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Original Article

Silencing of the long non-coding RNA RHPN1-AS1 suppresses the epithelial-to-mesenchymal transition and inhibits breast cancer progression

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Abstract: Breast cancer (BC) is a frequently diagnosed malignancy in women. Increasing evidence implicates mis-expression of the long non-coding RNA (lncRNA) RHPN1 antisense RNA 1 (RHPN1-AS1) in the development of multiple cancer types. However, little is known about the expression pattern and function of lncRNA RHPN1-AS1 in the pathobiology of BC. We evaluated the expression of RHPN1-AS1 in The Cancer Genome Atlas dataset, and analyzed associations between RHPN1-AS1 expression and clinicopathologic features of BC patients. Additionally, we compared the expression of RHPN1-AS1 between BC and breast non-tumor samples via quantitative real-time polymerase chain reaction, and in situ hybridization, and evaluated the prognostic value of RHPN1-AS1 in a BC tissue microarray. We examined the impact of RHPN1-AS1 knockdown on proliferation, migration, and invasion of BC cells *in vitro*, and tumor growth *in vivo*. Bioinformatics analyses were used to predict the function of RHPN1-AS1 in BC. RHPN1-AS1 expression was upregulated in BC and elevated RHPN1-AS1 expression was strongly associated with poor prognosis of BC patients. Moreover, both univariate and multivariate analyses revealed that RHPN1-AS1 was a significant and independent predictor of BC prognosis. Functionally, RHPN1-AS1 silencing attenuated BC cell proliferation, migration, and invasion *in vitro*, and reduced tumor growth in xenograft models. Furthermore, RHPN1-AS1 silencing was associated with a decrease in the expression of epithelial-to-mesenchymal transition (EMT) markers in the xenograft tumors, suggesting that RHPN1-AS1 promotes invasion in BC cells by enhancing EMT. These findings suggest that RHPN1-AS1 is a potential prognostic biomarker and therapeutic target for BC.

Keywords: lncRNA RHPN1-AS1, epithelial-to-mesenchymal transition, prognostic biomarker, breast cancer

Introduction

Breast cancer (BC) represents one of the most common malignancies in women worldwide [1]. In recent years, even though surgery and targeted treatments have improved the prognosis of BC patients, recurrence, metastasis, and drug resistance are clinical challenges that result in the poor overall survival (OS) of many BC patients [2-6]. Therefore, in order to enable the personalized management of BC and improve patient outcomes, it is essential to identify robust and reliable prognostic biomarkers and novel therapeutic targets.

In recent years, long non-coding RNAs (lncRNAs) have gained tremendous attention as studies have shown that they are involved in numerous biological processes, including the development and progression of different cancers [7-12]. Several reports have showed that lncRNAs are aberrantly expressed in BC, and their mis-expression is significantly correlated with aggressive tumor phenotypes and clinical behavior. For example, the lncRNA OIP5-AS1 is highly overexpressed in BC, and OIP5-AS1 silencing suppresses the malignant phenotypes of BC cells [13]. Zhang et al revealed that lncRNA HOTAIR was overexpressed in BC and promoted tumor metastasis through its interaction with polycomb repressive complex 2 (PRC2) [14]. Thus, deciphering the role of lncRNAs in BC may enhance our understanding of the molecular mechanisms driving BC progression.

The lncRNA RHPN1 antisense RNA 1 (RHPN1-AS1) is a 2030-nucleotide-long transcript originating from human chromosome 8q24 [15].

The functions of lncRNA RHPN1-AS1 and the molecular pathways it regulates in tumors are largely unknown. Studies indicate that expression of RHPN1-AS1 is often elevated in aggressive tumors, including non-small cell lung cancer and uveal melanoma [16, 17]. Additionally, overexpression of RHPN1-AS1 was significantly associated with malignancies and poor prognosis; conversely, down-regulation of RHPN1-AS1 inhibited proliferation and metastasis of cancer cells [18]. However, the expression level of RHPN1-AS1 in BC, and its role, if any, in the development and progression of BC has not been explored so far.

In this study, we found that the expression of lncRNA RHPN1-AS1 was significantly upregulated in BC, and elevated expression of RHPN1-AS1 correlated significantly with clinicopathological factors normally associated with unfavorable prognoses, and was a significant predictor of poor OS in BC patients, in both univariate and multivariate analyses. Functional analyses showed that down-regulation of RHPN1-AS1 inhibits BC cell proliferation, migration, and invasion *in vitro*, and tumor growth *in vivo*. Mechanistically, we found a significant positive correlation between the expression of RHPN1-AS1 and epithelial-to-mesenchymal transition (EMT) phenotypes, which was further confirmed via *in vitro* functional assays and *in vivo* xenograft models. Taken together, our data are the first to suggest that upregulation of RHPN1-AS1 serves as a potential prognostic biomarker in BC, and promotes the proliferation and invasion of BC cells, at least in part by enhancing EMT.

Materials and methods

Clinical specimens and construction of the Zhengzhou University (ZZU) cohort tissue microarray

Formalin-fixed paraffin-embedded breast tumor resection samples (n=160) and breast non-tumor tissues (n=60) were obtained from the First Affiliated Hospital of Zhengzhou University, China, for the construction of tissue microarrays (TMAs). BC tissues were obtained from patients who were consecutively diagnosed with BC between 2009 and 2013, and for whom tissue samples and detailed clinicopathologic annotation were available. TMAs were constructed using 1.5-mm-diameter cores. All aspects of this study were reviewed and approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University and all patients signed informed consents regarding the use of their tumor specimens for research.

The Cancer Genome Atlas (TCGA) dataset analysis

To explore the relationship between RHPN1-AS1 expression and clinicopathologic features of BC patients, including 1079 breast cancer tumor samples and 104 non-tumor breast tissue samples, we downloaded the mRNA expression and clinical data from The Cancer Genome Atlas Project (TCGA, http://tcga-data.nci.nih.gov/).

In situ hybridization (ISH) staining of TMAs

The RHPN1-AS1 detection probe (BOSTER, Wuhan, China) was used for ISH. Chromogenic visualization of probe-target hybrids was performed following the manufacturer's protocol. Finally, sections were counter-stained using hematoxylin, dehydrated, mounted, and imaged using a microscope. The intensities of RHPN1-AS1 staining were scored in the range of 1-4 as follows: 1 (no staining), 2 (weak staining), 3 (medium staining), and 4 (strong staining). Samples were evaluated by two pathologists blinded to each other's observations as well as to the clinicopathologic features of the samples. Samples with scores of 1 and 2 were considered low-expressing samples, and samples with other scores were considered to show high expression of lncRNA RHPN1-AS1.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) assays

Total RNA was extracted from BC tissue samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol. The RNAs were reverse transcribed with the PrimeScript RT Master Mix Perfect Real Time (Takara, Shiga, Japan). The qRT-PCR was performed according to the protocol of SYBR Green PCR Master Mix (TaKaRa). GAPDH was used as internal control and the relative expression of RHPN1-AS1 was calculated by using $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Table S3.

