# LncRNA EPB41L4A-AS1 Regulates Cell Proliferation, Apoptosis and Metastasis in Breast Cancer

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**Research**

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# Abstract

Background: Breast cancer is a leading global public health problem. In our previous study, we identified that lncRNA EPB41L4A antisense RNA 1 (EPB41L4A-AS1) was significantly downregulated in breast cancer. However, the functional role of EPB41L4A-AS1 in breast cancer has not been clarified. Here, we further confirmed the expression and biological function of EPB41L4A-AS1 in breast cancer.

Methods: To demonstrate the role of EPB41L4A-AS1 in breast cancer, we transfected breast cancer lines with pcDNA3.1-EPB41L4A-AS1 expression vector to induce ectopic overexpression of EPB41L4A-AS1. Then, to explore the role of EPB41L4A-AS1 overexpression in breast cancer cell growth, cell cycle, apoptosis, invasion and migration capacity, we performed CCK-8 assay, colony formation assay, flow cytometry analysis, wound recovery and transwell assay, respectively. We also constructed a co-expression network to explore the potential effect mechanism of EPB41L4A-AS1.

Results: Our research showed EPB41L4A-AS1 expression was significantly lower in tumor tissues than in adjacent non-cancerous tissues. overexpression of EPB41L4A-AS1 significantly reduced the proliferation of breast cancer cells. Flow cytometric analysis showed that forced expression of EPB41L4A-AS1 significantly increased the apoptosis rate of breast cancer cells. In addition, we found that upregulated EPB41L4A-AS1 significantly inhibited the migration and invasive ability of breast cancer. Functional analysis of co-expressed mRNAs suggested that EPB41L4A-AS1 may be involved in ribosomal, cell cycle, spliceosomal and p53 signaling pathways.

Conclusions: Our findings suggest that EPB41L4A-AS1 is a tumor suppressor gene in breast cancer.


# Background

Breast cancer is a leading global public healthcare problem, having been second cause of leading death in US. New breast cancer accounts for 30% in all women cancer diagnoses[1]. Breast cancer is also a leading cancer cause of mortality in both low- and middle-income nations[2]. Despite the continuous development of new drugs to treat breast cancer, the outcome of breast cancer treatment remains to be improved. In addition, the treatment outcome of breast cancer patients with similar stage grading varies greatly. Therefore, to explore the deeper mechanism and find effective molecular markers of breast cancer are significant.

Long non-coding RNAs (lncRNAs) are a category of transcripts that are lengths longer than 200bp and lack the capacity to encode proteins[3]. A growing number of studies have shown that lncRNAs are involved in a wide variety of bio-processes, including epigenetics, translational, post-transcriptional and translation[4]. Deregulation of lncRNAs has also been demonstrated to be involved in the development and progression of breast cancer[5, 6]. Identification of cancer-related lncRNAs and investigation of the biological functions of them can provide more insights into the occurrence and progression of cancer.

In our previous study, we explored the pattern of lncRNAs expression in breast cancer utilizing RNA sequencing[7–9]. We discovered numerous differentially expressed lncRNAs among breast cancer and non-tumorigenic breast tissue, among which a lncRNA, EPB41L4A antisense RNA 1 (EPB41L4A-AS1), was remarkably down-regulated in breast cancer. Nevertheless, the functional role of EPB41L4A-AS1 in breast cancer has not been clarified. Herein, we further confirmed the expression and the biological functions of EPB41L4A-AS1 in breast cancer.

# Methods

Tissue specimens and ethics statement

The date of EPB41L4A-AS1 expression levels of breast cancer was download from The Cancer Genome Atlas date portal(TCGA). To validate whether EPB41L4A-AS1 was a lncRNA, the Coding Potential Calculator 2 Tool (CPC2, http://cpc2.cbi.pku.edu.cn.) was employed to predict its protein-coding potential[10]. All breast cancer tissue specimens and paired was collected from the Department of Breast Surgery of the First Affiliated Hospital of Wenzhou Medical University. After surgical resection, the tissues were immediately transferred to Liquid nitrogen tank and stored at the − 80℃ refrigerator. All processes were authorized by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Cell culture

The breast cancer cell lines (MCF-7, MDA-MB-231) were acquired from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The MDA-MB-231 cells were cultured in RPMI 1640 medium (Invitrogen, USA), while the MCF-7 cells were cultured in DMEM medium (Invitrogen, USA). All cells were cultured at 37°C and 5% CO2 and nourished by the medium containing 10% FBS (Gibco, Grand Island, NY, USA), as well as 100 UI/ml penicillin with 100 μg/ml of streptomycin, mixed and kept at 4°C. The experiments consisted of three groups. The group design consisted of a non-transfected blank control group (Blank group), an empty vector negative control group (NC group) and EPB41L4A-AS1 overexpression plasmid transfection group (Overexpression group).

