

Original Article

uc.38 induces breast cancer cell apoptosis via PBX1

Lin-Xin Zhang*, Lu Xu*, Chen-Han Zhang*, Yi-Han Lu, Tian-Hao Ji, Li-Jun Ling

*Breast Disease Division, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China. *Equal contributors.*

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Abstract: Long non-coding RNAs (lncRNAs) are transcripts longer than 200 bp with no protein-coding capacity. Transcribed ultraconserved regions (T-UCRs) are a type of lncRNA and are conserved among human, chick, dog, mouse and rat genomes. These sequences are involved in cancer biology and tumorigenesis. Nevertheless, the clinical significance and biological mechanism of T-UCRs in breast cancer remain largely unknown. The expression of uc.38, a T-UCR, was down-regulated in both breast cancer tissues and breast cancer cell lines. However, uc.38 was expressed at significantly lower levels in larger tumours and tumours of more advanced stages. Based on the results of in vitro and in vivo experiments, up-regulation of uc.38 expression inhibited cell proliferation and induced cell apoptosis. Thus, uc.38 suppressed breast cancer. Additional experiments revealed that uc.38 negatively regulated the expression of the pre-B-cell leukaemia homeobox 1 (PBX1) protein and subsequently affected the expression of Bcl-2 family members, ultimately inducing breast cancer cell apoptosis. Describing the uc.38/PBX1 axis has improved our understanding of the molecular mechanisms involved in breast cancer apoptosis and has suggested that this axis is a potential therapeutic target for breast cancer.

Keywords: Long non-coding RNA, proliferation, pre-B-cell leukaemia homeobox 1, breast cancer, apoptosis

Introduction

Breast cancer is the most common type of tumour in women [1]. The incidence of breast cancer ranks first out of the various tumours in females worldwide, and the mortality rate currently ranks second. In the USA, one in eight women will develop breast cancer in her lifetime, and approximately 252,710 new cases and 40,610 breast cancer-associated deaths occurred in 2017 [2]. Although the mortality rate has declined with developments in medical science, the incidence and mortality of breast cancer are currently very high, particularly in many developed countries. Based on gene expression profiles, breast cancer is defined as basal-like, human epidermal growth factor receptor 2 (HER-2)-enriched, luminal A, or luminal B types [3]. Traditional prognostic markers are not sufficient to discriminate between different biological and clinical outcomes [4]. Therefore, the search for new ideal biomarkers and novel therapeutic targets and investigations into the underlying molecular mechanisms may contribute to the development of new treatments and prognostic monitoring strategies for breast cancer.

Long non-coding RNAs (lncRNAs), which are longer than 200 bp, are a subtype of non-coding RNAs [5]. They lack protein-coding capacity; however, they participate in human cancer development and progression. Based on accumulating evidence, lncRNAs are useful as diagnostic and/or prognostic tumour biomarkers [6]. For example, colon cancer-associated transcript 2 (CCAT2) is a prognostic marker of colon cancer and breast cancer [7, 8].

Transcribed ultraconserved regions (T-UCRs) are a type of lncRNA [9]. They are transcribed from regions longer than 200 bp, and they are absolutely conserved among human, chick, dog, mouse and rat genomes [10]. T-UCRs act as antisense inhibitors of protein-coding genes or other non-coding RNAs (e.g., microRNAs) [11]. They are also involved in cancer biology and tumorigenesis [12]. For example, uc.206 is up-regulated in cervical cancer, and over-expression of uc.63 is correlated with a poor prognosis of patients with luminal A-type breast cancer [13, 14]. As a result, T-UCRs are potentially useful as diagnostic or prognostic markers and represent potential new therapeutic targets for cancer treatment.

Nevertheless, the clinical significance and biological mechanism of T-UCRs in the progression of breast cancer remain largely unknown. In this study, we characterized one T-UCR, uc.38. The ultraconserved region (224 bp) was found to be located on chr1:161127332-161-127555 and its expression was remarkably down-regulated in breast cancer tissues and breast cancer cell lines. Its role in breast cancer development was also analysed.

Materials and methods

Specimens and cell lines

Breast tissue specimens were obtained from the Department of Breast Disease, the First Affiliated Hospital of Nanjing Medical University (NJMU). The study was approved by the Research Ethics Committee of Nanjing Medical University, and written informed consent was obtained from all patients. Samples were frozen in liquid nitrogen immediately after surgical removal. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The SUM1315 human breast cancer cell line was kindly provided by Dr. Stephen Ethier (University of Michigan, Ann Arbor, MI, USA). MCF-10A cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Sigma, St. Louis, MO, USA) supplemented with 5% horse serum (Gibco), 20 ng/ml epidermal growth factor (EGF) (R&D Systems), 0.5 mg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 10 mg/ml insulin (Gibco) and penicillin/streptomycin. The MCF-7, ZR-75-1, BT-474, MDA-MB-231, SUM1315 and SK-BR-3 cell lines were cultured in DMEM (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco) and antibiotics (1% penicillin/streptomycin, Gibco). All cell lines were grown in a humidified chamber supplemented with 5% CO₂ at 37°C.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the breast tissue specimens or cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentrations were quantified using a NanoDrop 2000 spectrophotometer (Nanodrop, Wilmington, DE, USA). For qRT-PCR, RNA samples were reverse transcribed to cDNA using the Reverse Transcription Kit (TaKaRa, Dalian, China). The qRT-PCR

experiment was performed using the 7500 Real-Time PCR System (Applied Biosystems, USA) with the Fast Start Universal SYBR Green Master (Roche, USA). The results were normalized to GAPDH expression. Specific primers are listed in [Supplementary Table 1](#). All procedures were performed in triplicate.

Cell proliferation assays

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocol. For the cell proliferation assay, transiently transfected cells were seeded into 96-well plates at a density of approximately 2000 cells per well, and cell proliferation was tested approximately every 24 h. After incubation with 10 µl of the CCK-8 reagent (Beyotime Institute of Biotechnology, Shanghai, China) for 2 or 4 h, the absorbance of each well was measured at 450 nm. Each sample was assayed in five replicate wells, and three parallel experiments were conducted.

Colony-formation assay

For the colony-formation assay, transfected cells (400 cells/well) were seeded into each well of a six-well plate and maintained in medium containing 10% FBS in an incubator with 5% CO₂ at 37°C. After two weeks, colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Colony formation was determined by counting the number of stained colonies. Triplicate wells were measured for each treatment group.