Cell lines

BC cell lines MDA-MB-231 and MCF-7, as well as the human breast epithelial cell line MCF-10A, were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) in a humidified incubator at 37°C with 5% CO_2. Cell lines used in this study are shown in Table S1.

Immunofluorescence (IF) staining

Immunofluorescent staining was performed as described previously [19]. Briefly, cultured cells were seeded into 24-well plates. When the cells attained about 50% confluency, they were fixed with 4% paraformaldehyde and treated with 0.2% Triton X-100. The cells were then blocked in 2% bovine serum albumin (BSA) at room temperature for 1 hour followed by incubation with anti-RHPN1-AS1 primary antibody (dilution 1:200) at 4°C overnight. Subsequently, after treating with secondary antibodies for 1 hour and DAPI for 5 minutes, cells were rinsed, mounted, and imaged using a fluorescence microscope.

ShRNA construction and transfection of cell lines

The following shRNAs were utilized in this study: RHPN1-AS1 (sh-RHPN1-AS1): 5'-CCGAATCTC-TTTACTTCCA-3'; Negative control shRNA (sh-NC): 5'-CTCAAACTTTGAGGGTCAT-3'; Lentiviruses harboring sh-RHPN1-AS1 and control shRNAs were generated according to the standard protocol.

Stable transfectants harboring sh-RHPN1-AS1 or sh-NC were generated in MDA-MB-231 and MCF-7 BC cells via lentiviral transduction of a plasmid (synthesized by GenePharma, Shanghai, China) carrying the corresponding shRNA sequence and the gene encoding firefly luciferase. Stably transfected cell lines were selected using puromycin (100 μg/ml) for 4 weeks.

Cell proliferation and DNA synthesis assays

Stably transfected MDA-MB-231 and MCF-7 cells were seeded into 96-well plates at a density of 1×10^4 cells per well. Cells were treated with 10 μL Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) solution at the indicated times after the cells were seeded. Cells were incubated for an additional 4 hours at 37°C before measuring absorbance at 450 nm using a spectrophotometer (Molecular Devices, Washington, DC, USA).

To evaluate the effects of RHPN1-AS1 knockdown on DNA replication in proliferating cells, stably transfected MDA-MB-231 and MCF-7 BC cells harboring RHPN1-AS1 knockdown (sh-RHPN1-AS1) or negative control shRNA (sh-NC) were incubated in DMEM containing 50 μM 5-ethynyl-2'-deoxyuridine (EdU; RiboBio, Guangzhou, China) in triplicates. DNA was stained using the Hoechst 33342 stain, and cells were visualized using an inverted fluorescence microscope (Olympus, Tokyo, Japan). For each EdU experiment, five random fields were imaged at 100× magnification. Images were processed and analyzed using the Image J software. The percentage of EdU-positive cells among the total number of cells in each field was determined.

Transwell cell invasion and migration assays

For the transwell cell migration and invasion assays, stably transfected MDA-MB-231 and MCF-7 BC cells were resuspended in FBS-free medium at a density of 1×10^6 cells/mL. For each cell line, 300 μL of cell suspension was added into the top compartment of the transwell chamber (BD Biosciences, Sparks, MD, USA) and 500 μL of medium containing 10% FBS was added into the bottom compartment to act as a chemoattractant. After incubation for 24 hours, the transwell insert was removed, and the side of the insert facing the upper compartment was carefully cleansed with a cotton swab to remove culture medium and cells that had not migrated through the insert. Cells that had migrated through the filter pores to the underside of the insert were fixed, stained with 0.1% crystal violet, and counted using a microscope (Olympus, Tokyo, Japan).

Western blot assay

The protocols of protein isolation and western blotting have been described before [20]. In brief, Protein lysates were separated on 12% SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) electrophoresis, and transferred into a nitrocellulose (NC) membrane (Millipore, Billerica, MA, USA). Then, the membrane was blocked with

5% non-fat milk and incubated with primary antibodies at 4°C for 12 hours. After washing with PBST, the membrane was treated with secondary antibodies for 2 hours at room temperature. The signal quanti-fication was visualized and analyzed by using the Odyssey infrared imaging system (LI-COR Biosciences). Primary antibodies used in this study are displayed in Table S2.

Immunohistochemical (IHC) staining and scoring

In brief, deparaffinization, rehydration, and antigen retrieval of the tissue slides was performed according to the protocol. Subsequently, tissue slides were blocked with 5% bovine serum albumin (BSA) (Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. Sections were incubated with primary antibodies overnight at 4°C and then probed with biotinylated goat anti-rabbit secondary antibodies (AmyJet Scientific, Wuhan, China). To visualize staining, slides were detected by a SignalStain® DAB Substrate Kit (CST, USA) and counterstained with hematoxylin, dehydrated, and mounted. Semi-quantitative of IHC scores was performed as described previously [21].

Tumor xenograft experiments

Male BALB/c nude mice (Vital River Laboratory Technology, Beijing, China) were injected subcutaneously in the flank with 1×10^7 MCF-7 cells that were stably transfected with sh-RHPN1-AS1 or sh-NC. Tumor growth was measured every week using non-invasive bioluminescent imaging. After 5 weeks, mice were sacrificed. Tumors were weighed, and tissue specimens were harvested for immunohistochemical staining. All animal studies were performed in compliance with University Committee on Use and Care of Animals protocols.

Statistical analysis

Comparisons between groups were performed using chi-square test for nominal variables and Student's t-test for continuous variables. Kaplan-Meier Survival Analyses were used to evaluate how the expression of lncRNA RHPN1-AS1 affected OS and progression-free survival (PFS) of patients. The statistical significance of between-group differences in OS and PFS was evaluated using log-rank tests (SPSS software version 23.0) and $P<0.05$ was considered to be statistically significant. All data are presented as the mean ± SD from at least three independent experiments.

