The EZH2–PHACTR2-AS1–Ribosome Axis induces Genomic Instability and Promotes Growth and Metastasis in Breast Cancer

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ABSTRACT

Aberant activation of histone methyltransferase EZH2 and ribosome synthesis strongly associate with cancer development and progression. We previously found that EZH2 regulates RNA polymerase III–transcribed 5S ribosomal RNA gene transcription. However, whether EZH2 regulates ribosome synthesis is still unknown. Here, we report that EZH2 promotes ribosome synthesis by targeting and silencing a long noncoding RNA PHACTR2-AS1. PHACTR2-AS1 directly bound ribosome DNA genes and recruited histone methyltransferase SUV39H1, which in turn triggered H3K9 methylation of these genes. Depletion of PHACTR2-AS1 resulted in hyperactivation of ribosome synthesis and instability of ribosomal DNA, which promoted cancer cell proliferation and metastasis. Administration of PHACTR2-AS1-30nt-RNA, which binds to SUV39H1, effectively inhibited breast cancer growth and lung metastasis in mice. PHACTR2-AS1 was downregulated in breast cancer patients, where lower PHACTR2-AS1 expression promoted breast cancer development and correlated with poor patient outcome. Taken together, we demonstrate that PHACTR2-AS1 maintains a H3K9 methylation-marked silent state of ribosomal DNA genes, comprising a regulatory axis that controls breast cancer growth and metastasis.

Significance: These findings reveal that EZH2 mediates ribosomal DNA stability via silencing of PHACTR2-AS1, representing a potential therapeutic target to control breast cancer growth and metastasis.

Introduction

Ribosomal biogenesis is a highly ordered multistage process involving ribosomal DNA (rDNA) transcription, ribosomal protein synthesis, and ribosomal assembly (1). In humans, rDNA genes are tandemly repeated (300–400 copies) in nucleolar organizer regions, and approximately half of the copies are transcriptionally silent (2). Moderate silencing of rDNA transcription is critical for genome stability. Disrupting rDNA silencing induces abnormal stimulation of rRNA synthesis, leading to uncontrolled cell proliferation or malignant cell transformation, and eventual tumorigenesis (3–5). In cancer cells, some oncogenic and tumor-suppressive pathways modulate rDNA transcription (6–9).

Long noncoding RNAs (lncRNAs) comprise a large class of regulatory RNAs without protein-coding potential that are >200 nucleotides long (10, 11). LncRNAs regulate cancer development and progression by influencing cell proliferation, metastasis, metabolism, and self-renewal (12–15). Histone methylation is an important means of lncRNA-mediating gene expression (16). The histone methyltransferase SUV39H1 catalyzes H3K9 methylation at repetitive DNA sequences and regulates heterochromatin formation. RNA plays a critical role in targeting SUV39H1 to DNA sequences. Chromatin-associated RNA mediates the association of SUV39H1 with pericentric DNA (17). The telomeric lncRNA TERRA targets SUV39H1 to telomeres, promoting H3K9me3 accumulation at damaged telomeres, and modulating telomere structures (18). Heterochromatin is formed at pericentromeres, telomeres, and rDNA loci. However, whether lncRNAs participate in targeting SUV39H1 to rDNA repeats is unknown.

Here, we report that EZH2-regulated lncRNA PHACTR2-AS1 mediates SUV39H1 localization to rDNA repeats and induces H3K9 methylation of rDNA, helping to suppress rRNA transcription. Moreover, PHACTR2-AS1 displays efficacy against tumor growth and metastasis in vivo. Our findings provide insight into the potential of PHACTR2-AS1 as a therapeutic target in breast cancer.

Materials and Methods

Cell culture and transfection

MCF7, MDA-MB-231, Hs578T, and HEK-293A cell lines were obtained from the Cell Resource Center, Peking Union Medical College (the Headquarter of National Infrastructure of Cell Line Resource, NSTI). The species origin of the cell lines was confirmed with PCR. The identity of the cell lines was authenticated with short tandem repeat profiling. The cell lines were checked free of Mycoplasma contamination by PCR. Cell stocks were created within five passages, and all experiments were completed within ten passages. The same batch of cells was thawed every 1 to 2 months. The cells were grown in DMEM (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS at 37°C under 5% CO2 in a humidified incubator.
Chu et al.

Plasmids were transfected using Lipofectamine 2000 (#11668019, Invitrogen; Thermo Fisher Scientific). ASOs and siRNAs were transfected using RNAiMAX Transfection Reagent (#13778150, Invitrogen; Thermo Fisher Scientific). Sequences of shRNAs and siRNAs were listed:

- PHACTR2-AS1-shRNA-1: GCAGTAAATACCAGCACTCTCT
- PHACTR2-AS2-shRNA-1: CCACC TACTCCTGGGAAGA T; Negative Control: AACAGCTTATACC; PHACTR2-AS2-AO-1: GCTTTGCAAAGGTTAAT; PHACTR2-AS2-AO-2: AACTAGCAATTTAGTGG; PHACTR2-AS2-AO-3: CTTGAAATGAAAGG; SUV39H1 siRNA-1: UCUUGGCGAAAGAACGAGCG; SUV39H1 siRNA-2: UUCUUUGCCAAUUCUCACGGG; siRNA-pools of EZH2, c-myc, Twist, YY1, and E2F6 includes 4 DharmaFECT siRNAs (Thermo Fisher Scientific).