Detection of apoptosis by flow cytometry (FCM)

Transfected cells were harvested after transfection by trypsinization. APC-Annexin V/7-aminocoumarin-actinomycin (7-AAD) (BD Biosciences, CA, USA) was used to detect cell apoptosis. Cells were treated according to the manufacturer's instructions. Approximately 1-5 × 10⁵ cells were collected and resuspended in 500 µl of 1 × Binding Buffer. APC-Annexin V and 5 µl of 7-AAD were added and mixed gently. The reaction lasted for 15 minutes at room temperature in a light-proof environment. Samples were analysed using a flow cytometer (BD Biosciences, CA, USA). Finally, the ratio of apoptotic cells to total cells was calculated. Each test was repeated in triplicate.

The role of lncRNAs in breast cancer

Oligonucleotides and transfection conditions

The siRNA sequences targeting the pre-B-cell leukaemia homeobox 1 (PBX1) gene and the negative control RNA (NC) were purchased from Genescript Biotech (Shanghai, China). All siRNA sequences are presented in [Supplementary Table 1](#). Each siRNA was transfected at a concentration of 50 nM. All transfection procedures were performed using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were harvested for RNA and protein extraction after 72 h.

Lentivirus production and transduction

The lentiviral vectors containing the PBX1 DNA sequence (Lv-PBX1), the uc.38 sequence [15] (Lv-uc.38), the negative control sequence (Lv-NC), the uc.38 siRNA (Lv-uc.38siRNA) and the control siRNA (Lv-uc.38siRNA-NC, empty vector) were produced by Genescript (Shanghai, China). Lentiviruses carrying the vectors described above were transfected into HEK293T cells (ATCC) to generate retroviral particles. Stable cell lines were established by infecting ZR-75-1 and SUM1315 cells with lentiviral vectors and selection with puromycin. After one week, cell pools were obtained and expanded. The infection efficiency was confirmed by qRT-PCR and Western blotting.

Subcellular fractionation

The nuclear and cytosolic fractions were separated using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA immunoprecipitation (RIP)

The RIP experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Antibodies against PBX1 used for the RIP assays were purchased from Abcam. The results were confirmed by qRT-PCR and electrophoresis on a 1% agarose gel.

Western blotting assay and antibodies

A total protein extraction kit (KeyGen Biotech, Nanjing, China) was used to extract total proteins. The procedures were performed accord-

ing to the manual provided in the kit. Total protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and transferred to a polyvinylidene fluoride membrane (PVDF) (Merck Millipore, Darmstadt, Germany) using a standard wet transfer apparatus. Then, membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature for 2 h and incubated overnight with the primary anti-PBX1 (1:1,000 dilution, ab97994, Abcam), anti-Bcl2 (1:1,000, ab32124, Abcam), anti-Bax (1:1,000, ab77566, Abcam), anti-cleaved Caspase-3 (1:1,000, ab2302, Abcam), or anti-GAPDH antibody (1:1,000 dilution, AF0006, Beyotime); the latter antibody was used as an internal control. The membrane was washed with TBST three times for 5 minutes per wash. Then, the PVDF membrane was incubated with blocking buffer containing the diluted secondary antibody at room temperature for 2 h. Finally, the protein expression levels were detected using an electrochemiluminescence (ECL) assay (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The density of each band was quantified using Image-Pro Plus 6 software. The expression of target proteins was normalized to GAPDH levels.

Immunofluorescence

When the coverage of cells on the cover slips reached approximately 90%, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. After aspirating the fixative, samples were rinsed three times with 1 × PBS for 5 minutes per wash and blocked with Blocking Buffer (1 × PBS supplemented with 0.3% Triton X-100 (Sangon Biotech) and 5% normal goat serum (Life Technologies)) for 60 minutes. After an incubation with the primary antibody overnight at 4°C, samples were rinsed three times with 1 × PBS for 5 minutes per wash and then incubated with the anti-rabbit IgG secondary antibody (1:200) for 60 minutes at room temperature in the dark. Normal rabbit IgG (Life Technologies) was used as the negative control. The antibodies used for the immunofluorescence assay were rabbit anti-PBX1 (Abcam), rabbit anti-IgG (Merck Millipore), and anti-rabbit IgG (Alexa Fluor® 488 Conjugate) (Life Technologies). The nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI) (Sangon Biotech), and cells were visualized using a Ti-S fluores-

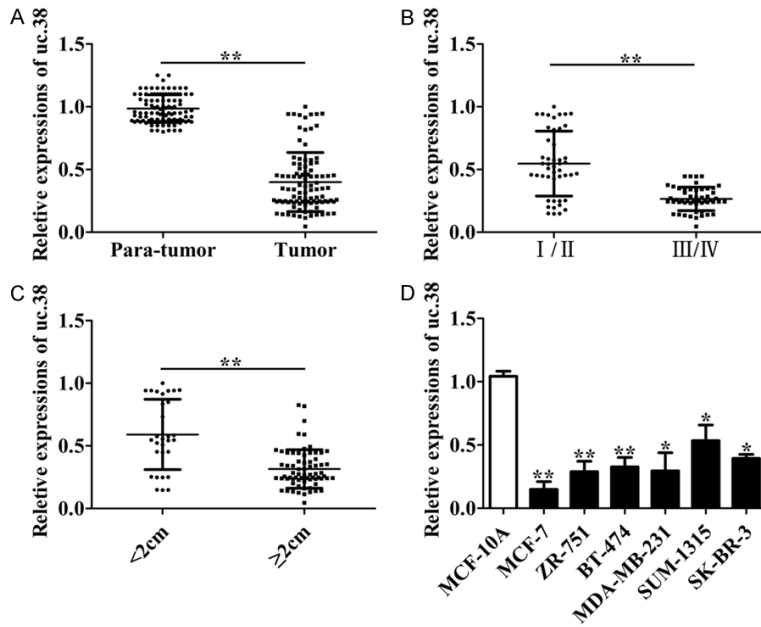


Figure 1. Expression of uc.38 in clinical specimens and breast cancer cell lines. A. Relative uc.38 expression levels were detected in 100 paired clinical specimens (para-tumour and tumour tissues) using qRT-PCR. Significantly lower expression levels of uc.38 were detected in tumour tissues than those in para-tumour tissues. B. lncRNA uc.38 was expressed at significantly lower levels in more advanced tumour stages. C. lncRNA uc.38 was expressed at significantly lower levels in larger tumours. D. qRT-PCR experiments showed significantly lower levels of uc.38 in breast cancer cell lines (MCF-7, ZR-75-1, BT474, MDA-MB-231, SUM1315 and SK-BR-3) than those in the breast epithelial cell line (MCF-10A). All uc.38 expression data were normalized to GAPDH. The results of qRT-PCR were presented as $2^{-\Delta\Delta CT}$ values in experimental groups relative to control groups. Data are presented as the mean \pm SD of triplicate experiments, and error bars represent the SD. * $P < 0.05$, ** $P < 0.01$.

cence microscope (Leica DM 5000B; Leica, Wechsler, Germany).

