biotinylated RNA at 4°C overnight. The biotin-coupled RNA complex was captured by streptavidin-coated magnetic beads (Invitrogen). The pull-down products were examined for EZH2 protein levels by western blot analysis.

Western Blot Analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. Cytoplasmic and nuclear extracts were prepared using a cell fractionation kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-EZH2, anti-p65, anti-lamin B1 (Cell Signaling Technology), anti-tubulin, or anti-GAPDH antibody (Santa Cruz Biotechnology, La Jolla, CA, USA) at 4°C overnight. After incubation with secondary antibodies, immunoreactive proteins were detected using the enhanced chemiluminescence (ECL) light-detecting kit (Amersham, GE Healthcare, Pittsburgh, PA, USA).

Cycloheximide (CHX) Chase Assay

For assessment of EZH2 protein turnover, MCF7 cells transfected with LOXL1-AS1-expressing plasmid or empty vector were treated with CHX (100 μg/mL; Sigma-Aldrich) for the indicated times. The cells were collected and subjected to western blot analysis.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed as previously described.[21] Briefly, MCF7 cells were transfected with LOXL1-AS1-expressing plasmid or empty vector and subjected to a ChIP assay using a ChIP assay kit (Upstate Biotechnology, Temecula, CA, USA) according to the manufacturer's specifications. Briefly, cells were cross-linked with 1% formaldehyde, lysed, and sonicated. Immunoprecipitation was carried out overnight at 4°C with anti-H3K27me3 or IgG control antibody (Abcam, Cambridge, MA, USA). Immunoprecipitated DNA was extracted and subjected to qPCR analysis. The PCR primers used are as follows: forward, 5′-GGTACTGTTGAGGGCTCTGC-3′, reverse, 5′-CCATTTTTAAATGCGGTCGT-3′.

Statistical Analysis

Data are represented as means ± standard deviation. Statistics were calculated using the Student's t test or one-way analysis of variance with a Tukey *post hoc* test. The correlation between LOXL1-AS1 and miR-708-5p expression in breast cancer was evaluated by Pearson correlation analysis. p values <0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.12.016.

AUTHOR CONTRIBUTIONS

P.X., F.J., and J.L. designed the experiments, processed the experimental data, and drafted the manuscript. H.-t.D., Q.L., T.Z., F.Y., Y.X., B.C., Y.W., and X.Z. conducted the experiments and analyzed

the data. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

1. Bertucci, F., Ng, C.K.Y., Patsouris, A., Droin, N., Piscuoglio, S., Carbuccia, N., Soria, J.C., Dien, A.T., Adnani, Y., Kamal, M., et al. (2019). Genomic characterization of metastatic breast cancers. Nature *569*, 560–564.

2. Racioppi, L., Nelson, E.R., Huang, W., Mukherjee, D., Lawrence, S.A., Lento, W., Masci, A.M., Jiao, Y., Park, S., York, B., et al. (2019). CaMKK2 in myeloid cells is a key regulator of the immune-suppressive microenvironment in breast cancer. Nat. Commun. *10*, 2450.

3. Sharma, S., Wu, S.Y., Jimenez, H., Xing, F., Zhu, D., Liu, Y., Wu, K., Tyagi, A., Zhao, D., Lo, H.W., et al. (2019). Ca^{2+} and CACNA1H mediate targeted suppression of breast cancer brain metastasis by AM RF EMF. EBioMedicine *44*, 194–208.

4. Cominetti, M.R., Altei, W.F., and Selistre-de-Araujo, H.S. (2019). Metastasis inhibition in breast cancer by targeting cancer cell extravasation. Breast Cancer (Dove Med. Press) *11*, 165–178.

5. McClements, L., Annett, S., Yakkundi, A., O'Rourke, M., Valentine, A., Moustafa, N., Alqudah, A., Simões, B.M., Furlong, F., Short, A., et al. (2019). FKBPL and its peptide derivatives inhibit endocrine therapy resistant cancer stem cells and breast cancer metastasis by downregulating DLL4 and Notch4. BMC Cancer *19*, 351.

6. Mathias, C., Zambalde, E.P., Rask, P., Gradia, D.F., and de Oliveira, J.C. (2019). Long non-coding RNAs differential expression in breast cancer subtypes: what do we know? Clin. Genet. *95*, 558–568.

7. Wang, C.J., Zhu, C.C., Xu, J., Wang, M., Zhao, W.Y., Liu, Q., Zhao, G., and Zhang, Z.Z. (2019). The lncRNA UCA1 promotes proliferation, migration, immune escape and inhibits apoptosis in gastric cancer by sponging anti-tumor miRNAs. Mol. Cancer *18*, 115.