**Tumor specimens and in situ hybridization**

The tissue microarrays of patients with breast cancer were purchased from National Human Genetic Resources Sharing Service Platform 2005DKA21300 (Shanghai Outdo Biotechnology Company Ltd.). Tissue sections were deparaffinized and rehydrated gradually. After being digested with pepsin, tissue samples were hybridized with 5′ biotin-labeled L NatM-modified PHACTR2-AS1 probe (Exiqon) at 54°C overnight, and subsequently streptavidin conjugated to Poly-Horseradish Peroxidase (Poly-HRP) detecting kit was applied (SP-9002, Zhong Shan Jin Qiao, Beijing, China). The scoring of staining was performed by three investigators including a pathologist. Criteria for assessing staining intensities of PHACTR2-AS1 were classified into four levels: 0 = no staining, 1 = low staining, 2 = media staining, and 3 = strong staining.

**Tumor xenografts and IVIS Lumina imaging**

MDA-MB-231-Luc-D3H2LN cells were infected with lentiviruses carrying empty LV5 vector (GenePharma) or PHACTR2-AS1-LV5. Cells were inoculated orthotopically onto the abdominal mammary fat pad (1.5 × 10⁶ cells) or injected into the lateral tail vein (5 × 10⁶ cells) of 6-week-old female NOD-SCID mice. After 4 weeks, bioluminescence imaging was performed. Mice were injected with D-luciferin (100 mg/mL) for 15 minutes, then 2 × 10⁶ cells were lysed in 500 μL lysis buffer (9). After 30-minute incubation on ice, the samples were centrifuged at 13,000 rpm at 4°C for 10 minutes. For fractionation, the lysates were loaded on 15%–45% sucrose gradients and separated by ultracentrifugation with a SW40 rotor (Beckman) at 39,000 rpm at 4°C for 3 hours. Linear sucrose gradients were prepared with a Gradient Master (Biocomp). The distribution of ribosomes on the gradients was recorded using BIOCOMP Mike Gradient Fractionator equipped with BIO-RAD ECONO UV Monitor (set at 260 nm).

**RNA pull-down assay**

Linearized plasmids of pcDNA3.1-PHACTR2-AS1 truncation mutants/antisense were used as DNA templates for transcription in vitro. MEGAscript T7 Kit (Ambion) with biotin-16-UTP (Ambion) was used to produce biotin-labeled RNA and MEGAclear Kit (Ambion) was then applied to purify those RNA transcripts. Biotinylated RNA in RNA structure buffer (19) was heated to 95°C for 2 minutes, put on ice for 3 minutes, and then left at room temperature for 30 minutes to allow proper secondary structure formation. Folded RNA was then mixed with preclreated cell lysates in binding buffer and then incubated at room temperature for 1 hour. Streptavidin Dynabeads (Invitrogen) were added to each binding reaction and further incubated at room temperature for 1 hour. Beads were washed briefly with binding buffer for five times and boiled in SDS buffer. Then, the retrieved proteins were detected by Western blot analysis.

**Western blot analysis**

Western blot assay was performed as described previously (20). We used anti-EZH2 (Cell Signaling Technology), anti-GAPDH (ZSBG-BIO), anti-H3K9me2 (Cell Signaling Technology), anti-H3K9me3 (Cell Signaling Technology), anti-SUV39H1 (Cell Signaling Technology), anti-GST (ZSBG-BIO), anti-fibrisarin (Abcam), anti-YY1 (Santa Cruz Biotechnology), and anti-PHACTR2 (Abcam) antibodies.

**Native RNA immunoprecipitation assay**

RNA immunoprecipitation (RIP) was performed using EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (#17-700, Merck Millipore) according to manufacturer’s instructions using anti-Flag (Sigma-Aldrich), anti-HA (Abcam), anti-SUV39H1 (Active Motif), anti-mouse IgG, or anti-rabbit IgG antibodies.

**Chromatin isolation by RNA purification assay**

Chromatin isolation by RNA purification (ChIRP) assay was performed using EZ-Magna ChIRP RNA Interactome Kit (#17-10495, Merck) according to manufacturer's instructions. RPLP0 and TGFβ1
served as negative controls for rDNA. LacZ-antisense DNA probes were used as a negative control for PHACTR2-AS1 probes. The sequences of probes were listed: 1-TTGATAGTACGCCTTGGGTTTCAGCCTGCACCATTTAC-biotin; 2-GTAGTGGTGAGAGGAGTCAA-biotin; 3-AATACGGGC-TGATATACAGG-biotin; 4-GTGGGAAAGAAGTTGCAAGGG-biotin; 5-ATGTTGGAACCCTGCTGGGAAG-biotin; 6-CATTCTGCCCTTCTAGAGG-biotin; 7-CTTCTCCTCGTCACCTAAG-biotin; 8-TGCTTTGTCTGACATTTAC-biotin.

**Dual luciferase reporter assay**

Plasmids of pGL3-rDNA-ires and pRL-TK were cotransfected using Lipofectamine 3000 Reagent (Invitrogen; Thermo Fisher Scientific). Firefly and Renilla luciferase activity was measured by the Dual-Luciferase Reporter Assay (#E1910, Promega), and Renilla activity was used to normalize firefly activity.