Xenograft study

All animal experiments were conducted according to the guidelines of the NJMU Institutional Animal Care and Use Committee. Thirty healthy five-week-old athymic BALB/c mice were maintained under specific pathogen-free conditions. Ten mice were randomly divided into two groups. A total of 2×10^6 SUM1315 cells were infected with each lentivirus (Lv-uc.38-NC and Lv-uc.38) in 0.2 ml of PBS and injected into the right shoulder pads of nude mice. Tumour volumes were examined every 4 days after the tumours started to grow. Four weeks after the injection, the mice were sacrificed, and the tumour volumes (length \times width² \times 0.5) and weights were measured. The primary tumours were excised, collected and prepared for IHC staining. The other 20 mice were injected with

different SUM1315 cells (Lv-uc.38-NC and Lv-uc.38) and randomly divided into two groups for an analysis of the overall survival (OS) time.

Immunohistochemistry

For the immunohistochemistry analysis, MaxVision™ techniques (Maixin Bio, China) were used according to the manufacturer's protocol. Fresh specimens were cryopreserved and routinely processed into frozen sections. Then, 4- μ m-thick sections were prepared, and immunohistochemical staining was performed using rabbit antibodies against PBX1, Bcl2 and Bax and a streptavidin-biotin immunoperoxidase assay. Sections were imaged under a light microscope (Leica, Germany) at 200 or 400 \times magnification. Brown staining in the cells was considered a positive signal.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software (IBM, SPSS, Armonk, NY, USA). Clinicopathological findings were compared using an unpaired t-test or Pearson's χ^2 test. One-way ANOVA was performed for data with a normal distribution that passed the equal variance assumption test. The OS rates were calculated using the Kaplan-Meier method, with the log-rank test applied for comparison. The results are expressed as the mean \pm SD, and $P < 0.05$ was considered significant.

Results

Expression of lncRNA uc.38 in breast cancer tissues and breast cancer cell lines

Expression levels of lncRNA uc.38 were investigated in 100 breast cancer tissues and 100 matched normal breast tissues using qRT-PCR. The expression of lncRNA uc.38 was significantly down-regulated in the tumour tissues compared with that in the adjacent normal tis-

The role of lncRNAs in breast cancer

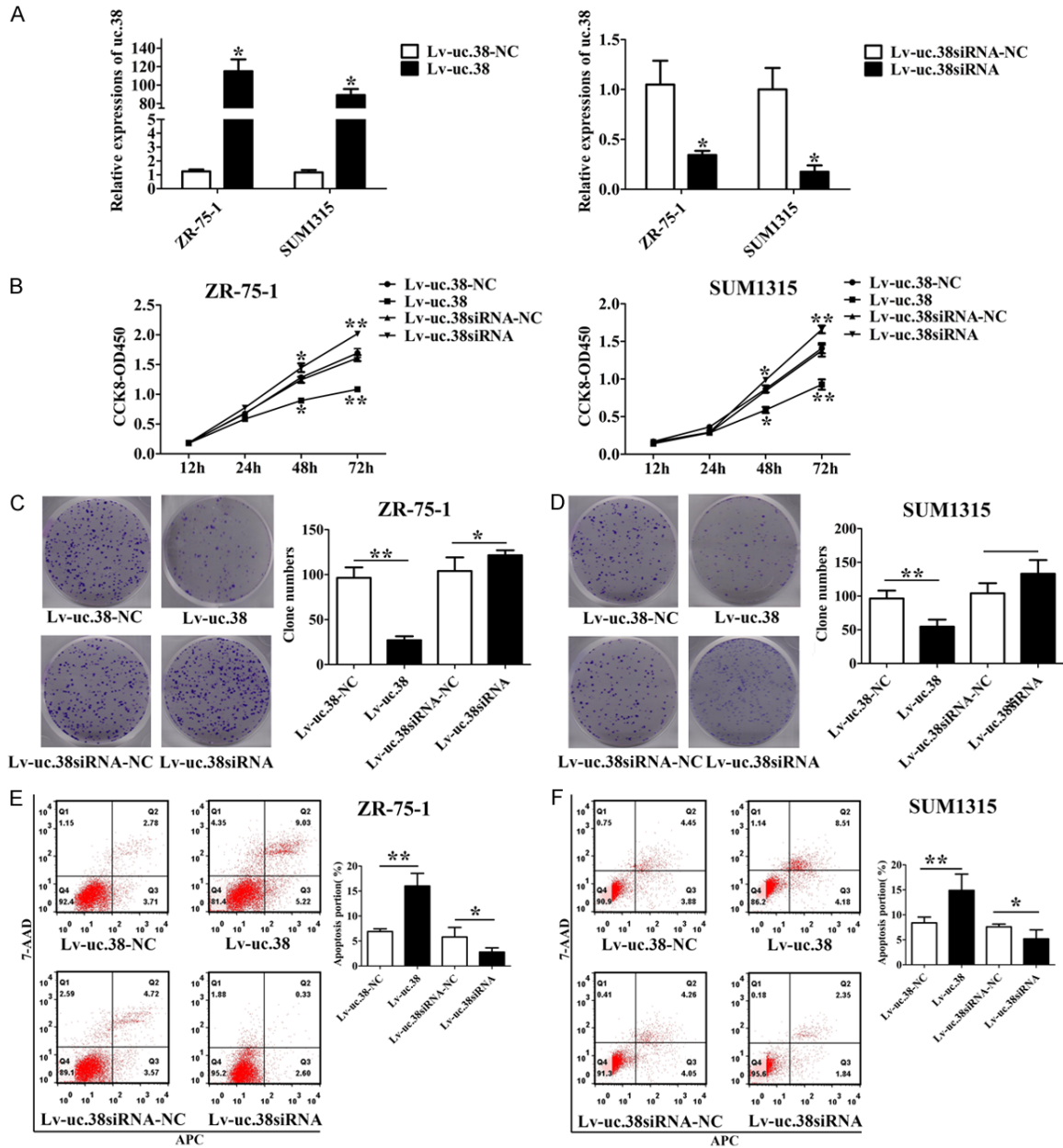


Figure 2. Effects of uc.38 expression on cell proliferation and apoptosis in breast cancer cell lines. A. Ectopic expression of uc.38 was confirmed by qRT-PCR after infection of ZR-75-1 and SUM1315 cells with a lentivirus. B. CCK-8 assays were used to assess the proliferation of different breast cancer cell lines and groups after transfection. C and D. Colony-formation assays were performed to determine the proliferation of ZR-75-1 and SUM1315 cells after transfection. E and F. FCM analyses showed that uc.38 induces apoptosis in ZR-75-1 and SUM1315 cells. Q3, early apoptotic cells. Q2, terminal apoptotic cells. Data are presented as the mean \pm SD of triplicate experiments, and error bars represent the SD. * $P < 0.05$, ** $P < 0.01$. Lv-uc.38 represents breast cancer cells stably over-expressing uc.38, Lv-uc.38siRNA designates breast cancer cells in which uc.38 expression was stably silenced, and Lv-uc.38-NC and Lv-uc.38siRNA-NC were used as negative controls.