Results

Upregulation of RHPN1-AS1 is associated with poor prognosis of BC patients in the TCGA dataset

To evaluate the expression pattern of lncRNA RHPN1-AS1 in a variety of cancers, we first analyzed mRNA expression data in the TCGA dataset. As shown in **Figure 1A**, the expression of lncRNA RHPN1-AS1 was dysregulated in 17 common kinds of cancers; notably, the expression of RHPN1-AS1 was upregulated in most cancers (14/17). RHPN1-AS1 expression was elevated in BC tissues compared with non-tumor breast tissues (**Figure 1B**), and upregulated RHPN1-AS1 expression was significantly associated with advanced TNM stages (III-IV stages) (**Figure 1C**). Moreover, among the four molecular subtypes of BC, RHPN1-AS1 expression in Her2 and LumB subtypes was significantly higher than in the LumA and Basal subtypes (**Figure 1D**). Kaplan-Meier analyses showed that the OS of BC patients with high RHPN1-AS1 expression was significantly lower than the OS of patients with low RHPN1-AS1 expression (**Figure 1E**). BC patients with both high TNM stage (III-IV) and high expression of RHPN1-AS1 were prone to have the shortest OS (**Figure 1F**). Taken together, these findings suggest that RHPN1-AS1 is upregulated in BC, and elevated expression of RHPN1-AS1 is associated with poorer prognosis of BC patients.

Elevated RHPN1-AS1 expression is associated with poor prognosis of BC patients in the ZZU cohort

To further verify the upregulated RHPN1-AS1 in BC, we conducted ISH analysis to investigate the expression of RHPN1-AS1 in the ZZU cohort TMAs (**Figure 2A**). Consistent with our findings in the BC dataset of TCGA, RHPN1-AS1 expression was elevated in BC tissues in the ZZU TMAs (**Figure 2B** and **2C**) and more strongly correlated with TNM III-IV stages than with I-II stages (**Figure 2D**). In addition, overexpression of RHPN1-AS1 was strongly associated with distant metastasis in BC (**Figure 2E**). Kaplan-Meier analyses showed that patients with low expres-

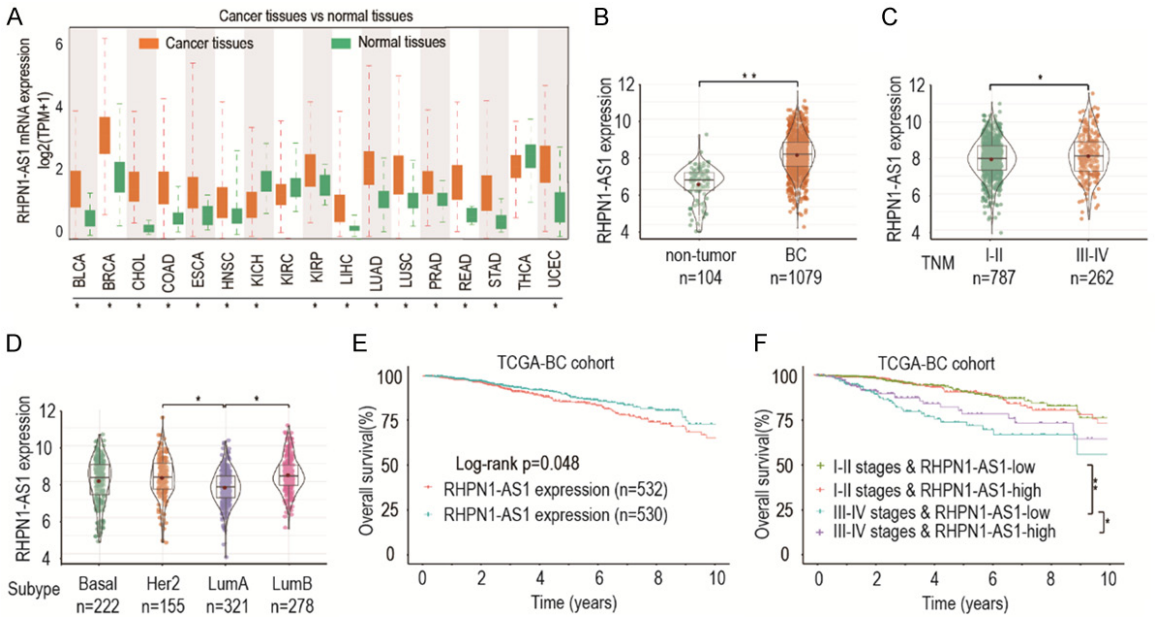

Figure 1. RHPN1-AS1 is upregulated in BC and RHPN1-AS1 expression is associated with prognosis of BC patients in the TCGA dataset. A. Comparison of lncRNA RHPN1-AS1 expression between different cancers and normal tissues in the TCGA dataset. B. Comparison of lncRNA RHPN1-AS1 expression between BC and non-tumor breast tissues in the TCGA dataset. RHPN1-AS1 expression was upregulated in BC tissues compared to non-tumor tissues. C. RHPN1-AS1 expression in breast tumor tissues according to TNM stage. RHPN1-AS1 expression was elevated in advanced TNM stages. D. Comparison of RHPN1-AS1 expression in BCs of different molecular subtypes. RHPN1-AS1 was overexpressed in Her2-enriched and LumB subtypes compared to LumA and Basal subtypes. E. Kaplan-Meier survival curves showing stratification of BC patients in the TCGA dataset based on lncRNA RHPN1-AS1 expression levels. BC patients with high RHPN1-AS1 expression had markedly shorter OS than those with low RHPN1-AS1 expression. F. Kaplan-Meier survival curves showing stratification of BC patients in the TCGA dataset based on both their lncRNA RHPN1-AS1 expression levels as well as their TNM stages. BC patients with high expression levels of RHPN1-AS1 and advanced TNM stages had the poorest OS. *P<0.05, **P<0.01. Abbreviations: BLCA: Bladder Urothelial Carcinoma; BRCA: Breast cancer; CHOL: Cholangiocarcinoma; COAD: Colon cancer; ESCA: Esophagus cancer; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Renal cancer; KIRP: Kidney renal papillary cell carcinoma; LIHC: Liver cancer; LUAD: Lung cancer; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; READ: Rectal cancer; STAD: Stomach cancer; THCA: Thyroid cancer; UCEC: Uterine Corpus Endometrial Carcinoma; OS, overall survival.

sion of RHPN1-AS1 had much longer OS and relapse-free (RFS) than those with high expression of RHPN1-AS1 (**Figure 2F** and **2G**). A summary of RHPN1-AS1 expression and clinicopathologic features of cases in the ZZU TMA is shown in **Table 1**.