**Chromatin immunoprecipitation**

A chromatin immunoprecipitation (ChIP) assay was performed using SimpleChIP Enzymatic Chromatin IP Kit (#9003, Cell Signaling Technology) according to the manufacturer’s instructions. Anti-EZH2 (Merck Millipore), anti-H3K27me3 (Cell Signaling Technology), anti-RTAP135 (Santa Cruz Biotechnology), and anti-H3K9me2 (Cell Signaling Technology), and anti-H3K9me3 (Cell Signaling Technology). Primers are listed: PHACTR2-AS1 CHIP1 F-CAGTAATGAAAGGACGGAC-TGACG; R-CTGCCATAGAGAAGAAAGA; PHACTR2-AS1 CHIP2 F-TGGCGATCTGCTGGC; R-GAGGTGTTTGCGTCAT; rDNA D0 F-TGGGTTGACCAGAGGGACC; R-GACACGGACACGGACACC; rDNA D8 F-AGTCGCTTGAGGTTGGGAAATGC; R-GCCITCAAGGT-ACTTTGTTGACT; rDNA D18 F-GTGAAGTAGAGAGGGAGGACGG; R-GAAATTGTTCTCCTCAAGCCTGTA.

**RNA reagents**

PHACTR2-AS1-30nt-RNA and negative control were chemically synthesized with modifications from Ribobio Co. All the 30 nucleotides were modified by 2'-O-methylation and 5'-Cholesterol for in vivo RNA delivery, which is long-lasting in mice. The negative controls were purchased from Ribobio Co. For delivery of methylation and cholesterol-conjugated RNA, 5 mmol RNA in 0.1 mL saline buffer was injected into tail vein of NOD/SCID mice once every 3 days for 2–4 weeks.

**Cell proliferation and colony formation**

Cells were plated into 96-well plates at 3,000 cells/well. Ten microliters of WST-1 (Roche) was added to the cells per well and incubated for 2 hours at 37°C. A chromatin immunoprecipitation (ChIP) assay was performed using SimpleChIP Enzymatic Chromatin IP Kit (#9003, Cell Signaling Technology) according to the manufacturer’s instructions. Anti-EZH2 (Merck Millipore), anti-H3K27me3 (Cell Signaling Technology), anti-RTAP135 (Santa Cruz Biotechnology), and anti-H3K9me2 (Cell Signaling Technology), and anti-H3K9me3 (Cell Signaling Technology). Primers are listed: PHACTR2-AS1 CHIP1 F-CAGTAATGAAAGGACGGAC-TGACG; R-CTGCCATAGAGAAGAAAGA; PHACTR2-AS1 CHIP2 F-TGGCGATCTGCTGGC; R-GAGGTGTTTGCGTCAT; rDNA D0 F-TGGGTTGACCAGAGGGACC; R-GACACGGACACGGACACC; rDNA D8 F-AGTCGCTTGAGGTTGGGAAATGC; R-GCCITCAAGGT-ACTTTGTTGACT; rDNA D18 F-GTGAAGTAGAGAGGGAGGACGG; R-GAAATTGTTCTCCTCAAGCCTGTA.

All data are presented as means ± SD of results from three independent experiments. Statistical significance was determined by the two-tailed Student t test. P < 0.05 was considered statistically significant.

**Results**

**LncRNA PHACTR2-AS1 is a target gene of EZH2**

Similar to protein-coding genes, IncRNAs are also subject to epigenetic regulation, especially H3K27 methylation mediated by EZH2 (21). To identify EZH2-regulated IncRNAs, we initially profiled the expression of IncRNAs in a pair of breast cancer cells (MCF7-control vs. MCF7-EZH2) and the top 10 downregulated IncRNAs were shown (Supplementary Fig. S1A). By detecting the level of the ten IncRNAs in human breast cancer tissues, PHACTR2-AS1 was markedly downregulated (Supplementary Fig. S1B), and low PHACTR2-AS1 expression was observed in a panel of cell lines with the mesenchymal phenotype (Supplementary Fig. S1C). To verify whether PHACTR2-AS1 was regulated by EZH2, we first estimated the levels of PHACTR2-AS1 with or without EZH2 overexpression. EZH2 overexpression led to inhibited PHACTR2-AS1 expression (Fig. 1A; Supplementary Fig. S1D). In contrast, EZH2 knockdown restored the expression of PHACTR2-AS1 (Fig. 1B and C, Supplementary Fig. S1E). Consistently, when cells were treated with GSK343, an EZH2 inhibitor, PHACTR2-AS1 expression was increased (Supplementary Fig. S1F and S1G). We then cloned the promoter of PHACTR2-AS1 into the upstream of firefly luciferase–coding region and examined the luciferase activity. Knockdown of EZH2 directly activated PHACTR2-AS1 promoter transcription (Supplementary Fig. S1H). Furthermore, to understand how PHACTR2-AS1 was regulated by EZH2, we detected the occupancy of both EZH2 and H3K27 trimethylation at PHACTR2-AS1 promoter and found that EZH2-mediated H3K27 trimethylation indeed occurred at PHACTR2-AS1 promoter (Fig. 1D).