sues (Figure 1A). Next, we evaluated the correlation between the uc.38 expression level and clinicopathological features in patients with breast cancer using a t-test. A significant positive correlation was observed between a low

uc.38 level and an advanced TNM stage and higher tumour burden (Figure 1B and 1C). Furthermore, we measured uc.38 expression in breast cancer cell lines and normal breast epithelial cells and found that uc.38 was expressed

The role of lncRNAs in breast cancer

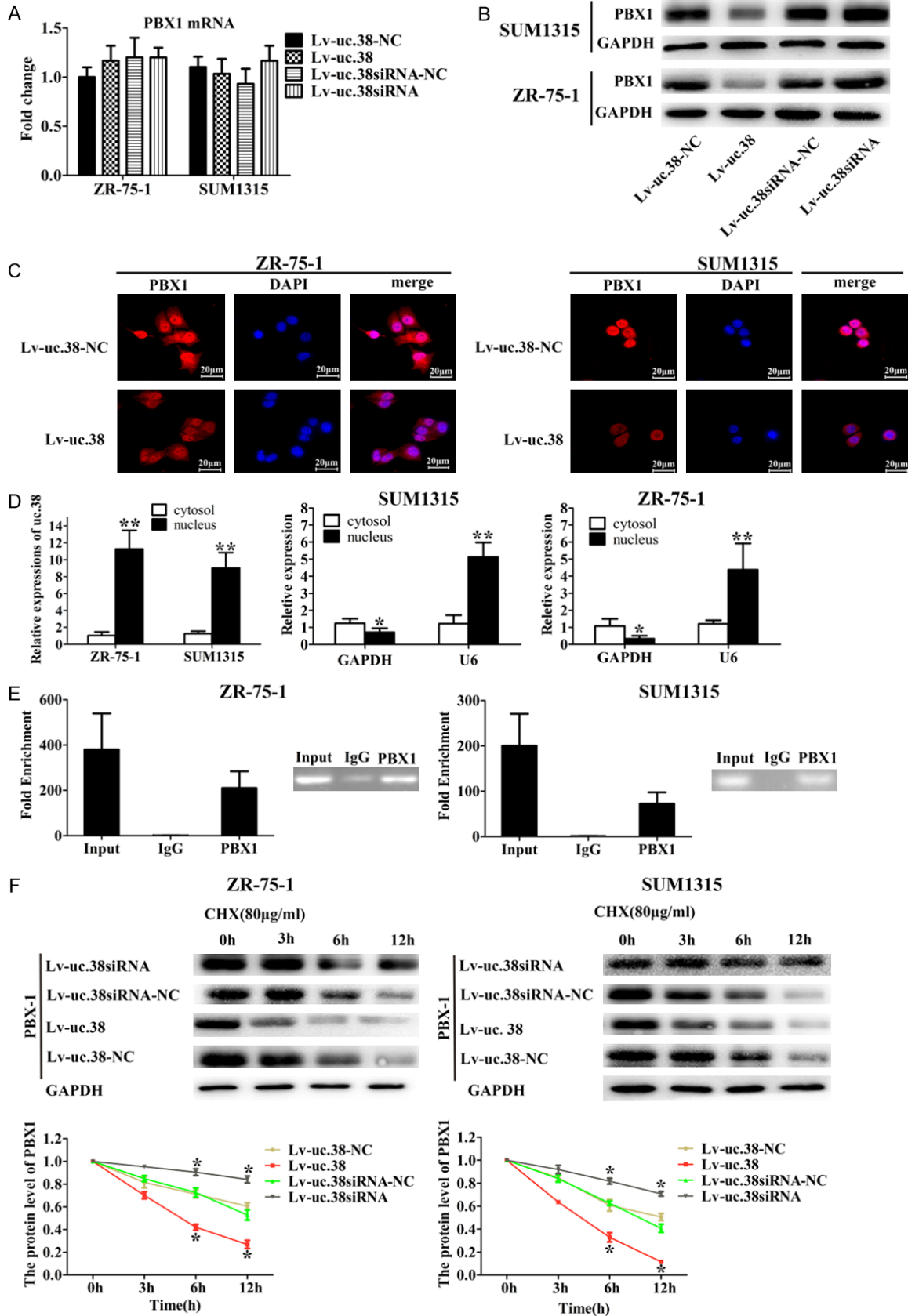


Figure 3. lncRNA uc.38 regulated PBX1 expression. A. Levels of the PBX1 mRNA were assessed by qRT-PCR after uc.38 expression was up-regulated and down-regulated in breast cancer cell lines (ZR-75-1 and SUM1315). B. After

The role of lncRNAs in breast cancer

over-expression and knockdown of uc.38 in ZR-75-1 and SUM1315 cells, Western blotting was used to determine levels of the PBX1 protein. C. After uc.38 over-expression in ZR-75-1 and SUM1315 cell lines, immunofluorescence staining was used to detect the levels of the PBX1 protein. D. The nuclear localization of uc.38 was identified by qRT-PCR in fractionated ZR-75-1 and SUM1315 cells. GAPDH was used as a cytosolic marker, whereas U6 was used as a nuclear marker. E. RIP results showed that lncRNA uc.38 interacted with the transcriptional activator PBX1. The agarose gel electrophoresis graph shows levels of the PCR products from the RIP assay. F. lncRNA uc.38 affected PBX1 protein turnover in ZR-75-1 and SUM1315 cells in response to cycloheximide treatment (CHX, 100 $\mu\text{g}/\mu\text{L}$) at the indicated intervals. Data are presented as the mean \pm SD of triplicate experiments, and error bars represent the SD. * $P < 0.05$, ** $P < 0.01$. Lv-uc.38 designates breast cancer cells stably over-expressing uc.38, Lv-uc.38siRNA designates breast cancer cells in which uc.38 expression was stably silenced, and Lv-uc.38-NC and Lv-uc.38siRNA-NC were used as negative controls.

