Lnc NR2F1-AS1 promotes breast cancer metastasis by targeting miR-25-3p/ZEB2 axis

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Abstract

Background: Long noncoding RNAs (LncRNAs) has been demonstrated to have a considerable effect on tumor metastasis and are aberrantly expressed in a variety of cancers. Nevertheless, its role in breast cancer (BC) remains incompletely inventoried.

Methods: The microarray assay of differentially expressed lncRNAs in epithelial-mesenchymal transition (EMT) and non-EMT cells was performed. The prognostic value of lnc NR2F1-AS1 expression in BC patients were analyzed in the TCGA database. Lnc NR2F1-AS1 expression level in different BC cell line were assessed by qPCR. The role of lnc NR2F1-AS1 in BC cell metastasis was investigated in vitro and in vivo. Dual luciferase reporter assay and RNA immunoprecipitation (RIP) were performed to uncover the relationship between lnc NR2F1-AS1, miR-25-3p, and ZEB2.

Results: High level of lnc NR2F1-AS1 was observed in BC cells undergoing EMT and was closely correlated with adverse prognosis in BC patients. Lnc NR2F1-AS1 knockdown notably inhibited BC cell migration, invasiveness in vitro and metastasis in vivo. Mechanistically, lnc NR2F1-AS1 competitively bind with miR-25-3p to impede the degradation of ZEB2, which is a positive EMT transcription factor in BC.

Conclusions: Taken together, our study reveals a novel lnc NR2F1-AS1/miR-25-3p/ZEB2 axis for BC metastasis and indicate that lnc NR2F1-AS1 may serve as a potential therapeutic target for breast cancer metastasis.

Introduction

The most prevalent cancer among women worldwide is breast cancer (BC) [1, 2]. Although therapeutic attempts have primarily focused on preventing progression of BC, the prognosis of metastatic BC patients remains poor. Metastasis is one of the pivotal causes of high BC mortality, which consist of epithelial-to-mesenchymal transition (EMT), migration, invasion and mesenchymal-to-epithelial transition (MET)[3]. Hence, it is crucial to investigate the underlying mechanisms triggering this process.

In the past, non-coding RNA was considered to be transcription noise. Recently, accumulated evidences have revealed that non-coding RNA plays an essential role in various physiological and pathological processes[4–6]. LncRNA has a length exceeding 200 nucleotides and is not capable of coding for proteins[7]. Researchers from all over the world have uncovered that IncRNAs are modulated in tumors and are closely related to the progression of several solid cancers. LncRNA MCM3AP-AS1 was found to promote hepatocellular carcinoma growth through miR-194-5p/FOXA1 pathway. Moreover, IncRNA MEG3 was shown to regulate melanoma growth, metastasis and formation[8–10]. Previously, we found that Inc NR2F1-AS1 could promote angiogenesis of BC by targeting IGF-1/IGF-1R/ERK axis[11]. However, the effects of Inc NR2F1-AS1 on the regulation of BC metastasis remain elusive.
In our present study, we explored the biological functions, molecular mechanism and prognostic value of lnc NR2F1-AS1 in BC. Our results have uncovered that lnc NR2F1-AS1 was significantly increased in BC cells, especially in invasive BC cells, and correlated with poor outcome of BC patients. Inhibition of lnc NR2F1-AS1 resulted in decreased migration, invasion and EMT capacities. On the contrary, overexpression of lnc NR2F1-AS1 found the opposite result. Mechanistically, lnc NR2F1-AS1 could act as a competing endogenous RNA (ceRNA) for miR-25-3p to upregulate ZEB2 expression. Our data demonstrated that lnc NR2F1-AS1/miR-25-3p/ZEB2 axis could be a promising therapeutic target for BC metastasis.

**Materials And Methods**

**Clinical sample**

Twenty-two pairs of breast cancer tissues and adjacent tissues were taken from patients who received treatment in the First Affiliated Hospital of Sun Yat-sen University. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. All enrolled patients signed the written informed consent form according to the relevant regulations. The tissues and matched adjacent-tumor controls were snap-frozen immediately in liquid nitrogen after being separated and stored at -80 °C before use.