To scrutinize how EZH2 was recruited to PHACTR2-AS1 promoter, we detected the regulation of some known EZH2-interacting transcription factors on PHACTR2-AS1 expression, including c-myc, Twist, YY1, and E2F6 (22). Among them, only YY1 knockdown can lead to the increase of PHACTR2-AS1 level (Fig. 1E). Transcription factor Yin Yang 1 (YY1) was reported to interact with EZH2 and lead to the increase of PHACTR2-AS1 level (23). Our data found that YY1 occupied the promoter of PHACTR2-AS1 (Fig. 1F). YY1 knockdown could decrease the occupancy of EZH2 and H3K27 trimethylation at PHACTR2-AS1 promoter (Fig. 1G), indicating that YY1 is involved in the recruitment of EZH2 to PHACTR2-AS1 promoter. All these results showed that EZH2 suppressed PHACTR2-AS1 expression, identifying PHACTR2-AS1 as a target gene of EZH2 in breast cancer cells.

**Ethics**

The Ethics Committee of Peking University Health Science Center has approved the mouse experiments (permit number: LA2014122) for this study. The handling of mice was conducted in accordance with the ethical standards of the Helsinki Declaration of 1975 and the revised version in 1983. We also referred to the procedures by Workman and colleagues.

**Statistical analysis**

All data are presented as means ± SD of results from three independent experiments. Statistical significance was determined by the two-tailed Student t test. P < 0.05 was considered statistically significant.

**Results**

**LncRNA PHACTR2-AS1 targets ribosome DNA and inhibits ribosome synthesis**

To explore the biological functions of PHACTR2-AS1, we first examined its subcellular localization. FISH followed by
immunoﬂuorescence showed a strong colocalization between PHACTR2-AS1 and nucleolar marker proteins (fibrillarin and UBF1), indicating that PHACTR2-AS1 was enriched in the nucleolus (Fig. 2A; Supplementary Fig. S2A). The nucleolus contains abundant rDNA repeats, suggesting that PHACTR2-AS1 may be associated with rDNA. We found a marked enrichment of PHACTR2-AS1 at the D0 region, demonstrating that PHACTR2-AS1 directly bound the rDNA promoter (Fig. 2B; Supplementary Fig. S2B). All rDNA promoter sequences in different chromosomes are identical. To discern whether PHACTR2-AS1 binding to rDNA blocked the occupancy of RNA Pol I at rDNA, we generated stable cell lines with PHACTR2-AS1 overexpression or downregulation (Supplementary Fig. S2C) and measured the effect of PHACTR2-AS1 on the occupancy of RPA135, a subunit of the Pol I complex. Indeed, the presence of PHACTR2-AS1 inhibited the occupancy of RNA Pol I at the rDNA promoter (Fig. 2C), whereas PHACTR2-AS1 depletion released the inhibition of RNA Pol I occupancy (Fig. 2D), strongly suggesting that PHACTR2-AS1 suppressed rRNA transcription.

Next, we examined the regulation of PHACTR2-AS1 on the 47S precursor rRNA (pre-rRNA), a direct product of rRNA transcription. PHACTR2-AS1 overexpression led to a pronounced reduction of pre-rRNA expression in breast cancer cell lines (Fig. 2E; Supplementary Fig. S2D). Treatment of PHACTR2-AS1 ASOs resulted in the increase of pre-rRNA, indicating the regulatory effect of PHACTR2-AS1 on pre-rRNA (Fig. 2F; Supplementary Fig. S2E). Furthermore, we detected the changes of nascent pre-rRNA and found PHACTR2-AS1 depletion led to increased nascent pre-rRNA transcription, suggesting that PHACTR2-AS1 speciﬁcally regulated pre-rRNA production (Fig. 2G). In agreement, luciferase reporter assays showed that PHACTR2-AS1 depletion resulted in signiﬁcant activation of rDNA promoter, indicating that PHACTR2-AS1 suppressed rRNA transcription (Fig. 2H).

Production of pre-rRNA in the nucleolus is the initiating step of ribosome biogenesis. Mature 80S ribosome, composed of a 40S and
Figure 2.
PACTR2-AS1 inhibits ribosome synthesis. A, Costaining of PACTR2-AS1 (RNA FISH, Alexa Fluor 488), fibrillarin, and UBF1 (Alexa Fluor 568) in Hs578T cells. B, Top, a schematic of an rDNA cluster. Bottom, ChIRP assay in PACTR2-AS1–overexpressing MDA-MB-231 cells. C and D, Lysates were prepared from Hs578T-control/ PACTR2-AS1 or MCF7-control/PACTR2-AS1-shRNA cells for ChIP assays. E, An empty or a PACTR2-AS1-overexpressing vector was transfected into Hs578T cells, followed by qRT-PCR. F, LNA-based ASOs were transfected into MCF7 cells, followed by qRT-PCR. The efficacy of ASOs was examined both in the nucleus and cytoplasm. G, ASO-treated MCF7 cells were pretreated with 5-FU for 4 hours and with 4sU for labeling nascent pre-rRNA during the last hour. Nascent pre-rRNA was measured by qRT-PCR and normalized to nascent GAPDH mRNA. H, Luciferase reporter assays were performed in ASO-treated MCF7 cells, and the values were normalized to those of Renilla. The above data represent the means ± SD from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. I, Absorbances of 40S, 60S, and 80S ribosomes were detected at 254 nm in the stable cells.
PHACTR2-AS1 promotes H3K9 methylation of rDNA by recruiting SUV39H1