8. Xu, S., Kong, D., Chen, Q., Ping, Y., and Pang, D. (2017). Oncogenic long noncoding RNA landscape in breast cancer. Mol. Cancer *16*, 129.

9. Di, W., Weinan, X., Xin, L., Zhiwei, Y., Xinyue, G., Jinxue, T., and Mingqi, L. (2019). Long noncoding RNA SNHG14 facilitates colorectal cancer metastasis through targeting EZH2-regulated EPHA7. Cell Death Dis. *10*, 514.

10. Mai, H., Zhou, B., Liu, L., Yang, F., Conran, C., Ji, Y., Hou, J., and Jiang, D. (2019). Molecular pattern of lncRNAs in hepatocellular carcinoma. J. Exp. Clin. Cancer Res. *38*, 198.

11. Wang, X., Chen, T., Zhang, Y., Zhang, N., Li, C., Li, Y., Liu, Y., Zhang, H., Zhao, W., Chen, B., et al. (2019). Long noncoding RNA Linc00339 promotes triple-negative breast cancer progression through miR-377-3p/HOXC6 signaling pathway. J. Cell. Physiol. *234*, 13303–13317.

12. Dong, H., Hu, J., Zou, K., Ye, M., Chen, Y., Wu, C., Chen, X., and Han, M. (2019). Activation of lncRNA TINCR by H3K27 acetylation promotes trastuzumab resistance and epithelial-mesenchymal transition by targeting microRNA-125b in breast Cancer. Mol. Cancer *18*, 3.

13. Sun, Q., Li, J., Li, F., Li, H., Bei, S., Zhang, X., and Feng, L. (2019). lncRNA LOXL1-AS1 facilitates the tumorigenesis and stemness of gastric carcinoma via regulation of miR-708-5p/USF1 pathway. Cell Prolif. *52*, e12687.

14. Wang, H., Li, L., and Yin, L. (2018). Silencing lncRNA LOXL1-AS1 attenuates mesenchymal characteristics of glioblastoma via NF-κB pathway. Biochem. Biophys. Res. Commun. *500*, 518–524.

15. Gao, R., Zhang, R., Zhang, C., Liang, Y., and Tang, W. (2018). lncRNA LOXL1-AS1 promotes the proliferation and metastasis of medulloblastoma by activating the PI3K/AKT pathway. Anal. Cell. Pathol. (Amst.) *2018*, 9275685.

16. Long, B., Li, N., Xu, X.X., Li, X.X., Xu, X.J., Liu, J.Y., and Wu, Z.H. (2018). Long non-coding RNA LOXL1-AS1 regulates prostate cancer cell proliferation and cell cycle progression through miR-541-3p and CCND1. Biochem. Biophys. Res. Commun. *505*, 561–568.

17. Zhang, B., Zhou, M., Zou, L., Miao, J., Wang, Y., Li, Y., Lu, S., and Yu, J. (2019). Long non-coding RNA LOXL1-AS1 acts as a ceRNA for miR-324-3p to contribute to cholangiocarcinoma progression via modulation of ATP-binding cassette transporter A1. Biochem. Biophys. Res. Commun. *513*, 827–833.

18. Senthil Kumar, K.J., Gokila Vani, M., Hsieh, H.W., Lin, C.C., Liao, J.W., Chueh, P.J., and Wang, S.Y. (2019). MicroRNA-708 activation by glucocorticoid receptor agonists regulate breast cancer tumorigenesis and metastasis via downregulation of NF-κB signaling. Carcinogenesis *40*, 335–348.

19. Chen, Y., Deng, X., Chen, W., Shi, P., Lian, M., Wang, H., Wang, K., Qian, D., Xiao, D., and Long, H. (2019). Silencing of microRNA-708 promotes cell growth and epithelial-to-mesenchymal transition by activating the SPHK2/AKT/β-catenin pathway in glioma. Cell Death Dis. *10*, 448.

20. Li, C.X., Li, H.G., Huang, L.T., Kong, Y.W., Chen, F.Y., Liang, J.Y., Yu, H., and Yao, Z.R. (2017). H19 lncRNA regulates keratinocyte differentiation by targeting miR-130b-3p. Cell Death Dis. *8*, e3174.

21. Ryu, S., McDonnell, K., Choi, H., Gao, D., Hahn, M., Joshi, N., Park, S.M., Catena, R., Do, Y., Brazin, J., et al. (2013). Suppression of miRNA-708 by polycomb group promotes metastases by calcium-induced cell migration. Cancer Cell *23*, 63–76.