Real-Time Polymerase Chain Reaction (qRT-PCR)

Extract RNA from cells and tissues according to the manufacturer's instructions using TRIzol reagent (Invitrogen, USA). Then, quantitative RNA was transcribed into cDNA, using Rever Tra Ace qPCR RT Kit (Toyobo). Real-time reactions run and analyzed by using a Real-Time PCR system (Applied Biosystems 7500), all data normalized to GAPDH levels in every sample. The primers sequences are presented as follow: EPB41L4A-AS1 forward□5’- AAGACAGTGAGGATGTGAAT − 3’ and reverse: 5’-TATGGTGACAGCAGTGAATA − 3’;GAPDH□forward: 5’- GTCTCCTCTGACTTCAACAGCG-3’ and reverse: 5’-ACCACCCTGTTGCTGTAGCCAA-3’.

Transfection

EPB41L4A-AS1 overexpression plasmid (pcDNA3.1- EPB41L4A-AS1) and a control vector were designed and constructed. The cloned plasmids were transfected into cells according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen) in Opti-MEM solution (Gibco). The negative control group and the blank group were transfected for the empty vector and without vector, but the same amount of Lipofectamine and Opti-MEM was used. The EPB41L4A-AS1 expression in cells after transfected by plasmids were confirmed by qPCR.

Colony forming assay

Transfected cells were seeded into 6-well plate, include 1500 cells each well, incubated at 37℃ with 5% CO2. Observing its growth state, harvesting when its cluster include more than 50 cells probably (about 7–10 days). At that time, colonies were fixed with 4% Paraformaldehyde Fix Solution and stained with 0.01% crystal violet solution.

Cell Counting Kit-8 - cell proliferation assay

Transfected cells (1500 cells/well) were seeded into 96-well plates and incubated at 37℃ with 5% CO2 for 24h, 48h, 72h and 96h, respectively. Then, 10 μl CCK-8 reagent was inserted into each well, repeated 5 times, and incubated for 2.5 h. The absorbance was then measured at 450 nm.

Cell cycle distribution assay

The transfected cells were collected and washed for three times with PBS. One milliliter of 70% ethanol was used to resuspend cells with soft stirring. The cells were stained with 300 μl of propidium iodide (PI), and then analyzed by Flow Cytometer.

Apoptosis detection analysis

Total cells were collected include free in medium and adhere in bottom. Centrifuging collected cells at 1000 rpm for 5 min and washing with 3ml PBS at 1000 rpm for 5 min, repeated three times. Finally, Using Annexin-V-FITC apoptosis detection kit according to the manufacturer’s instructions and analyzing by Flow Cytometer.

Transwell assay

The cells transfected and incubated in 6-well plate were trypsinized with Trypsin-EDTA Solution and ceased by 10%FBS DMEM. $3*10^4$ for MCF-7 or MDA-MB-231 resuspended in serum-free medium(300μl)were transferred into the upper chamber of transwell, while the bottom chamber was filled with RPMI DMEM culture medium（include 20%FBS）, then put into incubator about 24h. After24h, cells adhered to the upper surface were cleaned and then, fixed with 4% Paraformaldehyde Fix Solution for 15min, obliterated the exceptional cells with PBS, and at last, stained for 15 min with 0.01% crystal violet solution. The pictures were collected by photomicroscope.

Gene ontology (GO) analysis and pathway analysis

GO analysis was performed to define the biology of EPB41L4A-AS1. GO annotations were obtained by downloading from NCBI and GO databases[11]. Pathway analysis was used to identify pathways significantly enriched for EPB41L4A-AS1 according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[12]. Fisher's exact test was applied to identify significant GO categories and to select significant pathways.

# Statistical analysis

Statistical analysis was established using SPSS 23.0 statistical software packages (SPSS, Inc., Chicago, IL, USA). Statistical significance between groups was analyzed using student's *t*-test or a one-way ANOVA. $P < 0.05$ were considered statistically significant.

## Results

EPB41L4A-AS1 was a down-regulated lncRNA in breast cancer

In the sequencing cohort, we identified that the expression of lncRNA EPB41L4A-AS1 was markedly lower in breast cancer tissues than in adjacent normal tissues (fold change = 0.3, $P < 0.001$, Fig. 1A). To further verify whether EPB41L4A-AS1 was a lncRNA, we adopted the Coding Potential Calculator 2 tool to predict its protein coding potential because of its high predictive accuracy[10]. The result showed that the sequence of EPB41L4A-AS1 had low coding probability which means it was a bona fide lncRNA (Fig. 1B). The classical of lncRNA HOTAIR and the classical of mRNA GAPDH were used as negative and positive controls, separately. To further validate the dysregulation of EPB41L4A-AS1, the expression of EPB41L4A-AS1 in 24 pairs of breast cancer tissues and adjacent non-cancerous tissues was measured by qRT-PCR. As demonstrated in Fig. 1C, the expression of EPB41L4A-AS1 was remarkably lower in tumor tissues than in adjacent non-cancerous tissues ($P < 0.001$, Fig. 1C). In additional, we verify the deregulation of EPB41L4A-AS1 in The Cancer Genome Atlas (TCGA) database, which contained 837 breast cancer and 105 non-tumor tissues. Consistently, the expression of EPB41L4A-AS1 also showed a significant reduction in the TCGA cohort in breast cancer tissues ($P < 0.001$, Fig. 1D). Based on the RNAfold web server tool, we predicted secondary structure of EPB41L4A-AS1[13]. As showed in Fig. 1E, the free energy of the thermodynamic ensemble is -440.63 kcal/mol which suggested EPB41L4A-AS1 owned a highly structural stability. In conclusion, these results implied that EPB41L4A-AS1 was a down-regulated lncRNA and may exert a tumor suppressor role in breast cancer.