**Bioinformatics**

The KM plotter (http://kmplot.com/analysis/) was used to evaluate the survival prognosis of TCGA BC patients. StarBase (http://starbase.sysu.edu.cn/) were used to predict the lncRNA-miRNA interactions, the miRNA target genes and their correlations. KEGG pathway genome enrichment analysis was conducted by R language.

**Animal model**

The breast cancer metastasis assay was conducted in female nude mice (5~6 weeks old). All the procedures involving the animal experiments were performed in accordance with the Guide for the Administration of Affairs Concerning Experimental Animals. $1 \times 10^6$ cells were injected into the tail vein of nude mice. The lungs were removed 3 weeks after inoculation and metastatic nodules were counted. The whole lung was removed and fixed in paraformaldehyde, embedded in paraffin and subjected to hematoxylin-eosin staining. Numbers of lung metastatic foci were calculated to evaluate the development of pulmonary metastasis.

**Cell Culture**

The human breast epithelial cell line (MCF-10A) and breast cancer cell lines (MDA-MB-231, BT549, MCF-7, ZR-75-1) were obtained from ATCC (Rockville, MD, USA) and cultured in DMEM culture medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA). All cells were captured in the atmosphere.
containing 5% CO₂ and 37 °C. The overexpression plasmids, pcDNA3.1-NR2F1-AS1 and pcDNA3.1-ZEB2, pGPH1-sh-NR2F1 -AS1 and pGPH1-si-ZEB2 were purchased from GenePharma, Co., Ltd. The mimics and inhibitor of hsa-miR-25b-3p were provided by RiboBio. Cell transfection was operated using Lipo3000 reagent according to the manufacturer’s instructions.

**RNA isolation and real-time quantitative PCR (RT-qPCR)**

LncRNAs and mRNAs were extracted from cells by Trizol (Thermo Fisher Scientific, Inc.). MiRNAs were extracted by the miRNA Extraction Kit purchased from Tiangen according to the instruction. A PrimeScript RT Reagent kit (Takara Bio Inc.) was used to synthesize cDNA from lncRNA and mRNA. miRNAs were reverse transcribed by miRNA reverse transcription kit (Takara Bio Inc.). RT-qPCR analysis was performed in triplicate for each sample using TB Green® Fast qPCR Mix (Takara Bio Inc.) with GAPDH/U6 as the endogenous control. A two-step cycling condition was selected on Roche 480II following the instruction. Data was analyzed using the $2^{-\Delta\Delta Cq}$ method. The primers used are shown in Supplementary Table.

**Subcellular fractionation and fluorescence in situ hybridization (FISH)**

Nuclear and cytoplasmic lncRNA was separated using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Invitrogen, USA), and was subject to quantitative using RT-qPCR assay. For FISH assays, cells were immobilized, permeabilized and hybridized with 50 μl LGALS8-AS1 probe mix (RiboBio, China). Cells nuclei were stained with DAPI (Sigma-Aldrich). Intracellular distribution was observed by fluorescence microscopy (Olympus IX81, Japan). The probe is shown in Supplementary Table.

**Western blotting**

Total protein extracted from breast cancer cells and tissues were lysated by lysis buffer (Beyotime, China) with protease inhibitor. Protein lysates were separated by 10% SDS gel electrophoresis, and transferred to a PVDF membrane (Millipore, USA). After blocking in 5% BSA for 1 h at room temperature, the membrane was incubation with primary antibodies at 4°C overnight and followed by 1 h second antibodies at room temperature. The bands were imaged by ECL assay reagents (Beyotime, China). The primary antibodies were as follows: anti-E-cadherin (1:500; 20874-1-AP; Proteintech Group, Inc.), anti-N-cadherin (1:500; 22018-1-AP; Proteintech Group, Inc.), anti-Vimentin (1:1000; 10366-1-AP; Proteintech Group, Inc.), anti-ZEB1 (1:1000; 21544-1-AP; Proteintech Group, Inc.), anti-ZEB2 (1:1000; 67514-1-Ig; Proteintech Group, Inc.).