Given that various epigenetic modifications occur within rDNA loci, we first analyzed the presence of histone modifications at rDNA in breast cancer cells. Among those modifications, H3K9me2 and H3K9me3 were markedly enriched throughout the rDNA-repeat region (Supplementary Fig. S3A). Regions rich in H3K9me2/3 display heterochromatin-mediated gene silencing (24). We therefore reasoned that H3K9 methylation may mediate the PHACTR2-AS1–dependent silencing of rRNA genes. We observed that PHACTR2-AS1 overexpression enhanced the abundance of H3K9me2/3 at rDNA promoter (Fig. 3A; Supplementary Fig. S3B). Conversely, PHACTR2-AS1 knockdown in the nucleus led to reduced occupancy of both H3K9me2/3 at rDNA promoter (Fig. 3B; Supplementary Fig. S3C). These results indicated that PHACTR2-AS1 suppressed rRNA transcription by enhancing the occupancy of methylated H3K9 at rDNA loci.

SUV39H1 was reported to catalyze the methylation of H3K9 (25). To determine how PHACTR2-AS1 promotes H3K9me, the association of PHACTR2-AS1 and SUV39H1 was detected. First, RIP assays confirmed the interaction between PHACTR2-AS1 and SUV39H1 (Fig. 3C), and their colocalization was found in the nucleolus (Fig. 3D). Next, RNA pull-down assays showed that SUV39H1 directly interacted with PHACTR2-AS1 (Fig. 3E). Analysis of truncation mutants revealed that the middle region of SUV39H1 was responsible for binding with PHACTR2-AS1 (Fig. 3F), and PHACTR2-AS1 knockdown caused the formation of phosphorylated histone H2AX (yH2AX) foci, which is critical for DNA damage responses and genome stability (27). SUV39H1 depletion resulted in a global increase of yH2AX foci in the nucleolus (Fig. 4D and E; Supplementary Fig. S4C and S4D). In contrast, overexpression of PHACTR2-AS1 led to the decrease both of fragmented nucleoli and yH2AX foci (Supplementary Fig. S4E–S4H). Furthermore, ChIP analysis indicated that PHACTR2-AS1 knockdown led to a significant increase in yH2AX levels at rDNA repeats (Fig. 4F), suggesting that PHACTR2-AS1 depletion potentially resulted in increased DNA damage. Furthermore, the number of rDNA copies decreased significantly in PHACTR2-AS1–shRNA cells compared with control cells (Fig. 4G), indicating that PHACTR2-AS1 depletion may disrupt rDNA stability. We also found that PHACTR2-AS1–depleted cells showed marked genomic abnormalities, including micronuclei and abnormal mitoses (Fig. 4H and I). These findings suggested that PHACTR2-AS1 depletion disrupted the rDNA integrity, resulting in genome instability.

PHACTR2-AS1 suppresses breast cancer cell growth and metastasis

Hyperactivation of ribosome biogenesis is associated with uncontrolled cancer cell proliferation and genome instability induces cellular malignant transformation (6, 28). PHACTR2-AS1 depletion markedly promoted proliferation, migration, and invasion of breast cancer cells (Fig. 5A–D). In contrast, PHACTR2-AS1 overexpression significantly inhibited cell proliferation, migration, and invasion (Fig. 5E–H). Furthermore, a transcriptional factor of rDNA UB1 was overexpressed to activate ribosome synthesis (Supplementary Fig. S5A). PHACTR2-AS1 overexpression overcame the increase of cell proliferation and migration resulting from UB1 overexpression (Supplementary Fig. S5B and S5C). And the increase of cell migration induced by PHACTR2-AS1 depletion can be rescued by actinomycin D, an inhibitor of rDNA transcription (Supplementary Fig. S5D), suggesting that dysregulation of rDNA synthesis mediates the role of PHACTR2-AS1 in cancer cells. Enhancement of genome instability induced by SUV39H1 deficiency promotes tumorigenesis. PHACTR2-AS1 overexpression could rescue the effect of SUV39H1 knockdown (Supplementary Fig. S5E and S5F), indicating that SUV39H1 is involved in the role of PHACTR2-AS1 in cancer cells.

To examine the role of PHACTR2-AS1 in vivo, control cells and PHACTR2-AS1–overexpressing cells were orthotopically implanted
PHACTR2-AS1 Inhibits Ribosome Synthesis