# Influence of EPB41L4A-AS1 on the cell proliferation

To demonstrate the effect of EPB41L4A-AS1 in breast cancer, we transfected breast cancer lines with pcDNA3.1-EPB41L4A-AS1 expression vector to induce ectopic overexpression of EPB41L4A-AS1. The post-transfection expression level of EPB41L4A-AS1 was significantly increased in the cell lines. ($P <$

0.001, Fig. 2A). The negative control group and the blank group were transfected for the empty vector and without vector. CCK-8 assays suggested EPB41L4A-AS1 overexpression significantly reduced the growth rate of both cell lines ($P < 0.001$, Fig. 2B). Colony formation assays also indicated that EPB41L4A-AS1 overexpression greatly impaired the number of colonies in both MDA-MB-231 cells and MCF-7 cells breast cancer cells (Fig. 2C).

# Enforced expression of EPB41L4A-AS1 promoted breast cancer cells apoptosis

To investigate whether the EPB41L4A-AS1 effect on breast cancer cell proliferation reflects cell cycle arrest further, we have examined the cell cycling progression by flow cytometry analysis. The results showed that there was no effect on cell cycle in breast cancer cells transfected with EPB41L4A-AS1 vector (Fig. 3A). To determine if the proliferation of breast cancer cells was influenced by apoptosis, we conducted a flow cytometric analysis. Flow cytometry analysis showed that enforced expression of EPB41L4A-AS1 remarkably increased the apoptotic rate of MCF-7 cells as well as MDA-MB-231 cells (Fig. 3B). In conclusion, these results suggest that EPB41L4A-AS1 plays a key role in apoptosis of breast cancer cells.

EPB41L4A-AS1 regulated migration and invasion of breast cancer cells

The degree of malignancy was associated not only with proliferation, but also migration and invasion. Wound recovery was significantly delayed when overexpression of EPB41L4A-AS1 compared with control groups in MDA-MB-231cells ($P < 0.01$, Fig. 4A). To confirm further the role for EPB41L4A-AS1 in breast cancer, we next examined the migration and invasion functions by transwell assay. The results revealed that the upregulated EPB41L4A-AS1 significantly suppressed the migration and invasive ability of breast cancer cells as compared to the negative control group and blank control group ($P < 0.001$, Fig. 4B and 4C).

Potential effect mechanism of EPB41L4A-AS1

To further clarify the mechanism by which EPB41L4A-AS1 exerted its biological function, we screened 118 mRNAs whose expression levels were highly correlated with EPB41L4A-AS1 based on the preliminary RNA sequencing data. Then, function analysis of co-expression mRNAs of EPB41L4A-AS1 was shown in Table 1. The results of GO analysis suggested that the co-expressed mRNAs were participating in biological processes such as translation initiation, SRP-dependent co-translation, and viral transcription. The pathway analysis, on the other hand, revealed that these co-expressed mRNAs were involved in multiple signaling pathways involving ribosome, cell cycle, replisome, and p53 signaling pathways. Collectively, our analysis might partly explain its effect mechanism.

Table 1
Function analysis of co-expression mRNAs of EPB41L4A-AS1

| Database | Term | ID | Corrected P value |
|---|---|---|---|
| Gene Ontology | translational initiation | GO:0006413 | 4.96E-48 |
| Gene Ontology | SRP-dependent cotranslational protein targeting to membrane | GO:0006614 | 4.46E-46 |
| Gene Ontology | viral transcription | GO:0019083 | 6.44E-44 |
| Gene Ontology | nuclear-transcribed mRNA catabolic process, nonsense-mediated decay | GO:0000184 | 3.75E-43 |
| Gene Ontology | translation | GO:0006412 | 1.40E-40 |
| Gene Ontology | structural constituent of ribosome | GO:0003735 | 8.63E-40 |
| KEGG PATHWAY | Ribosome | hsa03010 | 5.78E-38 |
| KEGG PATHWAY | Cell cycle | hsa04110 | 0.009092 |
| KEGG PATHWAY | Spliceosome | hsa03040 | 0.010583 |
| KEGG PATHWAY | p53 signaling pathway | hsa04115 | 0.016371 |
| KEGG PATHWAY | Huntington disease | hsa05016 | 0.026894 |
| KEGG PATHWAY | Thermogenesis | hsa04714 | 0.041765 |