**Luciferase reporter assay**

Sequences of the wild-type or mutant NR2F1-AS1 fragment or ZEB2 3′-UTR containing the predicted binding sites of miR-25-3p were subcoloned into a psiCHECK2 dual-luciferase vector (Promega Corporation). The luciferase reporter plasmids were co-transfected into HEK293T with miR-25-3p mimics or negative control (NC) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer’s instructions and the transfected cells were cultured at 37°C in a humidified incubator.
with 5% CO₂ for 36 h. Luciferase signals were measured using the Dual-Glo® Luciferase Assay System (Promega Corporation) according to the manufacturer’s instructions. The activity of luciferase was detected by Synergy 2 Multidetector Microplate Reader (BioTek Instruments Inc., USA).

**Transwell assay**

Proper number of cells with serum-free conditioned medium were seeded into upper chamber coated with Matrigel (BD Biosciences, USA). Complete medium was added into the lower chambers. After maintaining for 24 h at 37°C, cells on the upper chamber were removed and the invaded cells were treated with 4% paraformaldehyde (FD, China) and stained with crystal violet. The cells were observed and counted under a microscope.

**Wound healing assay**

Cells were seeded in 6-well plates and cultured to the sub-confluent state. After starvation in serum-free DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) for 24 h, a straight wound was scratched at the bottom of the plate with a 200 μL sterile pipette tip. The cells were cultured in serum-free medium for 24 h and observed at 0 and 24 h, with an inverted light microscope. Scratch-healing (%) = (initial scratch area-final scratch area)/initial scratch area x 100.

**RNA immunoprecipitation (RIP) assay**

RIP assay was carried out using Magna RIP RNA-Binding Protein Immunoprecipitation Kit following manufacturer’s protocol (Millipore, USA). In brief, cells were lysed in RIP Lysis buffer, then the supernatant was transferred to a nuclease-free tube on ice, and added the resuspended beads, which were incubated with Ago2 or IgG. The bead-bound immunoprecipitated was eluted with elution buffer and the purified RNA fraction was analyzed by RT-qPCR.

**Statistical analysis**

Data in this research were presented as mean ± standard deviation (SD). Comparisons between two groups were conducted using unpaired Student’s t-tests. One-way or two-way ANOVA followed by Tukey’s post hoc test was used for comparing differences between more than two groups. The results were presented using GraphPad Prism 9.0. All experiments were repeated three times. P<0.05 was considered as statistically significant.

**Results**

**Lnc NR2F1-AS1 is upregulated and correlated with poor prognosis of BC patients**

We first sought to identify essential lncRNAs that potentially involve in BC metastasis. Our previous lncRNA microarray (GSE201574) showed that lnc NR2F1-AS1 is upregulated in BC cells undergoing EMT. We then validated the expression of lnc NR2F1-AS1 in normal breast cell (MCF-10A), non-invasive BC cell
(MCF-7, ZR-75-1) and invasive BC cell (MDA-MB-231, BT549). RT-qPCR results showed that Inc NR2F1-AS1 was significantly increased in BC cells, especially in invasive BC cells (Fig 1A). Further prognostic analysis of Inc NR2F1-AS1 suggested that high level of Inc NR2F1-AS1 is associated with adverse OS (overall survival) and RFS (Recurrence free survival) of BC patients depending on Kaplan-Meier analysis (Fig 1B). The subcellular distribution assay showed that Inc NR2F1-AS1 was mainly located in the cytoplasm (Fig 1C), which was further validated by FISH assay (Fig 1D). These findings demonstrated that Inc NR2F1-AS1 might participate in the progression of BC.