ssed at a lower level in the breast cancer cell lines (MCF-7, ZR-75-1, BT474, MDA-MB-231, SUM1315 and SK-BR-3) than that in the breast epithelial cell line MCF-10A (**Figure 1D**). Based on these results, a lower uc.38 expression level may be an important factor in breast tumorigenesis and progression.

lncRNA uc.38 affected breast cancer cell proliferation by regulating apoptosis in vitro

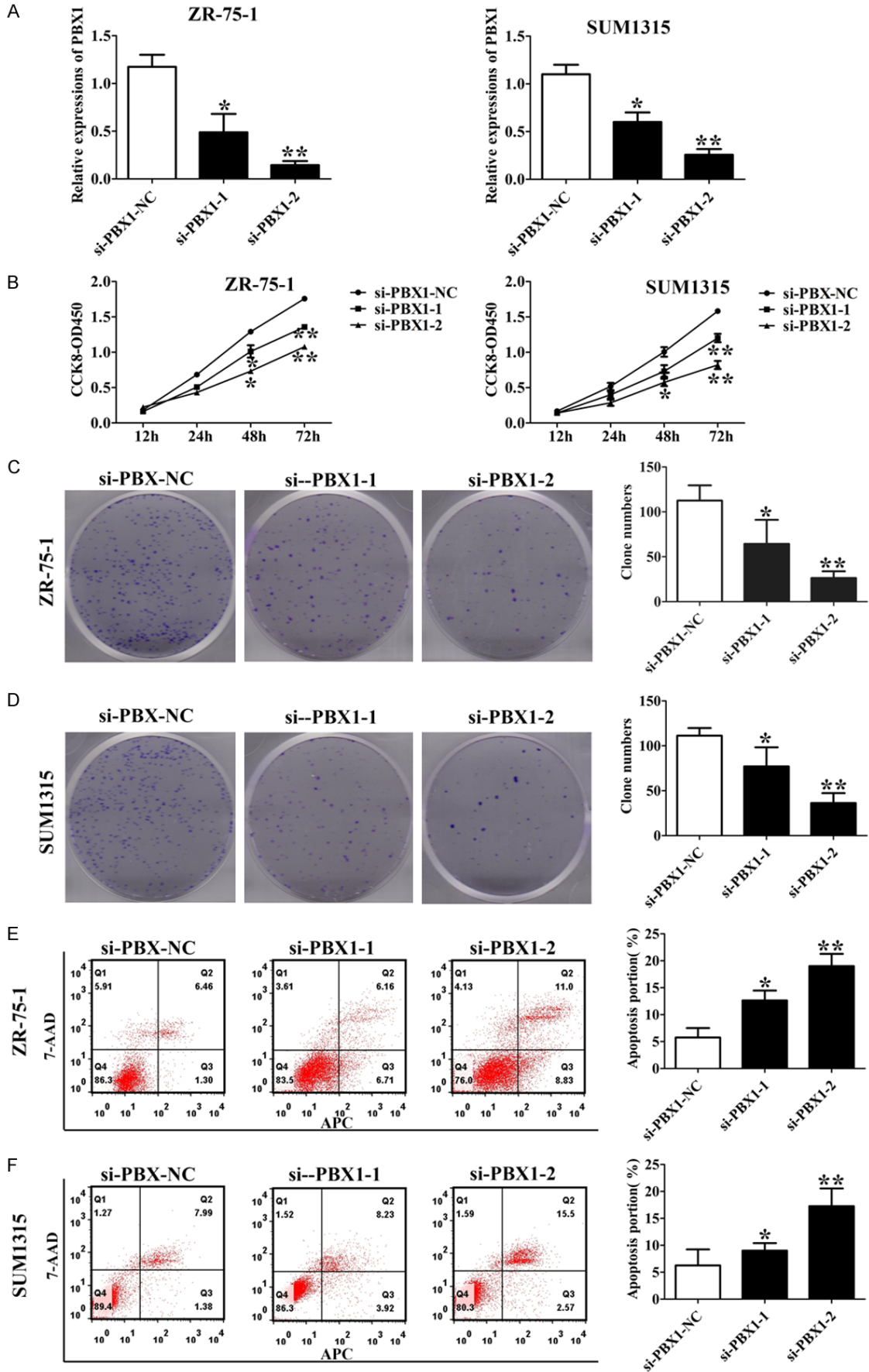
We established stable ZR-75-1 and SUM1315 cell lines in which uc.38 was over-expressed and knocked down using lentivirus infection to investigate the biological role of uc.38 in breast cancer; uc.38 expression was confirmed by qRT-PCR (**Figure 2A**). We selected si-uc.38-3 for lentivirus packing. Then, cell proliferation was measured using the CCK-8 and colony-formation assays. According to the results of the CCK-8 assay, uc.38 over-expression led to a significant decrease in the proliferation of both cell lines. Conversely, knockdown of uc.38 expression in Lv-uc.38siRNA cells led to a significant increase in tumour cell proliferation compared to the control group in both the ZR-75-1 and SUM1315 cell lines (**Figure 2B**). Similarly, the colony-formation assays revealed a significant decrease in the colony-formation ability following uc.38 over-expression, whereas knockdown of uc.38 expression increased the colony-formation ability of both the ZR-75-1 (**Figure 2C**) and SUM1315 (**Figure 2D**) cell lines. An FCM analysis was performed to probe the potential mechanisms underlying the effects of uc.38 over-expression or knockdown on proliferation. The percentage of apoptotic cells over-expressing uc.38 was significantly increased compared with cells transfected with the control vector. Conversely, knockdown of uc.38 expression reduced cell apoptosis in both the ZR-75-1 (**Figure 2E**) and SUM1315 (**Figure 2F**) cell lines. However, the results did not reveal significant changes in cell cycle progression

between the cell lines (data not shown). Thus, lncRNA uc.38 may have a strong role in controlling the proliferation of breast cancer cell lines.

lncRNA uc.38 affected the growth and apoptosis of breast cancer cells by regulating PBX1 expression

lncRNAs can regulate their neighbouring protein-coding genes [16, 17]. The PBX1 gene is located downstream of uc.38. PBX1 has been identified as a novel oncogene in human breast cancer [18]. Recently, high levels of PBX1 expression were correlated with a poorer prognosis for patients with ER-positive luminal breast tumours, independent of amplification [19]. Furthermore, PBX1 is related to cancer and cancer metastasis [19]. Thus, we hypothesized that PBX1 might be regulated by lncRNA uc.38. To test this hypothesis, we performed qRT-PCR and Western blotting to evaluate the levels of the PBX1 transcript and protein upon uc.38 over-expression. After lentiviral transfection, we examined the PBX1 mRNA level in the cells but did not observe a significant variation in the Lv-uc38 and Lv-uc.38 siRNA groups (**Figure 3A**). However, the PBX1 protein expression level was significantly decreased by uc.38 over-expression, whereas the PBX1 protein expression level was significantly increased upon knockdown of uc.38 expression (**Figure 3B**). Based on our results, PBX1 expression is down-regulated by lncRNA uc.38 at the translational level. Immunofluorescence (IF) staining revealed a reduction in the level of the PBX1 protein following over-expression of uc.38 (**Figure 3C**). We assessed the location of uc.38 using qRT-PCR by amplifying separate cytoplasmic and nuclear RNA fractions extracted from ZR-75-1 and SUM1315 cells. GAPDH was used as a cytosolic marker, and U6 was used as a nuclear marker. We detected a considerable increase in uc.