Figure 3.
PHACTR2-AS1 promotes H3K9 methylation of ribosome DNA by recruiting SUV39H1. A and B, Lysates were extracted from stable Hs578T-control/PHACTR2-AS1 or MCF7-control/PHACTR2-AS1-shRNA cells for ChIP assays. C, Endogenous SUV39H1 from MCF7 cells was immunoprecipitated with an anti-SUV39H1 antibody for RIP assay. RIP enrichment was determined as the amount of RNA recovered in association with SUV39H1, relative to that recovered with an IgG. The above data represent the mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001, Student t test; ns, nonsignificant. D, Costaining of PHACTR2-AS1 (RNA FISH, Alexa Fluor 488), SUV39H1 (immunofluorescence, Alexa Fluor 568). SUV39H1 (immunofluorescence, Alexa Fluor 568) in Hs578T cells. E, Purified GST-SUV39H1 protein was incubated with biotin-labeled PHACTR2-AS1 RNA for RNA pull-down assays. Biotin-labeled antisense RNAs against PHACTR2-AS1 and GAPDH mRNA were used as negative controls. F, Top, different regions of GST-SUV39H1. Different fusion proteins were incubated with biotin-PHACTR2-AS1 for RNA pull-down assays. G, Top, different PHACTR2-AS1 fragments. RNA pull-down assays were performed by incubating GST-SUV39H1 protein with biotin-PHACTR2-AS1 fragments. H, An empty or PHACTR2-AS1–overexpressing plasmid was transfected into Hs578T cells in the presence of control or SUV39H1 siRNA, followed by qRT-PCR and Western blot analysis. I, PHACTR2-AS1–overexpressing cells were treated with different doses of chaetocin for 48 hours, followed qRT-PCR and Western blot analysis.

into mammary fat pad of mice (Fig. 3I). After 4 weeks, tumor sizes were measured. Tumors induced by PHACTR2-AS1–overexpressing cells were significantly smaller than those developed from control cells. Tumor volumes and weights were monitored weekly, revealing that PHACTR2-AS1 significantly inhibited tumor growth. Next, control cells and PHACTR2-AS1–overexpressing cells were injected
Figure 4. PHACTR2-AS1 depletion induced genome instability. A, Immunofluorescence of fibrillarin in stable MCF7-control/PHACTR2-AS1-shRNAs cells. The images represent a confocal Z stack. Arrows, cells with fragmented nucleoli. B, Cells with fragmented nucleoli were counted for statistical analysis ($n = 100$). C, Fibrillarin expression was measured by Western blot. D and E, Immunofluorescence of γH2A.X was measured to assess the formation of γH2A.X foci. Representative images and statistical analysis are shown. F, ChIP assays were performed using an anti-γH2A.X antibody. G, Genome DNA was extracted, and qPCR was performed to quantitate the amounts of rDNA repeats, with normalization to GAPDH. H and I, Stable MCF7-control/PHACTR2-AS1-shRNA cells were stained with DAPI. Micronuclei or abnormal chromosomes (including anaphase bridges and lagging chromatin) were measured from interval or anaphase cells ($n = 100$), respectively. Arrows, individual abnormalities. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
intravenously into mice, and lung metastasis was determined at 6 weeks post-injection (Fig. 5J). PHACTR2-AS1 overexpression led to decreased lung metastasis of cancer cells. The efficacy of PHACTR2-AS1 overexpression was measured (Fig. 5K). Taken together, all these results indicated that PHACTR2-AS1 suppressed breast cancer growth and metastasis in vitro and in vivo.
Figure 6.
PHACTR2-AS1-30nt-RNA inhibited cancer cell growth and metastasis. A and B, The empty, PHACTR2-AS1full length, or PHACTR2-AS11766–1795 plasmid was transfected into Hs578T cells, followed by qRT-PCR and ribosome detection. C–E, Hs578T cells were transfected with empty or PHACTR2-AS11766–1795 plasmid for cell proliferation, migration, and invasion assays. F, Cy3-labeled PHACTR2-AS1-30nt-RNA was transfected into Hs578T cells. Cells were fixed and stained with an anti-fibrillarin. G, MDA-MB-231-Luc-D3H2LN cells were inoculated onto the mammary fat pad of mice (n = 8/group). After 2 weeks, the mice were divided into two groups and injected with control RNA or PHACTR2-AS1-30nt-RNA through the tail vein once every 3 days. Representative in vivo bioluminescence images. H, Bioluminescence-based quantitation of primary tumor sizes. I, Ribosomes were extracted and fractionated from tumor specimens. J, MDA-MB-231-Luc-D3H2LN cells were injected into mice through the tail vein, followed by tail vein injection of control RNA or PHACTR2-AS1-30nt-RNA once every 3 days (n = 8/group). Representative bioluminescence images are shown. K, Bioluminescent quantitation of lung metastasis. L, Representative lung metastasis specimens were sectioned and stained with hematoxylin and eosin (H&E). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
PHACTR2-AS1 Inhibits Ribosome Synthesis

A synthesized PHACTR2-AS1 fragment efficiently inhibited tumor growth and lung metastasis

Given that the region of PHACTR2-AS1 from nucleotides 1766–1795 was responsible for recruiting SUV39H1, we investigated whether this short region can function similar to the full-length PHACTR2-AS1. Our results showed that PHACTR2-AS11766–1795 contributed to decreases in pre-rRNA and ribosome levels and inhibited cell proliferation, migration, and invasion (Fig. 6A–E). We also synthesized PHACTR2-AS1-30nt-RNA (nucleotides 1766–1795) labeled with fluorophore Cy3 and observed its location in the nucleolus (Fig. 6F).