Next, we evaluated if traditional clinicopathologic factors were associated with BC patient outcomes (OS and RFS) via univariable and multivariable analyses. Univariable analyses showed that TNM stage, RHPN1-AS1 expression, differentiation grade and lymph node status were potential prognostic factors for OS and RFS. Moreover, advanced TNM stages, positive lymph node status and high expression of RHPN1-AS1 were independent and statistically significant predictors of OS and RFS in multivariable analysis (**Table 2**). Collectively, our data suggest that lncRNA RHPN1-AS1 expression may serve as a novel prognostic biomarker for BC.

RHPN1-AS1 silencing inhibits BC cell proliferation and invasion in vitro

To gain insights into the role, if any, of lncRNA RHPN1-AS1 in the pathobiology of BC, we first performed IF staining to ascertain the subcellular localization and expression level of RHPN1-AS1 in BC cell lines. Interestingly, RHPN1-AS1 was mainly expressed in the cytoplasm of the cell lines we evaluated, and RHPN1-AS1 expression in MCF-7 cells was much higher than that in normal breast epithelial MCF-10A cells (**Figure 3A**). Then, to further confirm the

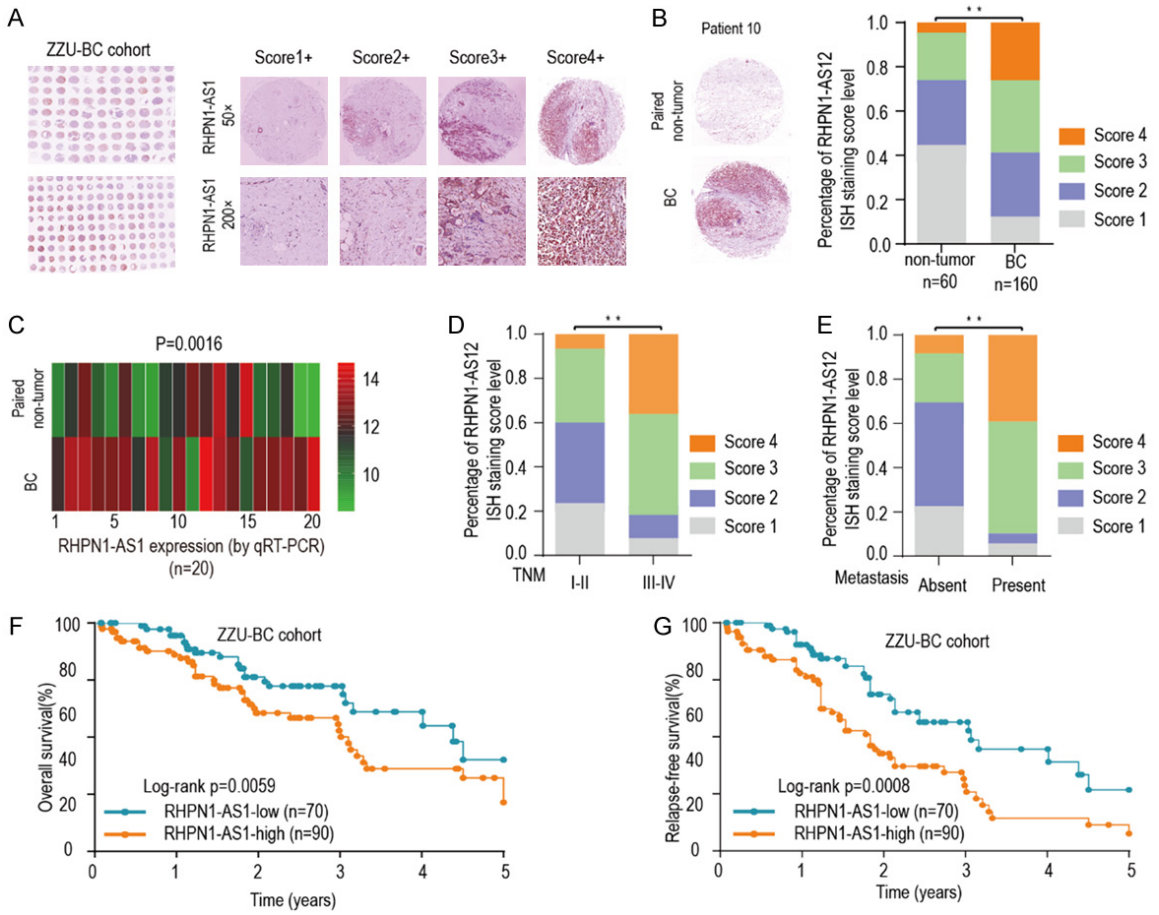

Figure 2. RHPN1-AS1 is overexpressed in BC tissues and high RHPN1-AS1 expression is associated with poorer OS in the ZZU cohort. A. Representative micrographs showing RHPN1-AS1 ISH staining patterns with different staining scores in BC tissues in the ZZU TMA. B. Representative images showing RHPN1-AS1 staining in BC tissues and paired non-tumor tissues in the ZZU TMA (left), and comparison of score distributions between BC tissues and paired non-tumor breast tissues in the ZZU TMAs (right). RHPN1-AS1 was overexpressed in BC tissues compared to non-tumor tissues. C. Comparison of RHPN1-AS1 expression in BC tissues and paired non-tumor breast tissues as assessed by qRT-PCR. RHPN1-AS1 expression was elevated in BC tissues compared to the expression levels in non-tumor breast tissues. D. Comparison of RHPN1-AS1 ISH staining score distributions based on patients' TNM stages. RHPN1-AS1 staining scores were notably higher in BC patients with advanced TNM stages. E. Comparison of RHPN1-AS1 ISH staining score distributions based on presence/absence of metastases. F, G. Kaplan-Meier survival curves showing stratification of patients in the ZZU TMA cohort based on expression of RHPN1-AS1 (orange: high RHPN1-AS1 expression; blue: low RHPN1-AS1 expression). BC patients with high RHPN1-AS1 expression had markedly shorter OS and RFS than those with low RHPN1-AS1 expression (P=0.0349). $^{**}P$<0.01. Abbreviations: BC: Breast cancer; OS, overall survival; RFS, relapse-free survival; ISH, in situ hybridization.

biological functions of RHPN1-AS1 in BC, we performed *in vitro* functional studies by using lenti-virus-mediated shRNA transduction to down-regulate RHPN1-AS1 expression in BC cell lines. The shRNA transfection efficiencies were confirmed by qRT-PCR (**Figure 3B**). Our CCK-8 assay showed that cell proliferation was significantly reduced in the sh-RHPN1-AS1 group compared to the sh-NC group (**Figure 3C**). The suppressive effect of RHPN1-AS1 knockdown on proliferation of MDA-MB-231 and MCF-7 cells was further assessed by the EDU assay, and results showed that DNA synthesis was markedly inhibited in the sh-RHPN1-AS1 group, compared to that in the sh-NC group (**Figure 3D**). Additionally, our transwell assay results demonstrated that the migration and invasion abilities of BC cells were significantly decreased in sh-RHPN1-AS1 group (**Figure 3E** and **3F**). These findings suggest that RHPN1-AS1 knockdown suppresses the proliferation, migration, and invasion of BC cells, and target-