Inhibition of Inc NR2F1-AS1 suppressed cell migration, invasiveness and tumor metastasis in BC

To investigate the functions of Inc NR2F1-AS1 in BC cells, we modulated Inc NR2F1-AS1 expression in MDA-MB-231 and MCF-7 cells by infection of lentivirus. RT-qPCR showed that Inc NR2F1-AS1 expression markedly downregulated in shLnc infected MDA-MB-231 cells and upregulated in ovlnc infected MCF7 cells compared with control (Fig 2A). Wound healing assay revealed that Inc NR2F1-AS1 inhibition notably reduced cell migration ability and Inc NR2F1-AS1 overexpression significantly promotes the potential of migration (Fig 2B-C). Moreover, we investigated whether Inc NR2F1-AS1 was participated in cell invasion. Transwell invasion assay indicated that cell invasion was dramatically decreased in Inc NR2F1-AS1 knockdown MDA-MB-231 cells and increased in Inc NR2F1-AS1 overexpression MCF-7 cells (Fig 2D-E). In animal model, our results also demonstrated that inhibition of Inc NR2F1-AS1 significantly suppressed BC lung metastasis (Fig 2F-G). Collectively, these results revealed that suppression of Inc NR2F1-AS1 inhibited BC metastasis in vitro and in vivo.

Lnc NR2F1-AS1 promotes EMT of BC cell

To further investigate how Inc NR2F1-AS1 regulate BC metastasis, we performed KEGG analysis. The results found that Inc NR2F1-AS1 is closely related to focal adhesion and TGF-beta pathway (Fig 3A). Since EMT is an essential downstream of TGF-beta pathway[12, 13], we tested whether Inc NR2F1-AS1 modulation affected EMT in BC cells. Our data showed that knockdown of Inc NR2F1-AS1 upregulated the expressions of epithelial markers (E-cadherin) and downregulated mesenchymal markers (N-cadherin, Vimentin, ZEB2). On the contrary, we observed that overexpression of Inc NR2F1-AS1 decreased epithelial markers expression and increased mesenchymal markers expression (Fig 3B-D). Moreover, Inc NR2F1-AS1 expression was positively associated with ZEB1 and ZEB2 expression in TCGA database analysis (Fig 3E-F). Taken together, these findings suggested that Inc NR2F1-AS1 promotes BC metastasis by inducing EMT.

Lnc NR2F1-AS1 acts as a molecular sponge for miR-25-3p in BC cells

A variety of researches have indicated that lncRNAs in cytoplasm could act as competing endogenous RNAs (ceRNAs) to modulate the expressions and activities of miRNAs in recent years[14, 15]. According to our data, Inc NR2F1-AS1 was mainly distributed in cytoplasm of BC cells (Fig 1C, D). Thus, we presumed that Inc NR2F1-AS1 might act as ceRNA to prevent miRNAs from degrading their target mRNAs. By using bioinformatic software, we identified miR-25-3p as a potential target of Inc NR2F1-AS1.
Additionally, there was an inverse correlation between lnc NR2F1-AS1 and miR-25-3p in TCGA database and our cohort (Fig 4A, B). To confirm the binding potential, dual luciferase reporter assay was conducted. Overexpression of miR-25-3p obviously decreased the luciferase activity of the pmirGLO-NR2F1-AS1-WT vector but not pmirGLO-NR2F1-AS1 mutant vector (Fig. 4C, D). We also observed that endogenous Inc NR2F1-AS1 was pulled down by AGO2 antibody and overexpression of miR-25-3p mimics significantly upregulated Inc NR2F1-AS1 binding to AGO2 antibody in the RNA immunoprecipitation assay (Fig. 4E-F). Furthermore, Inc NR2F1-AS1 inhibition promoted miR-25-3p expression, whereas Inc NR2F1-AS1 overexpression suppressed miR-25-3p expression (Fig 4G). Those data demonstrated that miR-25-3p is an inhibitory target of Inc NR2F1-AS1 in BC.