The role of lncRNAs in breast cancer



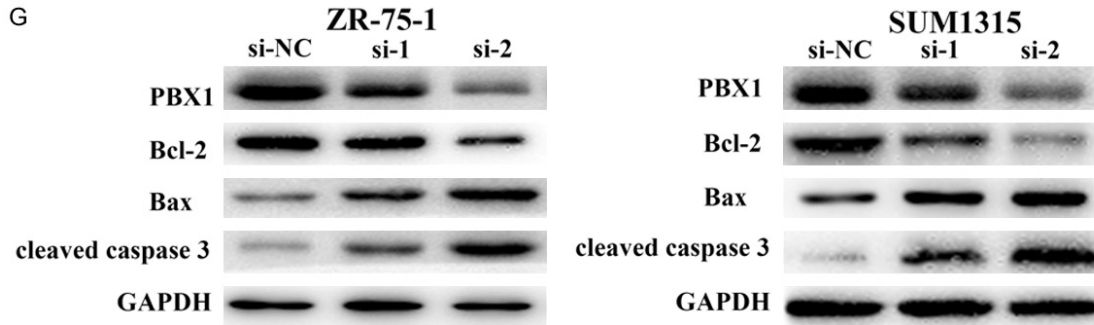


Figure 4. PBX1 depletion induced apoptosis by regulating the expression of Bcl-2 family members. A. Levels of the PBX1 mRNA were decreased in ZR-75-1 and SUM1315 breast cancer cell lines after transfection with 2 siRNAs targeting PBX1. B. CCK-8 assays were performed on ZR-75-1 and SUM1315 cells transfected with siRNAs. C and D. Clonogenic assays were performed to assess breast cancer cell proliferation in different groups. E and F. FCM analyses showing apoptosis in PBX1-silenced cells. Q3, early apoptotic cells. Q2, terminal apoptotic cells. G. After transfection with the PBX1 siRNA, levels of the PBX1, activated Caspase-3 and Bcl-2 family proteins (Bax and Bcl-2) were detected by Western blotting. The GAPDH protein was used as an internal control. NC, negative control. Data are presented as the mean \pm SD of triplicate experiments, and error bars represent the SD. * $P < 0.05$, ** $P < 0.01$. Note that si-PBX1-1 designates breast cancer cells transfected with siRNA1 targeting PBX1, si-PBX1-2 designates breast cancer cells transfected with siRNA2 targeting PBX1, and si-NC was used as a negative control.

38 expression in the nucleus compared with the cytosol (Figure 3D), suggesting that uc.38 may have a major regulatory function at the nuclear level. We performed an RIP assay with an antibody against PBX1 using cell extracts from ZR-75-1 and SUM1315 cells over-expressing uc.38 to verify the interaction between uc.38 and PBX1. The PBX1 protein specifically interacted with lncRNA uc.38. lncRNA uc.38 was enriched in the anti-PBX1 RIP fraction compared to that in the input and that in the IgG fraction in both the ZR-75-1 and SUM1315 cell lines (Figure 3E). Then, we determined whether lncRNA uc.38 expression affected PBX1 protein turnover. Cycloheximide (CHX) was used to block de novo protein synthesis, and the PBX1 protein level was examined at the indicated time points via Western blotting. Depletion of lncRNA uc.38 accelerated PBX1 protein turnover in both the ZR-75-1 and SUM1315 cells. Thus, lncRNA uc.38 interacts with the transcriptional activator PBX1, and over-expression of lncRNA uc.38 may accelerate PBX1 degradation (Figure 3F).

PBX1 depletion induced apoptosis by regulating Bcl-2 protein family expression

We used two siRNAs to knockdown PBX1 expression and investigate the biological function of PBX1 in breast carcinoma. The PBX1 expression level was confirmed by qRT-PCR and Western blotting after transfection with si-PBX1 or si-NC for 48 h. Levels of the PBX1

mRNA and protein were substantially down-regulated compared with the levels observed in the negative control group (Figure 4A). The CCK-8 assay showed a significant decrease in tumour cell proliferation following PBX1 knockdown (Figure 4B). The colony-formation assay showed a decrease in the colony-formation ability upon PBX1. Next, we investigated the effects of PBX1 on cell apoptosis using FCM (Figure 4C and 4D). The proportions of apoptotic cells were significantly increased in the PBX1 knockdown group compared with those in the negative control group (Figure 4E and 4F). We analysed the expression of the apoptosis-related Bcl-2 protein family members (Bax and Bcl-2) and Caspase-3 activation in the siPBX1-transfected ZR-75-1 and SUM1315 cell lines to elucidate the pathway downstream of PBX1 (Figure 4G). Knockdown of PBX1 increased levels of the pro-apoptotic protein (Bax) and decreased levels of the anti-apoptotic protein (Bcl-2), suggesting that down-regulation of PBX1 exerted the same biological effect. Hence, we concluded that PBX1 expression induced Caspase-3-dependent apoptosis in breast cancer cells and thus exerted a critical effect on breast cancer cell apoptosis.

lncRNA uc.38 exerts its pro-apoptotic effect through PBX1

We conducted rescue assays to determine whether PBX1 mediated the uc.38-induced decrease in breast cancer cell proliferation.