Table 1. Correlation of clinicopathological features with RHPN1-AS1 expression in the ZZU TMA BC cohort

Variables	Clinicopathological features	RHPN1-AS1 expression level		*P*-value
		Low (n, %)	High (n, %)	
Age (years)	≤50	46 (63.9)	49 (54.4)	0.150
	>50	24 (36.1)	41 (45.6)	
Her-2 status	Negative	50 (71.4)	74 (82.2)	0.105
	Positive	20 (28.6)	16 (17.8)	
PR status	Negative	42 (60.0)	56 (62.2)	0.775
	Positive	28 (40.0)	34 (37.8)	
ER status	Negative	47 (67.1)	48 (53.3)	0.078
	Positive	23 (32.9)	42 (46.7)	
TNBC status	TNBC	27 (38.6)	37 (41.1)	0.745
	Non-TNBC	43 (61.4)	53 (58.9)	
Tumor size	<20 mm	22 (31.4)	27 (30.0)	0.846
	≥20 mm	48 (68.6)	63 (70.0)	
Involved lymph node	Negative	32 (45.7)	26 (28.9)	0.028
	Positive	38 (54.3)	64 (71.1)	
Differentiation grade	Well & Moderate	55 (78.6)	43 (47.8)	0.000
	Poor	15 (21.4)	47 (52.2)	
TNM stage	Stage I and II	47 (67.1)	39 (43.3)	0.003
	Stage III and IV	23 (32.9)	51 (56.7)	

ing RHPN1-AS1 may be a promising therapeutic strategy for BC.

RHPN1-AS1 knockdown suppresses tumor growth in vivo

To further elucidate the effect of RHPN1-AS1 silencing on breast tumorigenesis and tumor growth *in vivo*, MCF-7 cells stably transfected with sh-RHPN1-AS1 or sh-NC were injected into nude mice. Results showed that RHPN1-AS1 knockdown profoundly inhibited tumor growth in murine xenograft models (**Figure 4A** and **4B**). Additionally, bioluminescent imaging revealed that the mean luciferase signal in sh-RHPN1-AS1 group was lower than that in sh-NC group (**Figure 4C**). Tumor weight and volumes were also lower in the sh-RHPN1-AS1 group compared with those in the sh-NC group (**Figure 4D** and **4E**). In addition, the expression of both RHPN1-AS1 and Ki-67 were significantly reduced in the sh-RHPN1-AS1 group (**Figure 4F**). Taken together, these results demonstrate that RHPN1-AS1 silencing decreases breast tumor growth *in vivo*.

RHPN1-AS1 is involved in cell cycle and EMT process in breast cancer

Subsequently, we conducted *in silico* bioinformatics analyses to gain insights into the molecular pathways and processes regulated by RHPN1-AS1 in BC. As shown in **Figure 5A**, there was a strong positive correlation between the expressions of Ki-67 and RHPN1-AS1, as well as the expression levels of proliferating cell nuclear antigen (PCNA) and RHPN1-AS1. Moreover, functional and pathway enrichment analyses showed that cell cycle pathways were markedly enriched in RHPN1-AS1-high BC samples compared to RHPN1-AS1-low samples (**Figure 5B**). We then performed GSEA analysis to investigate the relationship between RHPN1-AS1-high expression and cell cycle-related pathways, and found that the genes involved in cell cycle regulation were enriched in the RHPN1-AS1-high samples (**Figure 5C**).

Additionally, RHPN1-AS1-high samples were positively correlated with EMT phenotype signatures (**Figure 5D**). Therefore, we hypothesized that RHPN1-AS1 might promote tumor progression by enhancing the EMT process. As expected, the expression of key protein markers (E-cadherin, N-cadherin and Vimentin) of the EMT process, were significantly decreased in the sh-RHPN1-AS1 group by western blot analysis (**Figure 5E**). Similar results were obtained upon IHC analyses of mouse tumors from our xenograft experiments (**Figure 5F**). These results suggest that RHPN1-AS1 is involved in regulating the cell cycle and EMT processes in BC; thus, upregulated RHPN1-AS1 might function as an oncoRNA and contribute to BC progression, at least in part, through EMT regulation.

Discussion

BC is the second leading cause of cancer-related death among women worldwide. Despite the tremendous advances made in recent decades in BC therapeutic strategies, the prognosis for advanced patients is still dismal [22-25].

Table 2. Correlation of clinicopathological features with RHPN1-AS1 expression in breast cancer TMA cohort

Clinicopathological features	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Univariate and multivariate analyses of overall survival in breast cancer patients (n=160)						
Age (>50 vs <50)	1.120	0.719-1.439	0.692			
Her-2 status (Negative *vs* positive)	0.953	0.498-1.042	0.549			
PR status (Negative *vs* positive)	1.503	0.621-2.482	0.732			
ER status (Negative *vs* positive)	1.388	0.939-2.181	0.529			
TNBC status (Negative *vs* positive)	1.063	0.729-1.875	0.495			
Tumor size (>20 mm *vs* <20 mm)	1.249	1.029-2.054	0.260			
Involved lymph node (Negative *vs* positive)	2.160	1.584-4.827	0.028	2.226	1.801-2.995	0.020
Differentiation grade (Well & Moderate *vs* Poor)	2.279	1.651-3.742	0.014	1.771	1.252-2.223	0.068
TNM stage (I & II *vs* III & IV)	2.679	1.951-4.841	0.000	2.467	1.993-3.922	0.002
RHPN1-AS1 expression level	2.112	1.814-3.975	0.002	1.943	1.587-3.969	0.019
Univariate and multivariate analyses of relapse-free survival in breast cancer patients (n=160)						
Age (>50 vs <50)	1.232	0.778-2.212	0.761			
Her-2 status (Negative *vs* positive)	1.146	0.942-1.567	0.659			
PR status (Negative *vs* positive)	1.728	0.821-3.011	0.842			
ER status (Negative *vs* positive)	1.607	0.939-2.905	0.651			
TNBC status (Negative *vs* positive)	1.163	0.910-1.754	0.545			
Tumor size (>20 mm *vs* <20 mm)	1.327	0.729-2.154	0.286			
Involved lymph node (Negative *vs* positive)	2.592	1.781-4.211	0.034	2.833	2.245-3.997	0.023
Differentiation grade (Well & Moderate *vs* Poor)	2.734	1.957-4.609	0.017	1.539	1.166-2.901	0.093
TNM stage (I & II *vs* III & IV)	3.214	2.011-4.942	0.000	3.144	2.866-4.450	0.005
RHPN1-AS1 expression level	2.279	1.946-4.027	0.006	2.874	2.213-3.826	0.026

Therefore, there is an urgent need to uncover the molecular underpinnings of BC progression and find effective targets for advanced BC therapy.