Lnc NR2F1-AS1 promotes ZEB2 expression via suppression of miR-25-3p

Using starbase database, we discovered that ZEB2 was a potential target of miR-25-3p. ZEB2 was reported as a key transcription factor regulating EMT of BC[16, 17]. However, the relationship between Inc NR2F1-AS1/miR-25-3p and ZEB2 in BC remain poorly understood. We discovered that the expression of ZEB2 was negatively associated with miR-25-3p expression in TCGA databases (Fig 5A). We also found an inverse correlation between ZEB2 and miR-25-3p or Inc NR2F1-AS1 in our cohort (Fig 5B). Thus, ZEB2 was selected as target of miR-25-3p for further investigation. The predicted binding site of miR-25-3p on ZEB2 mRNA was shown in Fig 5C. Our luciferase assay indicated that miR-25-3p overexpression reduced the luciferase activity of the WT ZEB2 vector but not the mutant ZEB2 vector (Fig 5D). Moreover, mRNA level of ZEB2 was downregulated by Inc NR2F1-AS1 inhibition and was reversed by miR-25-3p inhibitor. We also observed that ZEB2 mRNA expression was increased by Inc NR2F1-AS1 overexpression and was retrogressed by miR-25-3p mimic (Fig 5E). Finally, transwell invasion assay and WB assay showed that miR-25-3p inhibition or ZEB2 overexpression significantly promoted BC invasion and EMT capacities suppressed by Inc NR2F1-AS1 knockdown. Meanwhile, we observed that miR-25-3p overexpression or ZEB2 inhibition dramatically decreased Inc NR2F1-AS1 overexpression induced BC invasion and EMT (Fig 5F-H). Overall, our above data revealed that Inc NR2F1-AS1 functions as a ceRNA to promote ZEB2 level by competitively binding to miR-25-3p, and ultimately enhance BC cell EMT, migration and invasion.

Discussion

The therapeutic methods effective to patients with metastatic BC are limited. Thus, there is a clinical need for comprehensively understanding the specific mechanisms behind BC metastasis[18, 19]. Recently, lncRNAs have been considered as essential pathogenic regulator in the pathogenesis of cancer progression. A growing body of evidences have revealed the functions and modulation of lncRNAs to identify potential targets for BC treatment[20–22]. Lnc NR2F1-AS1, an antisense lncRNA of NR2F1, is also known as COUP Transcription Factor I. High level of lnc NR2F1-AS1 has been demonstrated to promote progression of gastric cancer and hepatocellular carcinoma[23, 24]. In our current study, Inc NR2F1-AS1 was upregulated in BC tissues and cells, and high Inc NR2F1-AS1 expression was closely related to poor prognosis of BC patients. Functional studies discovered that inhibition of Inc NR2F1-AS1
could suppress migration, invasion and EMT of BC cell in vitro and lung metastasis in vivo, which suggesting a carcinogenic role of Inc NR2F1-AS1 in BC.

Subcellular distribution is crucial to understanding the biological function of IncRNAs. Researchers have reported that cytoplasmic IncRNAs is involved in posttranscriptional gene regulation, including by serving as ceRNA and preventing downstream target mRNA from degradation[25–27]. In cell cytoplasmic/nuclear fractionation and FISH assays, we found that Inc NR2F1-AS1 was mainly located in the cytoplasm of BC cells. Therefore, we presumed that Inc NR2F1-AS1 may act as a miRNA sponge in BC. MiR-25-3p was previously shown to be a tumor suppressor in colorectal cancer and cervical cancer[28, 29]. In BC, we found that miR-25-3p expression was negatively associated with Inc NR2F1-AS1 in BC tissues from TCGA database and our cohort. Next, we identified the specific binding sites between miR-25-3p and Inc NR2F1-AS1 by using three online bio-informatic tools (RNAhybrid, Starbase v2.0 and Targetscan), which was further validated by RIP assay and dual luciferase reporter assay. Moreover, miR-25-3p significantly reversed modulation effect of BC invasion and EMT induced by Inc NR2F1-AS1 inhibition or overexpression. Our results confirmed the antitumor effect of miR-25-3p in cancer, and investigated more about its functions in BC EMT.