The role of lncRNAs in breast cancer

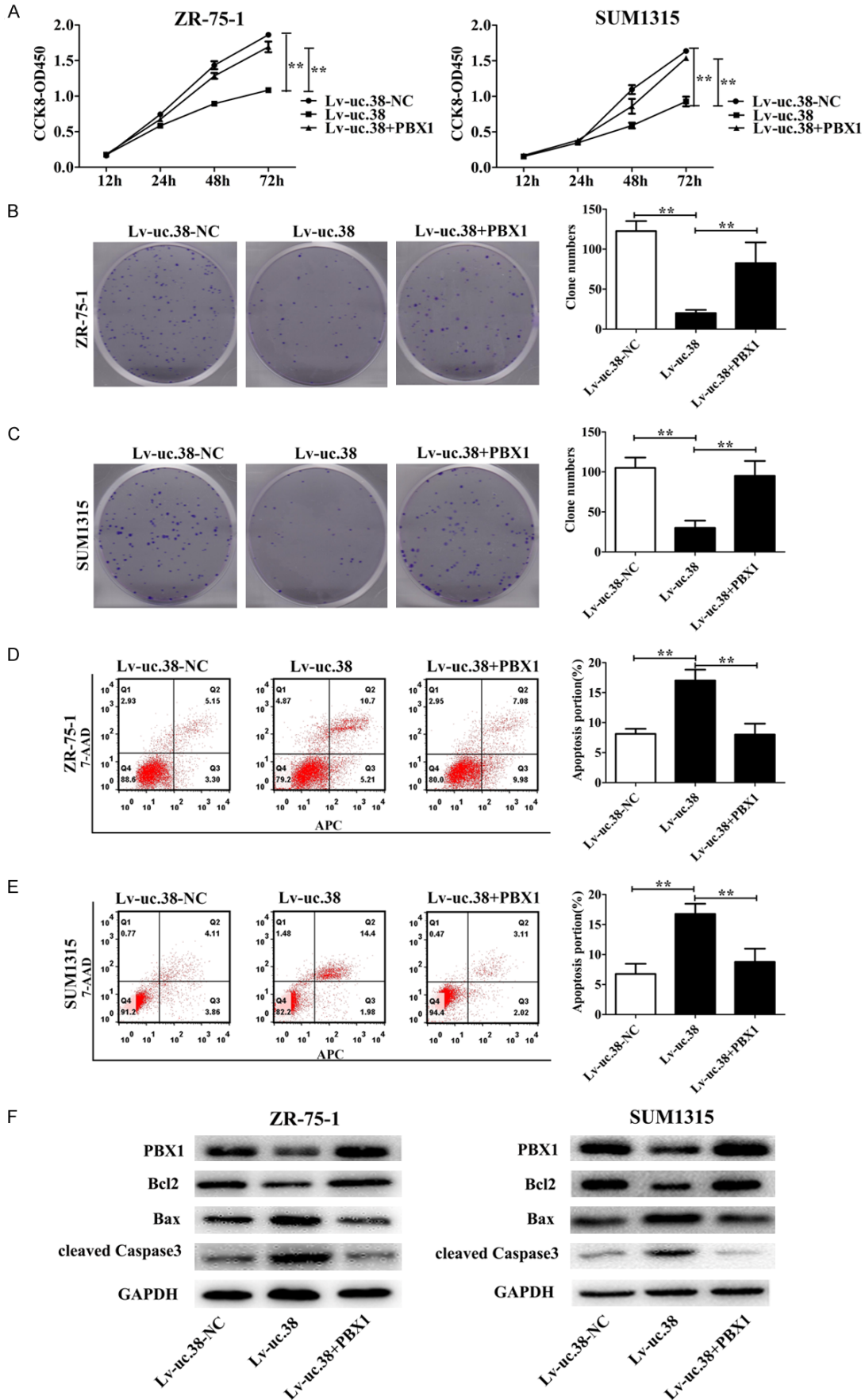


Figure 5. Over-expression of uc.38 induced breast cancer cell apoptosis that was partially abrogated by PBX1 over-expression. A. CCK-8 assays were performed to examine the proliferation of different breast cancer cell lines. B and C. Colony-forming assays were performed to determine the colony-formation abilities of different breast cancer cell lines. D and E. FCM analyses were used to detect the proportions of apoptotic cells in different breast cancer cell lines. Q3, early apoptotic cells. Q2, terminal apoptotic cells. F. Western blotting was used to detect the expression levels of PBX1, activated Caspase-3 and Bcl-2 family proteins (Bax and Bcl-2) in the rescue experiment. Data are presented as the mean \pm SD of triplicate experiments, and error bars represent the SD. * $P < 0.05$, ** $P < 0.01$. Lv-uc.38 designates breast cancer cells stably over-expressing uc.38; Lv-uc.38-NC was used as a negative control. Lv-uc.38+PBX1 designates breast cancer cells cotransfected with Lv-PBX1 after transfection with Lv-uc.38.

After transfection with Lv-uc.38, the ZR-75-1 and SUM1315 cells were cotransfected with Lv-PBX1. Western blotting showed that both cell lines cotransfected with Lv-PBX1 exhibited a rescue of the down-regulated expression of the PBX1 protein. The CCK-8 assay showed that PBX1 over-expression rescued the uc.38-induced increase in breast cancer cell proliferation (**Figure 5A**). Based on the results of the colony-formation assay, PBX1 over-expression increased clonogenic survival (**Figure 5B** and **5C**). Consistent with these findings, FCM revealed that PBX1 over-expression compromised the effects of uc.38 over-expression on breast cancer apoptosis (**Figure 5D** and **5E**). Western blotting was performed to measure the levels of activated Caspase-3 and Bcl-2 family proteins (Bax and Bcl-2) after Lv-PBX1 co-transfection and showed that PBX1 over-expression increased levels of the anti-apoptotic protein (Bcl-2) and decreased levels of the pro-apoptotic protein (Bax) (**Figure 5F**). Thus, over-expression of lncRNA uc.38 in breast cancer may reduce the level of the PBX1 protein by accelerating PBX1 protein turnover, and low PBX1 levels decrease Bcl-2 expression and increase Bax expression, thereby inducing breast cancer cell apoptosis.

Impact of uc.38 over-expression on tumorigenesis in vivo

SUM1315 cells stably overexpressing uc.38 (Lv-uc.38) and parallel control cells (Lv-uc.38-NC) were inoculated into female nude mice to investigate whether uc.38 affected tumorigenesis in vivo. All mice developed xenograft tumours at the injection site. As shown in **Figure 6A**, significantly slower tumour growth was observed in the Lv-uc.38 group than that in the Lv-uc.38-NC group at up to 4 weeks post-injection. The average tumour weights and tumour volumes in the Lv-uc.38 group were obviously less than the averages in the control group (**Figure 6B** and **6C**). The analysis of the OS time revealed a better survival rate for the mice transfected with the uc.38-expressing lentivi-

rus (**Figure 6D**). We also confirmed the uc.38-dependent decrease in PBX1 expression in vivo by analysing uc.38-over-expression in xenograft tumours and by performing immunohistochemical staining for PBX1 and apoptosis-related markers (**Figure 6E**).