# Discussion

The latest research shows that breast cancer is still the most common malignant tumor in women[14, 15]. In recent years, the treatment options for breast cancer have become more and more abundant, just like preoperative neoadjuvant chemotherapy, endocrine therapy, and targeted drug[16, 17]. Rates of long-term survival of breast cancer patients have grown steadily. All of above can be attributed to unremittingly explore the potential mechanism of pathogenesis. Nevertheless, breast cancer differs in its natural history and response to treatment[18]. Genomic variations are the major cause of breast cancer biodiversity. Hence, there is an urgent need to generate candidate biomarkers in order to stratify patients and personalize treatment to avoid excessive or inadequate treatment.

With the emergence of investigations associated with lncRNAs, it has been found that lncRNAs play significant contributions to the process of tumor initiation, progression, metastasis and recurrence[19]. Earlier studies have already revealed that the overexpression of LINK-A lncRNA boosted cell growth, inhibited apoptosis, and upregulated survivin expression, while knockdown of LINK-A lncRNA had the reverse effect in triple-negative breast cancer[20]. In addition, other scholars have previously reported that the silencing of LOC101060264 suppressed tumor proliferation in nude mice in vivo and suppressed the migration, invasion and proliferation of colon cancer cells in vitro[21]. Furthermore, lncRNAs are also quite prospering for clinical applications in oncology. For example, lncRNA-D16366 has been shown to be a potential biomarker with high diagnostic and prognostic value for the diagnosis of hepatocellular carcinoma[22].

EPB41L4A-AS1 is located in the 5q22.2 region of the genome, which is strongly linked to oncogenesis due to frequent DNA fragment deletions. Previous investigations have indicated that EPB41L4A-AS1 regulates glycolysis and glutaminolysis as well as inhibited tumor cell proliferation[23, 24]. Jie Bin et al. had reported that EPB41L4A-AS1 functions as an oncogene by regulating the Rho/ROCK pathway in colorectal cancer[25]. The role of EPB41L4A-AS1 in the development and progression of breast cancer, however, remains unknown. In this article, we examined EPB41L4A-AS1 expression in breast cancer tissues and demonstrated the function of EPB41L4A-AS1 in breast cancer. To our knowledge, the present study identified for the first time that EPB41L4A is involved in breast cancer. The precise mechanisms of EPB41L4A-AS1 remain unknown. Previous studies have covered the extensive existence of sense-nonsense transcripts in mammalian cells and noted that antisense RNA perturbation can alter the expression of sense genes[26]. However, our study showed that the expression level of EPB41L4A was not significantly altered after overexpression of EPB41L4A-AS1 (data not shown in the results). To explore the mechanisms of EPB41L4A-AS1, we constructed co-expression networks based on the preliminary RNA sequencing data. The function analysis of co-expression mRNAs had shown that EPB41L4A-AS1 might participated in Ribosome, Cell cycle, Spliceosome, p53 signaling pathway, etc. We will investigate the correlation of EPB41L4A-AS1 with these pathways in subsequent experiments to clarify the role and mechanism of EPB41L4A-AS1 in breast cancer.λ

In addition, we also observed a difference in p-value between the two experimental groups of MDA-MB-231 and MCF-7. There are two possible reasons to explain the different p-values between MDA-MB-231 and MCF-7. First, the two cell lines belong to different breast cancer subtypes. MDA-MB-231 belongs to a triple-positive breast cancer cell line, while MCF-7 belongs to an HR-positive breast cancer cell line. Therefore, there is a huge difference in their biological backgrounds. Second, overexpression of EPB41L4A-AS1 in MFC-7 was more effective and may have resulted in more diverse experimental results and lower p-values for MCF-7.

# Conclusions

We identified a significantly decreased expression level of EPB41L4A-AS1 in breast cancer and demonstrated enforced expression of EPB41L4A-AS1 promoted breast cancer cells apoptosis as well as inhibition of the proliferation, migration and invasion. Our findings indicated that EPB41L4A-AS1 was a breast cancer tumor suppressor gene. To our knowledge, the present study identified for the first time that EPB41L4A was involved in breast cancer. Further research is needed to elucidate the underlying mechanism of EPB41L4A-AS1 in breast cancer, as well as the signaling pathways involved.

# Abbreviations

EPB41L4A antisense RNA 1, EPB41L4A-AS1

Long non-coding RNAs, lncRNAs

Real-Time Polymerase Chain Reaction, qRT-PCR

Gene ontology, GO

Kyoto Encyclopedia of Genes and Genomes,KEGG

The Cancer Genome Atlas, TCGA

# Declarations

Ethics approval and consent to participate：This study was reviewed and approved by Institutional Review Board of the First Affiliated Hospital of Wenzhou Medical University. Informed consents were obtained from all participants in the study.