22. Zhang, D.Y., Zou, X.J., Cao, C.H., Zhang, T., Lei, L., Qi, X.L., Liu, L., and Wu, D.H. (2018). Identification and functional characterization of long non-coding RNA *MIR22HG* as a tumor suppressor for hepatocellular carcinoma. Theranostics *8*, 3751–3765.

23. Zhu, G., Wang, S., Chen, J., Wang, Z., Liang, X., Wang, X., Jiang, J., Lang, J., and Li, L. (2017). Long noncoding RNA HAS2-AS1 mediates hypoxia-induced invasiveness of oral squamous cell carcinoma. Mol. Carcinog. *56*, 2210–2222.

24. Jani, K.S., Jain, S.U., Ge, E.J., Diehl, K.L., Lundgren, S.M., Müller, M.M., Lewis, P.W., and Muir, T.W. (2019). Histone H3 tail binds a unique sensing pocket in EZH2 to activate the PRC2 methyltransferase. Proc. Natl. Acad. Sci. USA *116*, 8295–8300.

25. Zhang, Y., Tang, B., Song, J., Yu, S., Li, Y., Su, H., and He, S. (2019). Lnc-PDZD7 contributes to stemness properties and chemosensitivity in hepatocellular carcinoma through EZH2-mediated ATOH8 transcriptional repression. J. Exp. Clin. Cancer Res. *38*, 92.

26. You, B.H., Yoon, J.H., Kang, H., Lee, E.K., Lee, S.K., and Nam, J.W. (2019). HERES, a lncRNA that regulates canonical and noncanonical Wnt signaling pathways via interaction with EZH2. Proc. Natl. Acad. Sci. USA *116*, 24620–24629.

27. Yu, G., Zhou, H., Yao, W., Meng, L., and Lang, B. (2019). lncRNA TUG1 promotes cisplatin resistance by regulating CCND2 via epigenetically silencing miR-194-5p in bladder cancer. Mol. Ther. Nucleic Acids *16*, 257–271.

28. Jin, L., Cai, Q., Wang, S., Wang, S., Mondal, T., Wang, J., and Quan, Z. (2018). Long noncoding RNA MEG3 regulates LATS2 by promoting the ubiquitination of EZH2 and inhibits proliferation and invasion in gallbladder cancer. Cell Death Dis. *9*, 1017.

29. Cao, J., Han, X., Qi, X., Jin, X., and Li, X. (2017). TUG1 promotes osteosarcoma tumorigenesis by upregulating EZH2 expression via miR-144-3p. Int. J. Oncol. *51*, 1115–1123.

30. Ramchandani, D., Lee, S.K., Yomtoubian, S., Han, M.S., Tung, C.H., and Mittal, V. (2019). Nanoparticle delivery of miR-708 mimetic impairs breast cancer metastasis. Mol. Cancer Ther. *18*, 579–591.

31. Jiang, L., Ren, L., Zhang, X., Chen, H., Chen, X., Lin, C., Wang, L., Hou, N., Pan, J., Zhou, Z., et al. (2019). Overexpression of PIMREG promotes breast cancer aggressiveness via constitutive activation of NF-κB signaling. EBioMedicine *43*, 188–200.

32. Sirinian, C., Papanastasiou, A.D., Schizas, M., Spella, M., Stathopoulos, G.T., Repanti, M., Zarkadis, I.K., King, T.A., and Kalofonos, H.P. (2018). RANK-c attenuates

aggressive properties of ER-negative breast cancer by inhibiting NF-κB activation and EGFR signaling. Oncogene *37*, 5101–5114.

33. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. Methods *25*, 402–408.

34. Shi, L., Hong, X., Ba, L., He, X., Xiong, Y., Ding, Q., Yang, S., and Peng, G. (2019). Long non-coding RNA ZNFX1-AS1 promotes the tumor progression and metastasis of colorectal cancer by acting as a competing endogenous RNA of miR-144 to regulate EZH2 expression. Cell Death Dis. *10*, 150.

35. Zhao, C.C., Jiao, Y., Zhang, Y.Y., Ning, J., Zhang, Y.R., Xu, J., Wei, W., and Kang-Sheng, G. (2019). Lnc SMAD5-AS1 as ceRNA inhibit proliferation of diffuse large B cell lymphoma via Wnt/β-catenin pathway by sponging miR-135b-5p to elevate expression of APC. Cell Death Dis. *10*, 252.