Transcriptional factor ZEB1 and ZEB2, key regulator of cell invasion and EMT, have been investigated in several solid cancers including BC. ZEB1 and ZEB2 was shown to exert its EMT-inducing function through transcription repression on E-cadherin gene[30–32]. In our research, our data showed that Inc NR2F1-AS1 upregulated ZEB1 and ZEB2 expression in BC. Furthermore, we confirmed that ZEB2 was a direct target of miR-25-3p in our further validation. Lnc NR2F1-AS1 could positively modulate ZEB2 level through sponging to miR-25-3p. The biological effects induced by Inc NR2F1-AS1 inhibition could be reversed by overexpression of ZEB2 in BC cells, highlighting the Inc NR2F1-AS1/miR-25-3p/ZEB2 axis in promoting BC cell migration, invasion and EMT. Besides, we also tested the association between ZEB1 and miR-25-3p and gained negative result(data not shown). Thus, Inc NR2F1-AS1 may upregulate ZEB1 expression through other mechanisms, which needs to be further investigated.

Conclusions

In summary, we identified a novel Inc NR2F1-AS1, which was upregulated in BC and correlated with adverse prognosis of BC patients. Lnc NR2F1-AS1 functioned as a sponge for miR-25-3p and prominently contributed to BC EMT and metastasis by activating ZEB2 expression. The finding of the Inc NR2F1-AS1/miR-25-3p/ZEB2 axis might be a potential therapeutic target for BC metastasis.

Declarations

Acknowledgements

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Conflicts of interest

The authors declare no conflicts of interest.

References

15. Yang XZ et al (2018) LINC01133 as ceRNA inhibits gastric cancer progression by sponging miR-106a-3p to regulate APC expression and the Wnt/beta-catenin pathway. Mol Cancer 17(1):126

Figures

Figure 1
Lnc NR2F1-AS1 upregulation is associated with poor prognosis in BC. A RT-qPCR was used to detect lnc NR2F1-AS1 expression in normal breast cell line and BC cell line. GAPDH was the internal control (n=3 independent cultures). B Kaplan-Meier analysis demonstrated the relationship between lnc NR2F1-AS1 expression and overall survival (n=943) or recurrence-free survival (n=2032) of BC patients. C The expression level of lnc NR2F1-AS1 in the subcellular fractions of MCF7 cells and MDA-MB-231 cells was determined by qRT-PCR. GAPDH and U6 were used as cytoplasmic and nuclear markers, respectively. D The localization of lnc NR2F1-AS1 (Green) in MCF-7 and MDA-MB-231 cells was detected by FISH assay. Nuclei (blue) was stained by DAPI. Data are presented as the means±SEM, ***p<0.001 (unpaired, two-tailed Student’s t-test).

Figure 2

Lnc NR2F1-AS1 promotes cell migration, invasiveness and tumor metastasis in BC. A The expression level of lnc NR2F1-AS1 in MCF-7 and MDA-MB-231 cells after overexpression or inhibition of lnc NR2F1-AS1 were detected by RT-qPCR (n=3 independent cultures). B, C The effects of lnc NR2F1-AS1 overexpression or knockdown on BC cell migration were determined by wound healing assay (n=3 independent cultures). D, E The level of invasiveness after overexpression or inhibition of lnc NR2F1-AS1 in MCF-7 and MDA-MB-231 cells were detected by transwell assay (n=3 independent cultures). F, G Lung metastasis of BC in mice was measured by HE staining after lnc NR2F1-AS1 knockdown (n=5 mice per group). Data are expressed as the means±SEM, *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 (unpaired, two-tailed Student’s t-test for A, E, G; one-way ANOVA with Tukey’ s post hoc test for C).