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

Competing interests: Not applicable

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Authors' contributions: Fan Yang is responsible for biological experiments and Shixu Lv is responsible for article writing.

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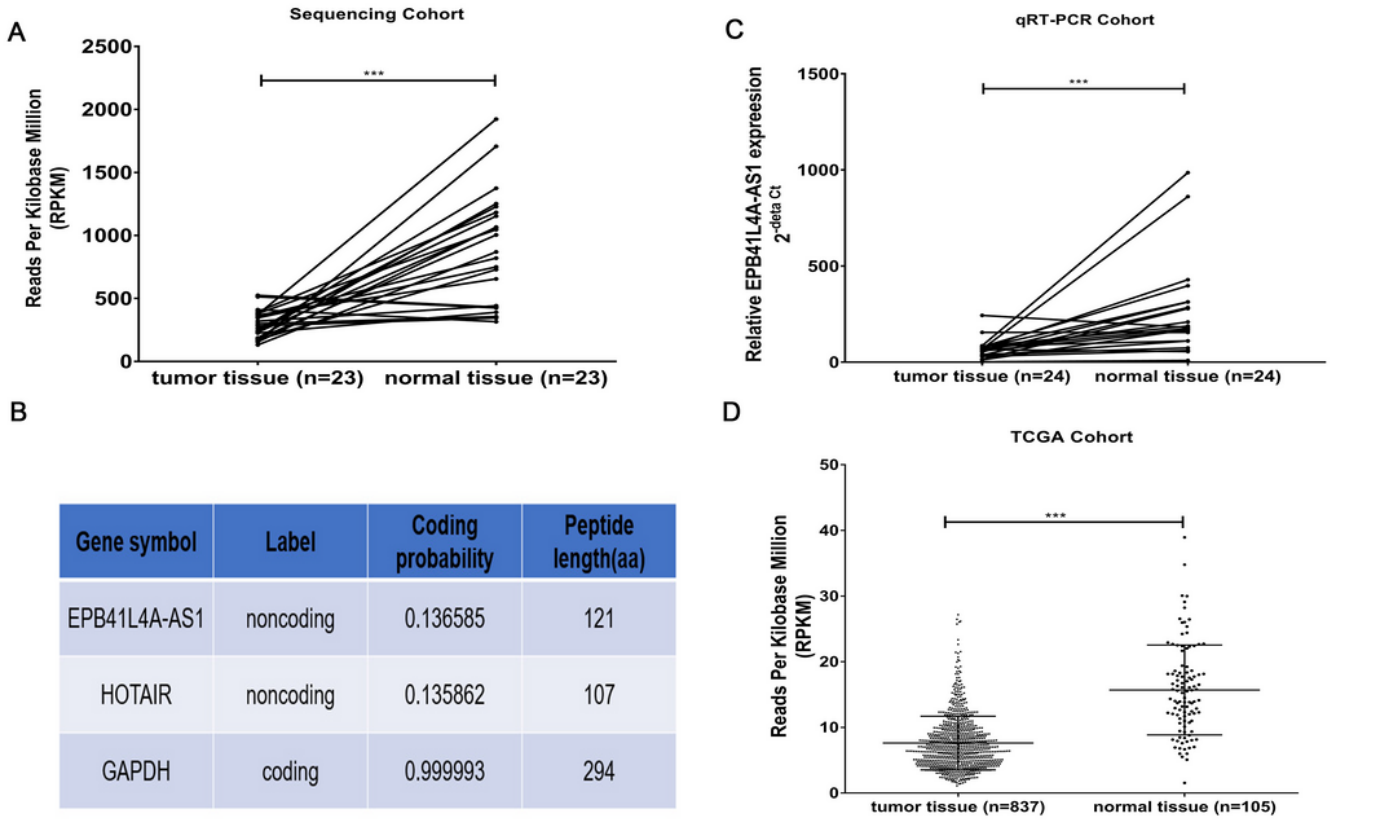
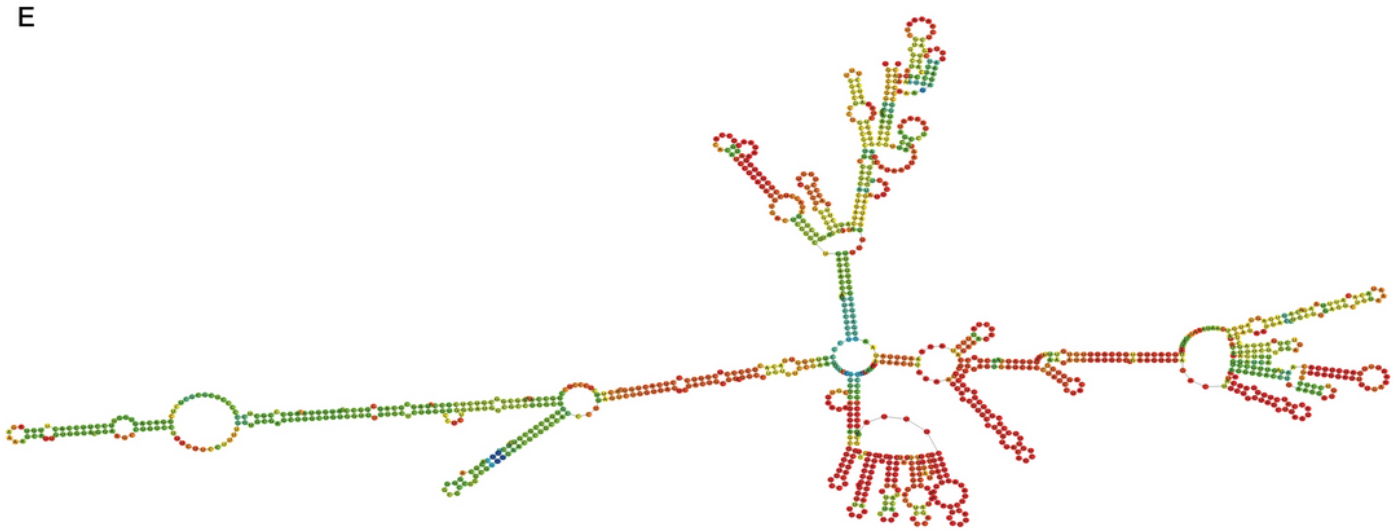
# Figures