LncRNAs can play essential roles in cancers, and are implicated in multiple processes associated with tumorigenesis and the regulation of tumor suppressor genes and oncogenes [26-28]. Studies revealed that lncRNAs influence cellular signaling cascades, transcriptional regulation, mRNA stability and/or translation. For example, several lncRNAs, including MALAT1, HOTAIR, and NLIPMT, are known to be dysregulated in BC [20, 29-31]. Peng J et al reported that 13 lncRNAs were associated with the expression level of estrogen receptor (ER) in BC, and many of these lncRNAs were transcribed from genomic regions in chromosome 1 [32]. It is therefore of paramount importance to understand how lncRNAs may drive breast tumorigenesis and/or progression so that their potential prognostic value may be harnessed, and new therapeutic strategies may be developed to improve BC outcomes.

LncRNA RHPN1-AS1 is a critical regulator of disease progression in multiple cancer types. Lu et al reported that overexpression of RHPN1-AS1 was observed in most uveal melanoma cell lines [17]. Consistent with the above findings, we found that the expression of the lncRNA RHPN1-AS1 was dysregulated in several cancers in the TCGA dataset. Notably, expression of RHPN1-AS1 was significantly upregulated in BC tissues in comparison with the levels observed in adjacent normal tissues. Additionally, high expression of RHPN1-AS1 was associated with late TNM stages and poor prognosis of patients with BC in the TCGA dataset. We obtained concordant results with our ISH analysis of the BC patients in the ZZU cohort, thereby affirming the strong association between elevated RHPN1-AS1 expression and an aggressive disease course in BC patients. By contrast, RHPN1-AS1 appears to be downregulated in non-small-cell lung cancer where it acts as a potential tumor suppressor [16]; these observations suggest that RHPN1-AS1 may exert an oncoRNA or tumor suppressor function depending on the cancer type and/or tissue context.

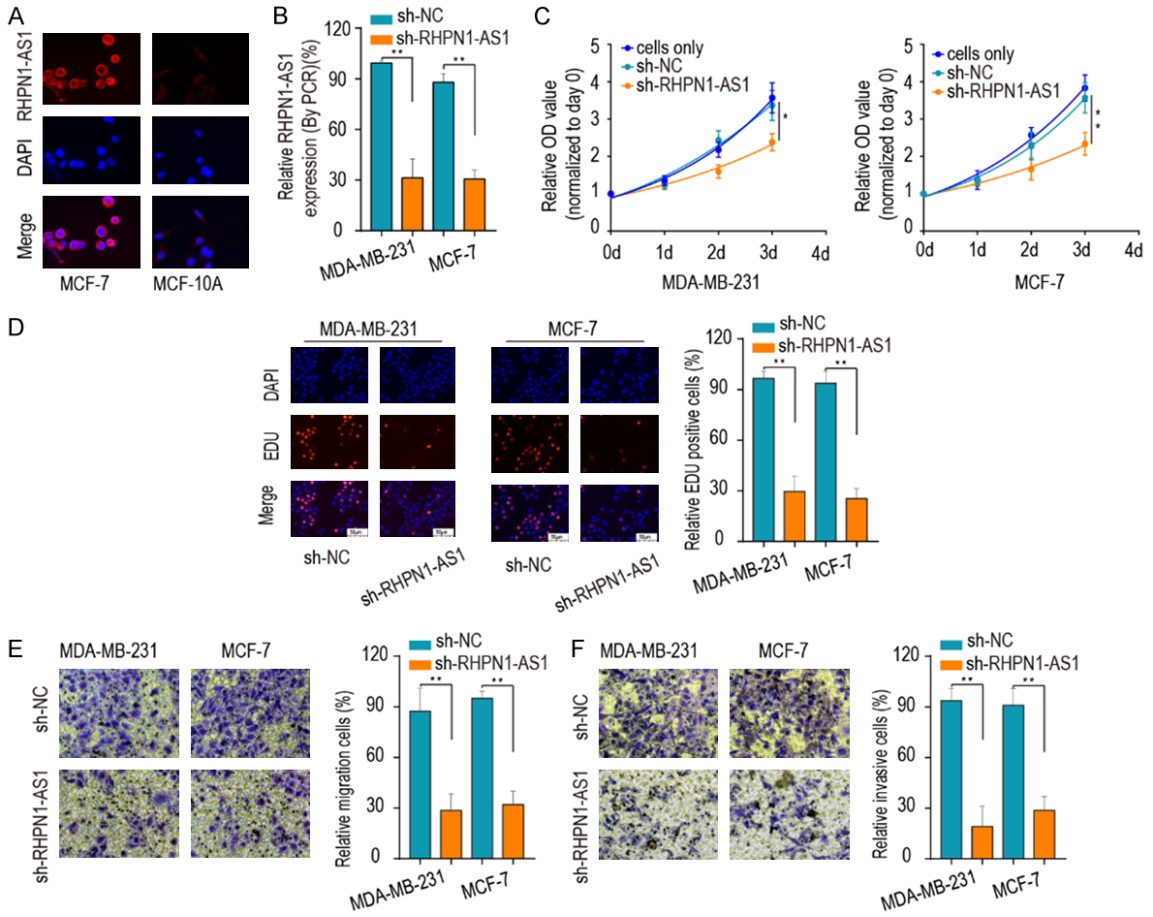

Figure 3. RHPN1-AS1 knockdown inhibits proliferation, migration, and invasion of BC cells *in vitro*. A. Immunofluorescent staining analysis of the localization of RHPN1-AS1 in MCF-7 and MCF-10A cells. RHPN1-AS1 was overexpressed in MCF-7 cells compared to MCF-10A cells. B. Verification of the efficiency of RHPN1-AS1 knockdown in BC cells via qRT-PCR. C. CCK8 assay showing that RHPN1-AS1 silencing attenuated proliferation of MDA-MB-231 and MCF-7 cells. D. Representative fluorescence micrographs (left) and bar graphical quantitation of the percentage of EDU-positive cells from the EDU assay (right) demonstrating that RHPN1-AS1 knockdown suppresses proliferation of MDA-MB-231 and MCF-7 cells. E, F. Representative micrographs (left) and bar graphical quantitation of migrated/invaded cells (right) from the transwell assay showing that RHPN1-AS1 silencing suppressed cell migration and invasion in MDA-MB-231 and MCF-7 cells. **P<0.01. Abbreviations: BC: Breast cancer.