To evaluate the possible therapeutic potential of PHACTR2-AS1-30nt-RNA, we synthesized a durable PHACTR2-AS1-30nt-RNA (Supplementary Fig. S6A). First, we injected the biotin-labeled PHACTR2-AS1-30nt-RNA into tail vein of mice bearing xenograft tumors. The location of biotin-PHACTR2-AS1-30nt-RNA was showed by avidin staining of freezing section. At about 72 hours, the intensity of biotin-PHACTR2-AS1-30nt-RNA in tumor cells reached a highest point, then began to decline and disappeared at 96 hours (Supplementary Fig. S6B and S6C), indicating that the retention-time of PHACTR2-AS1-30nt-RNA in tumor cells is about 72 hours. In addition, biotin-PHACTR2-AS1-30nt-RNA was clearly observed in tumor cells of lungs at 72 hours after injection (Supplementary Fig. S6D). These data showed that the biotin-PHACTR2-AS1-30nt-RNA can be taken up by the tumor cells of both mice xenograft tumors and lung metastasis.

Furthermore, MDA-MB-231-Luc-D3H2LN cells were orthotopically implanted into mammary fat pad of mice (Fig. 6G). Tumor growth was significantly slower in the PHACTR2-AS1-RNA-treated group compared with that in the control RNA-treated group (Fig. 6H), suggesting that long-lasting RNA fragments can effectively inhibit tumor growth. Importantly, PHACTR2-AS1-30nt-RNA decreased levels of 40S, 60S, and 80S ribosomes extracted from xenografts (Fig. 6I), indicating that ribosome synthesis mediated the inhibitory effect of PHACTR2-AS1 on tumor growth. In addition, 30-nt-RNA treatment enhanced the abundance of H3K9me2/3 at the loci (Supplementary Fig. S6E and S6F), indicating the specific activity and of the 30-nt-RNA.

To further examine the role of PHACTR2-AS1-30nt-RNA in lung metastasis, MDA-MB-231-Luc-D3H2LN cells were injected into tail vein of mice, followed by tail vein injection of control RNA or PHACTR2-AS1-30nt-RNA for 4 weeks (Fig. 6J). Blueluminescence imaging showed that PHACTR2-AS1-30nt-RNA decreased breast cancer cell metastasis to the lungs (Fig. 6K and L), suggesting that PHACTR2-AS1-30nt-RNA is of therapeutic value for blocking breast cancer lung metastasis.

PHACTR2-AS1 is downregulated in patients with breast cancer

To assess the role of PHACTR2-AS1 in patients with breast cancer, we stained PHACTR2-AS1 in 90 pairs of breast cancer tissues and normal tissues and found that normal breast tissues exhibited stronger PHACTR2-AS1 staining than cancer tissues (Fig. 7A and B). In addition, PHACTR2-AS1 levels were higher in normal tissues than in some types of cancers, including pancreatic, kidney, lung, and rectal cancers (Supplementary Fig. S7A). We also found that the patients with elevated PHACTR2-AS1 expression showed better overall survival than those with reduced PHACTR2-AS1 expression (Fig. 7C; Supplementary Fig. S7B). Collectively, these findings indicated that PHACTR2-AS1 expression was suppressed in breast cancer and that lower PHACTR2-AS1 expression may predict poor outcomes for patients with breast cancer.

Furthermore, we detected the EZH2 protein level by IHC staining and PHACTR2-AS1 RNA level by RNA in situ hybridization in human breast cancer tissue arrays. The expression of EZH2 was negatively correlated with the expression of PHACTR2-AS1 (Fig. 7D and E). The level of PHACTR2-AS1 is higher in the group of low expression of EZH2 than that in the group of high expression of EZH2 (Fig. 7F). Likewise, the level of EZH2 is also higher in the group of low expression of PHACTR2-AS1 than that in the group of high expression of PHACTR2-AS1 (Fig. 7F), suggesting that the protein level of EZH2 is negatively correlated with the RNA level of PHACTR2-AS1 in breast cancer tissues.

Furthermore, we used 17 pairs of fresh breast cancer tissues and normal tissues to assess the RNA level of both PHACTR2-AS1 and EZH2 and the ribosomal level. PHACTR2-AS1 level was downregulated in breast cancer tissues compared with the normal tissues, whereas the RNA level of EZH2 was upregulated in cancer tissues (Fig. 7G), indicating the negative relation between PHACTR2-AS1 and EZH2 again (Fig. 7H). Next, we detected the ribosomal levels of the 17 patients and found that the ribosomes are markedly increased in 13 patients with breast cancer (Fig. 7I; Supplementary Fig. S7C). Collectively, these results suggested that EZH2 may enhance ribosome synthesis through silencing PHACTR2-AS1 expression to promote breast cancer development.

Discussion

Here, we report that PHACTR2-AS1 binds to rDNA and recruits SUV39H1, triggering H3K9 methylation at rDNA. H3K9 modification blocked the binding of RNA Pol I to rDNA, resulting in the suppression of ribosome synthesis. Upon depletion of PHACTR2-AS1 induced by EZH2-mediated H3K27 methylation, rRNA transcription is reactivated, leading to increased ribosome synthesis and genomic instability, both of which promote cancer proliferation and metastasis (Fig. 7J).