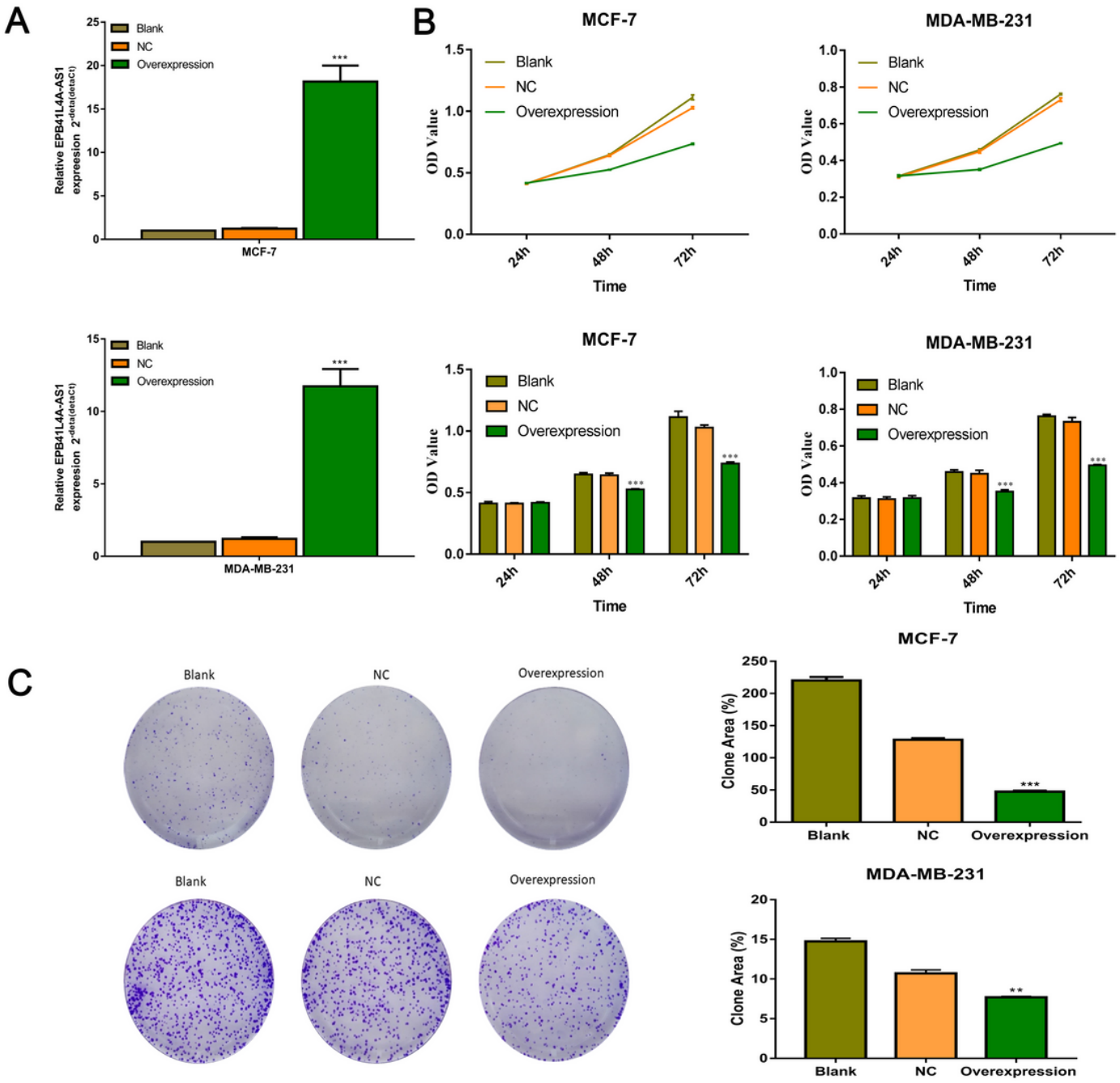



| Gene symbol | Label | Coding probability | Peptide length(aa) |
|---|---|---|---|
| EPB41L4A-AS1 | noncoding | 0.136585 | 121 |
| HOTAIR | noncoding | 0.135862 | 107 |
| GAPDH | coding | 0.999993 | 294 |