Collectively, these data suggest that dysregulated expression of RHPN1-AS1 could play essential roles in tumor progression.

To further our understanding about how overexpression of RHPN1-AS1 may drive BC progression, we evaluated the impact of RHPN1-AS1 silencing through a series of *in vitro* functional assays, and *in vivo* experiments in murine xenograft models. Our results showed that RHPN1-AS1 silencing suppressed cell proliferation, migration, and invasion *in vitro*, as well as tumor growth *in vivo*. In agreement with our findings, knockdown of RHPN1-AS1 was reported to inhibit tumor growth *in vivo* and cell migration *in vitro* in models of uveal melanoma [17].

Furthermore, our *in silico* bioinformatics analyses showed that RHPN1-AS1 overexpression was positively correlated with and enriched for gene ontologies and pathways related to cell cycle regulation and DNA replication; therefore, RHPN1-AS1 may contribute to BC progression via these essential pathways, and targeting RHPN1-AS1 could be a promising therapeutic approach for BC.

We also found that elevated RHPN1-AS1 overexpression was significantly associated with distant metastasis in BC. Several studies have unequivocally shown that EMT is an important process underlying tumor cell invasion of surrounding tissues and metastasis. Previous

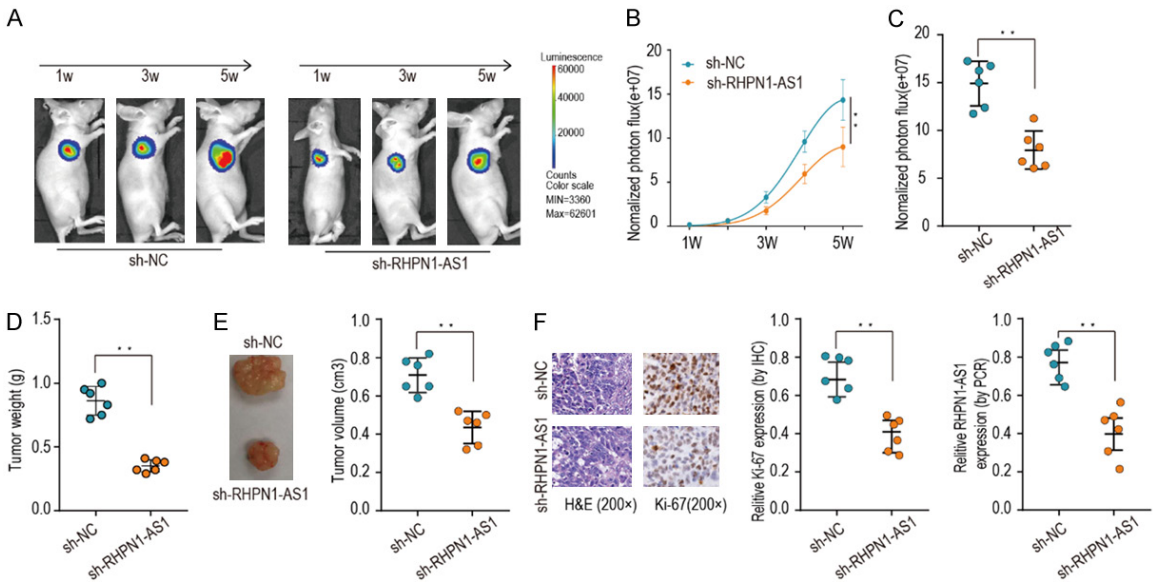

Figure 4. RHPN1-AS1 silencing suppresses breast tumor growth *in vivo*. A. BC cells stably transfected with sh-RHPN1-AS1 or sh-NC were injected into nude mice subcutaneously (n=12). Images of tumors that formed in the nude mice were captured using a live imaging system that detected a luciferase signal. B, C. The luciferase activity of the sh-RHPN1-AS1 tumors was lower than that of tumors in the sh-NC group. D, E. Tumor weights and volumes in the sh-RHPN1-AS1 group were markedly lower than those in the sh-NC group. F. Representative images of mouse (xenograft) tumor sections stained with hematoxylin and eosin (H&E), and immunohistochemically stained for Ki-67, as well as PCR results confirming knockdown of RHPN1-AS1 in mouse tumors. The expressions of both Ki-67 and RHPN1-AS1 were down-regulated in the sh-RHPN1-AS1 group compared to the sh-NC group. $^{**}P<0.01$. Abbreviations: BC: Breast cancer; NC, negative control.

studies have also implicated lncRNAs in EMT [33, 34]. For example, Wang et al found that lncRNA PVT1 increased cancer cell proliferation and migration through regulation of EMT in triple-negative BC [35]. Zhang et al reported that lncRNA NEAT1 regulates the EMT process to promote BC cell proliferation and metastasis [36]. Our GSEA analysis revealed that genes positively corelated with the EMT process were enriched in the group showing high expression of RHPN1-AS1; thus, RHPN1-AS1 may contribute to distant metastasis by enhancing the EMT process in BC. To further confirm this phenomenon, we performed western blot analysis and IHC assays to evaluate the expression level of EMT markers (E-cadherin, N-cadherin and Vimentin) in tumors excised from our murine xenograft models, and our results showed a marked decrease in the expression of these markers in the sh-RHPN1-AS1 group compared to their expression in the sh-NC group. Taken together, our findings make a case for a likely causative link between the upregulation of RHPN1-AS1, enhancement of proliferation and EMT in BC cells, and an aggressive clinical course which confers worse prognosis on BC patients.

Conclusion

In summary, this study demonstrated that RHPN1-AS1 was elevated in BC, and that silencing of RHPN1-AS1 significantly impairs cell proliferation, migration, and invasion, and EMT of BC cells. Therefore, RHPN1-AS1 could be a promising prognostic biomarker and a novel therapeutic target for BC.

Acknowledgements

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Disclosure of conflict of interest

None.