Epigenetic regulation of RNA genes is involved in dysregulating ribosome biogenesis during the malignant transformation of cells (29, 30). NoRC (nucleolar remodeling complex) is critical for maintaining the constitutively silent state of an rDNA cluster by recruiting DNM1 and HDAC (2). eNoSC (energy-dependent nucleolar silencing complex) contains SIRT1, SUV39H1, and NML (31). Under glucose starvation, eNoSC triggers rDNA silencing, thereby protecting cells from energy deprivation–dependent apoptosis. Thus, eNoSC is mainly responsible for precisely regulating rRNA synthesis under different energy conditions. Although lucRNA PHACTR2-AS1 interacts with SUV39H1, PHACTR2-AS1 is not required for rRNA gene silencing in response to glucose deprivation (Supplementary Fig. S7D), suggesting that PHACTR2-AS1 is not a component of the eNoSC complex. Here, we found EZH2-mediated H3K27me3 inhibited PHACTR2-AS1 expression. EZH2 has been regarded as a biomarker for aggressive breast cancer (32). Blocking EZH2-mediated H3K27me3 disrupts the silent state of rRNA genes associated with PHACTR2-AS1, uncovering the relationship between EZH2 and rRNA synthesis.

Hyperactivation of ribosome biogenesis was reported to play significant roles in cancer initiation and progression (33, 34). Cancer tissues have lower rDNA copy numbers than normal tissues, despite increased rRNA synthesis and proliferation (35). Indeed, depletion of PHACTR2-AS1 led to the decreased rDNA copies, although rRNA synthesis was activated. It is possible that depletion of PHACTR2-AS1 induced the switch of silent rDNA to active rDNA or upregulated the transcription activity of active rDNA copies. PHACTR2-AS1 depletion can reduce SUV39H1 recruitment to rRNA genes, resulting in
Figure 7.
The PHACTR2-AS1 is downregulated in patients with breast cancer. A and B, Detection of PHACTR2-AS1 expression in human breast cancer compared to the matched adjacent normal breast tissues by chromogenic in situ hybridization (CISH) with paraffin-embedded tissues \((n = 90)\). Representative CISH staining of PHACTR2-AS1. Statistical analysis of differential expression is shown. C, Kaplan-Meier survival analysis of PHACTR2-AS1 expression by CISH in tissue microarrays of patients with breast cancer. D and E, Both PHACTR2-AS1 expression (CISH) and EZH2 expression (IHC) were detected in paraffin-embedded tissues. Representative images are shown. Spearman and Pearson correlation tests were performed. F, Left, analyses of expression level of PHACTR2-AS1 in the two groups of high and low expression of EZH2. Right, analyses of expression level of EZH2 and PHACTR2-AS1 staining index (CISH) \((n = 98)\). G and H, Levels of PHACTR2-AS1 RNA and EZH2 mRNA were measured in fresh human breast cancer tissues and matched adjacent normal breast tissues \((n = 17)\) by qRT-PCR. Pearson correlation tests were performed. I, Ribosomes were measured in three representative patients. J, A working model of PHACTR2-AS1. * \(P < 0.05\).
heterochromatin relaxation at rDNA. Heterochromatin relaxation may cause fragmentation of nuclear structure, DNA double-strand breaks, and loss of rDNA repeats, all of which lead to genome instability (36). Thus, PHACTR2-AS1 depletion enhances protein synthesis rates and increases genomic instability, both of which contribute to malignant cell transformation. Consistent with PHACTR2-AS1, depletion of TIPS (a large subunit of NoRC) induced genomic instability and concurrently enhanced rRNA transcription, leading to malignant transformation (37).

Chen and colleagues reported that PHACTR2-AS1 (also known as NR027113) regulated PTEN/PI3K/AKT signaling pathway in hepato-cellular carcinoma. Recently, they reported that this lncRNA also regulated ERK and AKT signaling and renamed it as LncIHS according its function in hepatocellular carcinoma (38, 39). Since no obvious nucleolar localization was showed in HCC cells, PHACTR2-AS1 may not effect on ribosome synthesis in HCC, which can explain the dichotomous role of PHACTR2-AS1 in hepatocellular cancer and breast cancer. We have demonstrated that rDNA is the direct target of PHACTR2-AS1 in breast cancer cells. However, due to functional diversity of lncRNAs, we cannot exclude that there are other targets of PHACTR2-AS1 in breast cancer cells, which leaves an opportunity for future study.

Ribosome biogenesis has been confirmed as a potential target for cancer treatment (40–42). Several features of lncRNAs determine their potentials as therapeutic targets (43). One of the most used strategies is to block cancer-upregulated lncRNA function by applying specific ASOs against lncRNA (44, 45). Another strategy of lncRNA targeting is to restore lncRNA levels with synthetic RNA molecules, which is aimed to cancers with downregulated lncRNAs. However, for now this strategy cannot be well applied, due to limitations in the lengths of lncRNAs. In this study, we explored the functional fragment of an lncRNA and synthesized a durable PHACTR2-AS1-30nt-RNA molecule. Synthetic PHACTR2-AS1-30nt-RNA molecules formed a hairpin structure and mimicked the full-length lncRNA by inhibiting tumor growth and lung metastasis, providing insight into the potential of PHACTR2-AS1 as a therapeutic target in breast cancer.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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The EZH2–PHACTR2–AS1–Ribosome Axis induces Genomic Instability and Promotes Growth and Metastasis in Breast Cancer

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