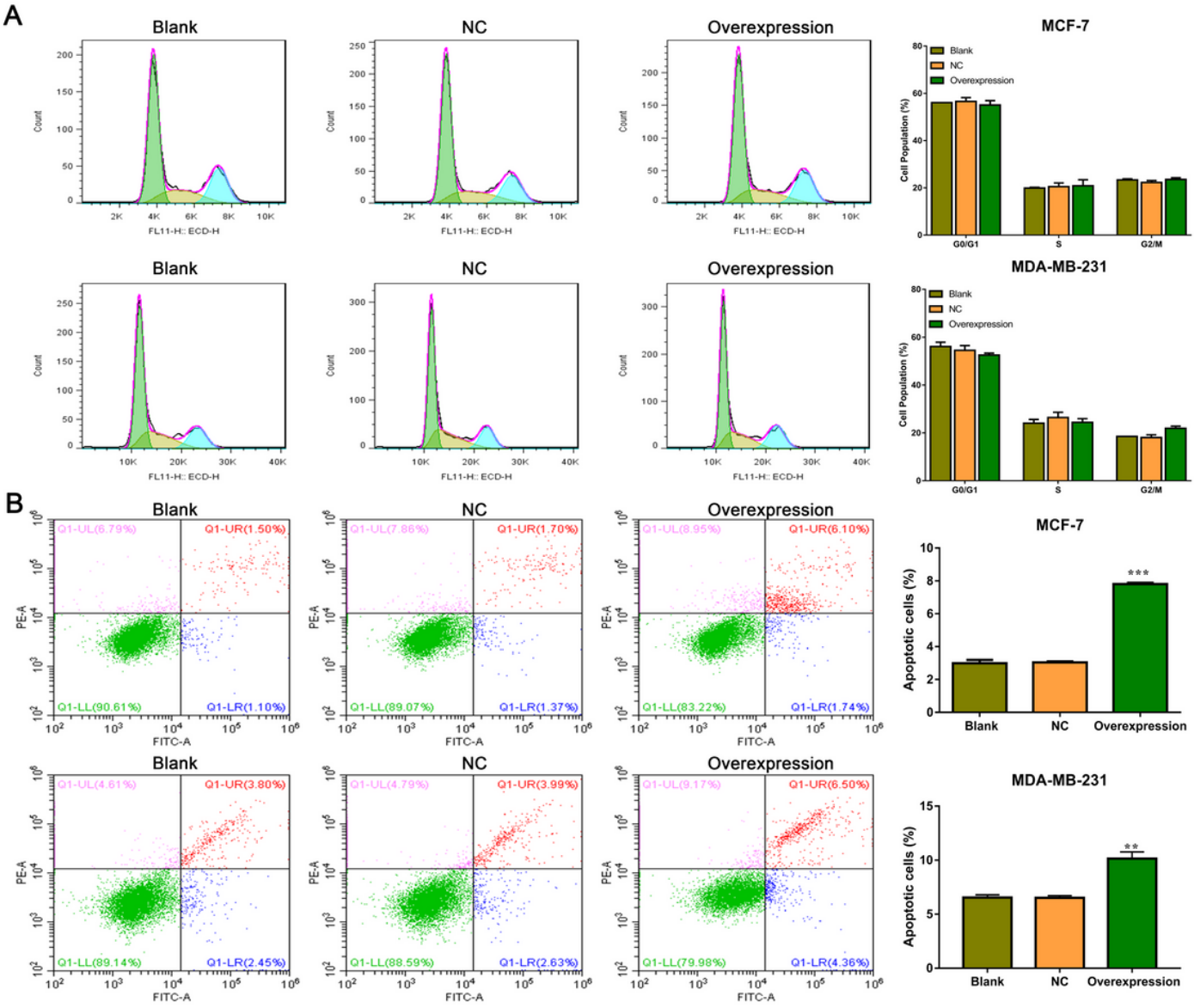

## Figure 1

EPB41L4A-AS1 is a down-regulated lncRNA in breast cancer. (A) EPB41L4A-AS1 expression in the sequencing cohort. (B) Protein-coding potential of the RNA predicted by the coding potential calculator2. (C) The EPB41L4A-AS1 expression in 23 pairs of cancer tissues and adjacent normal tissues detected by qRT-PCR. (D) The EPB41L4A-AS1 expression in the TCGA cohort. (E) Secondary structure of EPB41L4A-AS1 predicted by coding potential calculator 2 RNAfold web server tool.