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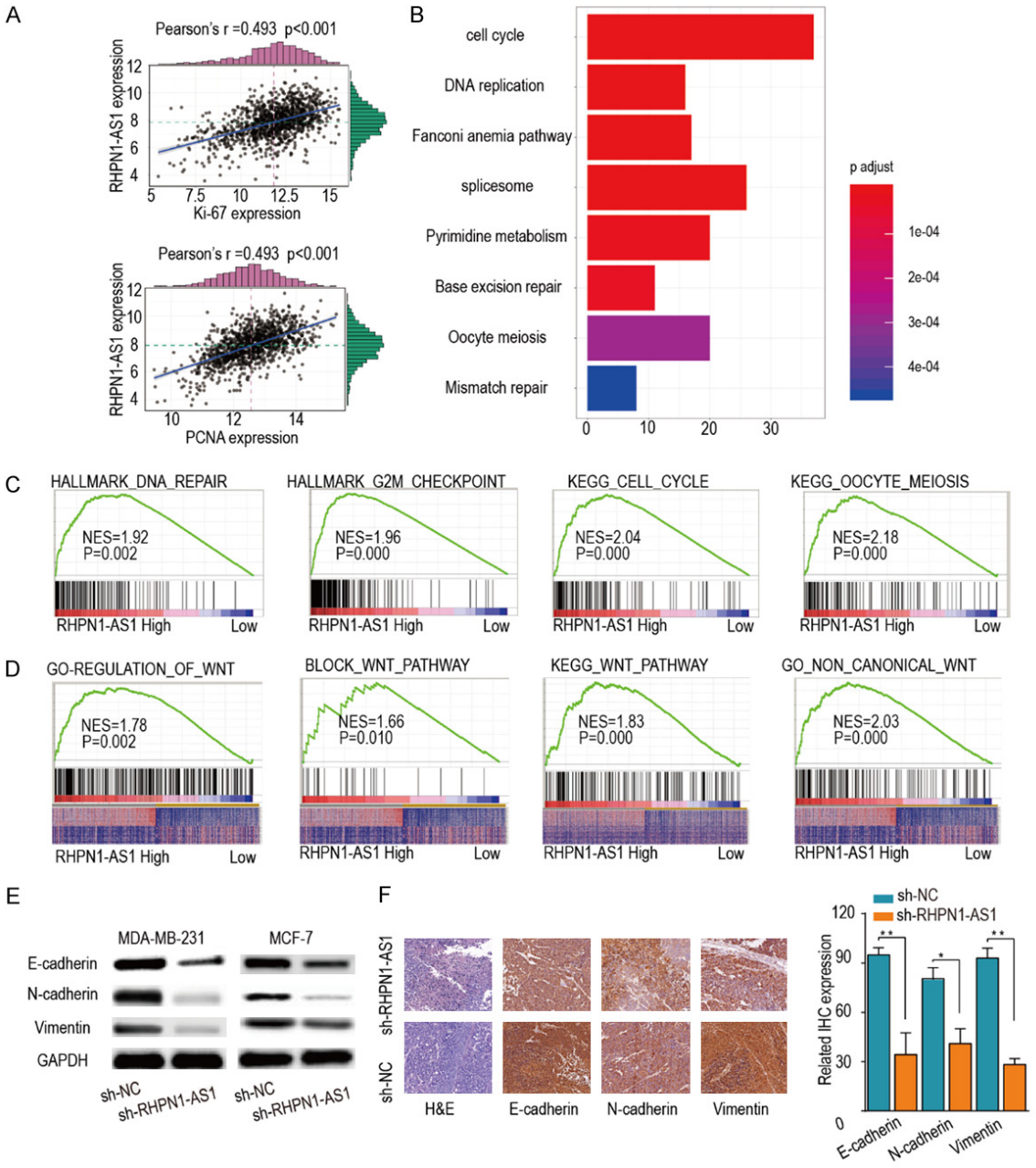

Figure 5. Functional and pathway enrichment analysis. A. Analysis of Pearson correlations between the expressions of RHPN1-AS1 and Ki-67/PCNA. B. Functional and pathway enrichment analyses showing gene ontologies upregulated in BC samples with high expression of RHPN1-AS1. Cell cycle and DNA replication pathways were enriched in RHPN1-AS1-high expressing samples. C. GSEA Plots showing enrichment scores for the indicated gene sets in RHPN1-AS1-high versus RHPN1-AS1-low samples. DNA repair, G2/M checkpoint, cell cycle and oocyte meiosis pathway-related genes are upregulated in samples with high expression of RHPN1-AS1. D. GSEA Plots showing enrichment scores for the indicated gene sets in RHPN1-AS1-high versus RHPN1-AS1-low samples. Canonical and non-canonical Wnt signaling pathway-related genes (that are known to drive EMT) are upregulated in samples with high expression of RHPN1-AS1. E. Western blot analysis showing that the expressions of key EMT marker proteins (E-cadherin, N-cadherin and Vimentin) is reduced in the sh-RHPN1-AS1 group compared to their expression in the sh-NC group. F. Representative images of immunohistochemically stained mouse tumor sections (left) and bar graphical quantitation of IHC expression scores (right) showing that key EMT marker proteins (E-cadherin, N-cadherin and Vimentin) are down-regulated in the sh-RHPN1-AS1 group compared to the sh-NC group. *P<0.05, **P<0.01. Abbreviations: BC, Breast cancer; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; EMT, epithelial-to-mesenchymal transition.

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Table S1. Cell lines used in this study

Cell lines	Cell type	Source	Country
MDA-MB-231	Breast cancer cell	Cell Bank of the Chinese Academy of Science	Shanghai, China
MCF-7	Breast cancer cell	Cell Bank of the Chinese Academy of Science	Shanghai, China
MCF-10A	Breast epithelial cell	Cell Bank of the Chinese Academy of Science	Shanghai, China

Table S2. Information on antibodies used in this study

Antibody	WB	IHC	Specificity	Company
GAPDH	1:5000	/	Mouse monoclonal	Proteintech Group, China
E-cadherin	1:1000	1:100	Mouse monoclonal	CST, Shanghai, China
N-cadherin	1:1000	1:125	Rabbit monoclonal	CST, Shanghai, China
Vimentin	1:1000	1:100	Rabbit monoclonal	CST, Shanghai, China
Ki-67	/	1:500	Rabbit Polyclonal	Proteintech Group, China

Table S3. qRT-PCR Primer sequences used in this study

Name	Direction	Primer (5'-3')
RHPN1-AS1	Forward	GCTCCTGGTCATCAAGTTCCTCT
	Reverse	GCACAGGCACCAGAATGATCC
GAPDH	Forward	AGGTCGGAGTCAACGGATTTG
	Reverse	TGTAAACCATGTAGTTGAGGTCA