Figure 3

Lnc NR2F1-AS1 promotes EMT of BC cell. A KEGG pathway analysis of high and low lncRNA NR2F1-AS1 expression cells. B RT- qPCR analysis of the mRNA expression of epithelial markers (E-cadherin) and mesenchymal markers (N-cadherin, Vimentin, ZEB2) upon LncRNA NR2F1- AS1 overexpression or knockdown (n=3 independent experiments). C, D Protein level of epithelial markers and mesenchymal markers in MCF-7 and MDA-MB-231 cells after overexpression or inhibition of lnc NR2F1-AS1 were detected by Western blooting and immunofluorescence. E=F Pearson's correlation analysis was used to explore the association between expression of lnc NR2F1-AS1 and ZEB1 or ZEB2. Data are presented as the means±SEM, *p< 0.05, **p< 0.01, ***p< 0.001 (one-way ANOVA with Tukey’ s post hoc test).

Figure 4
**Lnc NR2F1-AS1 functions as a miR-25-3p sponge in BC cells.**

A, B Correlation analysis was applied to evaluate the relationship between expression of Lnc NR2F1-AS1 and miR-25-3p in BC tissues from TCGA database (n=1085) and our cohort (n=22). C The putative binding site for miR-25-3p in Lnc NR2F1-AS1 and mutant sequences of the binding sites were shown. D Luciferase assays in HEK293T cells cotransfected with WT or mutant Lnc NR2F1-AS1 and miR-25-3p NC or miR-25-3p mimic (n=3 independent experiments). E Anti-AGO2 RIP was performed in HEK293T cells, followed by RT-qPCR to examine the expression of Lnc NR2F1-AS1 associated with AGO2 (n=3 independent experiments). F RT-qPCR was used to determine the effect of miR-25-3p NC or miR-25-3p mimic on the expression of Lnc NR2F1-AS1 associated with AGO2. (n=3 independent experiments). G RT-qPCR analysis of the mRNA expression of miR-25-3p in Lnc NR2F1-AS1 overexpressed MCF-7 cells and Lnc NR2F1-AS1 inhibited MDA-MB-231 cells (n=3 independent experiments). Data are expressed as the means±SEM, **p< 0.01, ***p< 0.001, ****p< 0.0001 (unpaired, two-tailed Student’ s t-test for G; one-way ANOVA with Tukey’ s post hoc test for D, E, F).

**Figure 5**

**Lnc NR2F1-AS1 upregulates ZEB2 expression via inhibition of miR-25-3p.**

A Correlation analysis was applied to assess relationship between ZEB2 and miR-25-3p in BC tissues from TCGA database. B The association between ZEB2 and Lnc NR2F1-AS1 or miR-25-3p were determined by Pearson’s correlation analysis. C The putative binding site for miR-25-3p in ZEB2 and mutant sequences of the binding sites. D Luciferase reporter assays in HEK293T cells cotransfected with WT or mutant ZEB2 and miR-25-3p NC or miR-25-3p mimic (n=3 independent experiments). E, F The effects of ov Lnc NR2F1-AS1 and miR-25-3p mimic cotransfection or sh Lnc NR2F1-AS1 and miR-25-3p inhibitor cotransfection on ZEB2 expression were detected by qPCR (n=3 independent experiments). G, H The effects of ov Lnc NR2F1-AS1 and miR-25-3p mimic cotransfection or sh Lnc NR2F1-AS1 and miR-25-3p inhibitor cotransfection on cell invasiveness were measured by transwell assay (n=3 independent experiments). I Western blotting was used to detect protein level of epithelial markers and mesenchymal markers in MCF7 cells cotransfected with miR-25-3p mimic or siZEB2 and ov Lnc NR2F1-AS1 and MDA-MB-231 cotransfected with miR-25-3p inhibitor or ZEB2 and shlnc NR2F1-AS1. Data are presented as the means±SEM, ***p< 0.001, ****p< 0.0001 (one-way ANOVA with Tukey’s post hoc test).
Figure 6

Schematic diagram of lnc NR2F1-AS1 function in BC.

Supplementary Files

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