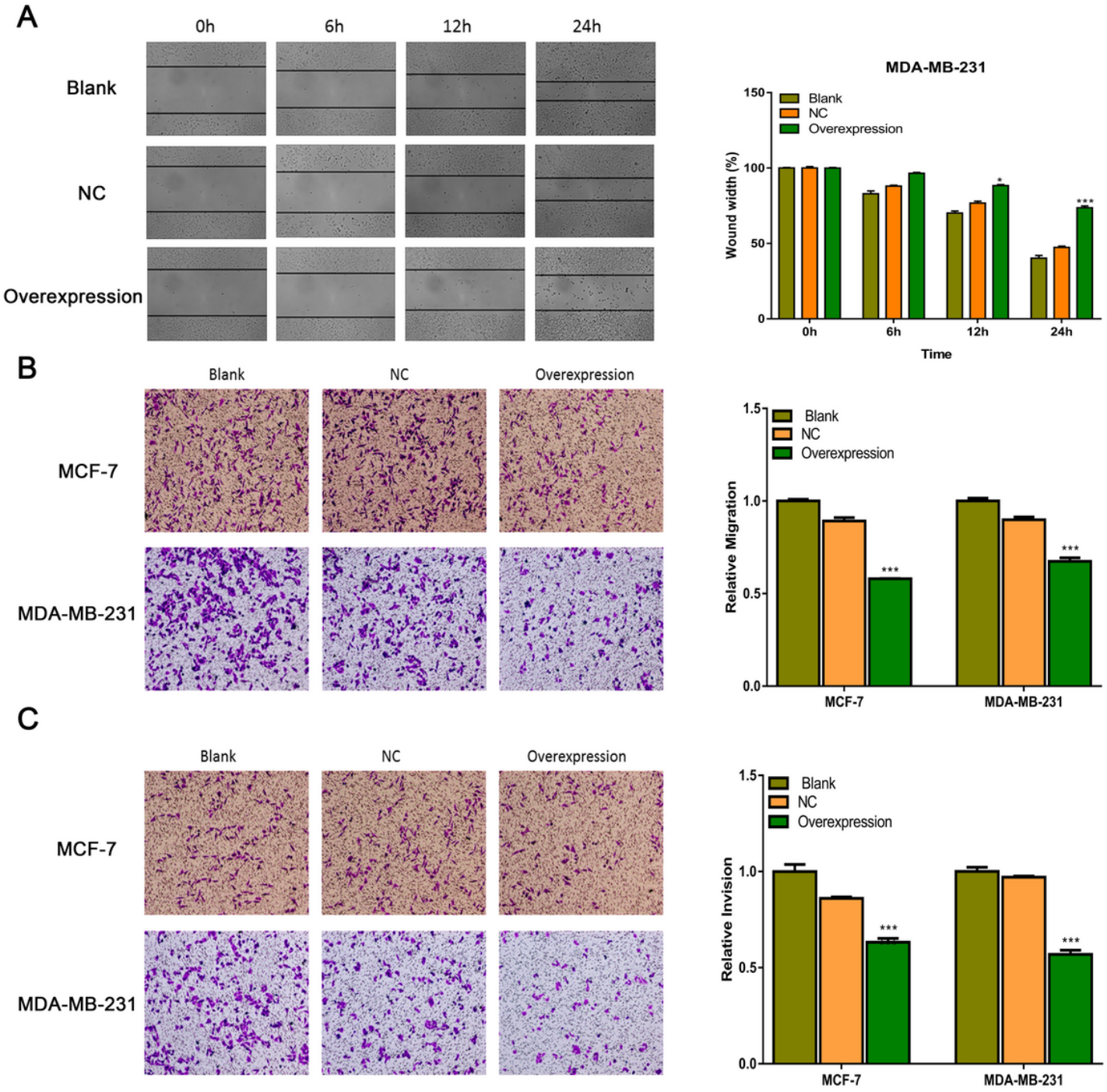

## Figure 2

Enforced expression of EPB41L4A-AS1 inhibits tumor cell proliferation. (A) Breast cancer lines MCF-7 and MDA-MB-231 were transfected with pcDNA3.1-EPB41L4A-AS1 expression vector to induce ectopic overexpression of EPB41L4A-AS1. (B) CCK-8 assays. (C) Colony formation assays. ** P<0.01, *** P<0.001.




## Figure 3

Enforced expression of EPB41L4A-AS1 promoted breast cancer cells apoptosis. (A) The cell cycling progression were measured by flow cytometry assays.(B) The cell apoptosis rate were measured by flow cytometry assays. ** P<0.01, *** P<0.001.




## Figure 4

EPB41L4A-AS1 regulated migration and invasion of breast cancer cells. (A) Wound recovery assays. (B) Migration assays. (C) Invasion assays. * P<0.05, *** P<0.001.