Discussion

lncRNAs have been confirmed to play crucial roles in human diseases including cancer. lncRNA growth arrest-specific 5 (GAS5) has been reported to promote apoptosis, and its expression is down-regulated in breast cancer [20, 21]. lncRNA HOTAIR regulates cancer invasiveness via a mechanism dependent on repressive complex 2 (PRC2) [22]. Additionally, lncRNA SPRY4-IT1 regulates breast cancer cell proliferation through a mechanism dependent on ZNF703 expression [23]. However, the functional roles of most lncRNAs remain obscure. lncRNA uc.38 was expressed at lower levels in breast cancer tissues than that in their adjacent normal counterparts in the present study. uc.38 was also expressed at lower levels in breast cancer cell lines than those in normal breast epithelial cells. Thus, uc.38 was negatively correlated with breast cancer.

T-UCRs have very important biological functions. The level of expression and type of T-UCR have been shown to be closely related to the survival rates of patients with cancer. Thus, T-UCRs have emerged as important players in cancer biology. Based on accumulating evidence, lncRNAs may function as oncogenes or tumour suppressors. lncRNA uc.38 was recently shown to inhibit the growth of hepatocellular carcinoma cells [24]. In this study, uc.38 was expressed at significantly lower levels in breast tumour tissues than those in paratumour tissues. It was also expressed at lower levels in breast cancer cell lines than those in normal breast epithelial cells. Based on these results, uc.38 was negatively correlated with breast cancer. Moreover, comparisons of uc.38 expression in clinical tumour specimens

The role of lncRNAs in breast cancer

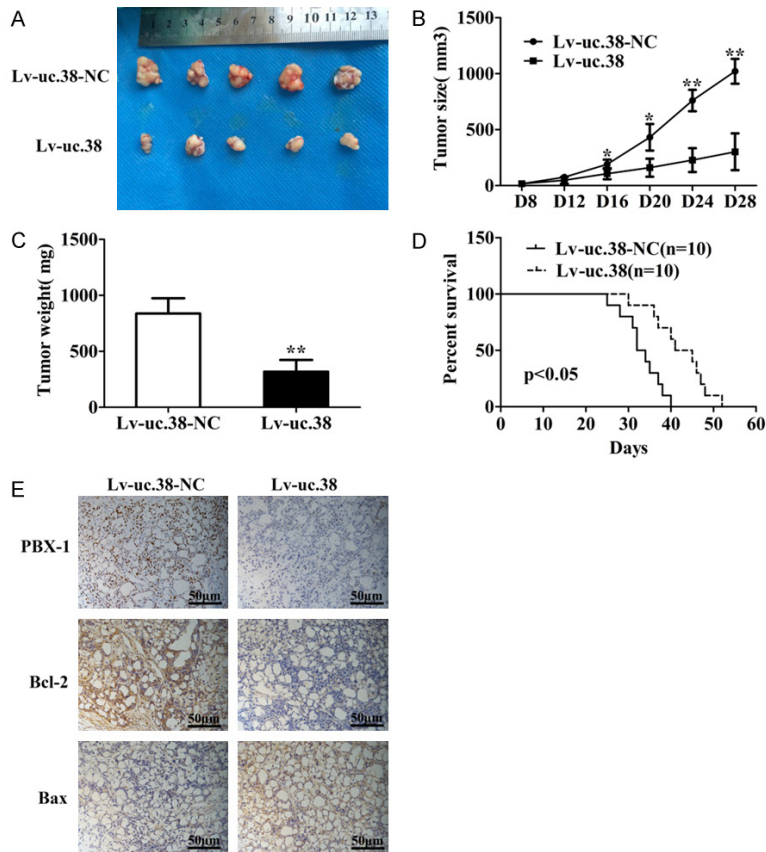


Figure 6. Over-expression of uc.38 inhibited tumour growth in vivo. A. SUM1315 breast cancer cells (2×10^6) stably over-expressing Lv-uc.38 or Lv-uc.38-NC were inoculated into nude mice. Four weeks later, primary tumours were obtained. B. Tumour volumes from Lv-uc.38 and Lv-uc.38-NC mice were compared. B and C. Tumour weights from Lv-uc.38 and Lv-uc.38-NC mice were compared. D. Kaplan-Meier analysis of OS of mice ($n=10$ mice per group). E. PBX1, Bcl-2 and Bax expression were examined by IHC staining in primary tumour specimens from the 2 groups. Data are presented as the mean \pm SD of triplicate experiments, and error bars represent the SD. * $P<0.05$, ** $P<0.01$.

showed that uc.38 was expressed at lower levels in larger tumours and tumours of more advanced stages, indicating that uc.38 might suppress breast cancer development. However, we lack sufficient clinical prognostic data. Then, lentiviruses were used to separately up-regulate and down-regulate uc.38 expression in breast cancer cell lines. Up-regulation of uc.38 expression in vitro inhibited cell proliferation and induced cell apoptosis. Meanwhile, uc.38 down-regulation produced the opposite results. Since uc.38 may suppress breast cancer, Lv-uc.38 breast cancer cell lines (which over-expressed uc.38) were used for in vivo experiments. The results supported the hypothesis that up-regulation of uc.38 inhibits tumour formation and ultimately contributes to increase

the OS time in mice. This evidence supports a suppressive effect of uc.38 on breast cancer.

PBX1, which belongs to the three amino acid loop extension (TALE) class homeodomain family, is located downstream of uc.38 [15]. According to the literature, PBX1 is required for diverse developmental processes, including haematopoiesis, skeleton patterning and pancreatic system organogenesis [25]. Although PBX1 is best known as an oncoprotein in pre-B-cell leukaemia, it contributes to prostatic, ovarian and oesophageal cancers as well [26]. In breast cancer, the PBX1 protein interacts with other homeodomain-containing nuclear proteins, such as HOX (homeotic genes) and MEIS (Meis homeobox), to form heterodimeric transcription complexes [27]. PBX1 then modulates the transcriptional activity of nuclear receptors and acts as an oncogene [28]. In the present study, neither up-regulation nor down-regulation of uc.38 altered the expression of the PBX1 mRNA in vitro. However, levels of the

PBX1 protein were significantly decreased in uc.38-over-expressing cells and increased in uc.38 silenced cells. Breast cancer cell lines over-expressing uc.38 were used to explore the location of PBX1. According to the qRT-PCR and immunofluorescence staining, uc.38 and PBX1 were both located in nucleus of breast cancer cells, and up-regulation of uc.38 expression decreased PBX1 expression. RIP and cycloheximide experiments proved that uc.38 over-expression promoted the degradation of the PBX1 protein. PBX1 expression was silenced with an siRNA to explore the role of PBX1 in breast cancer. PBX1 silencing inhibited the proliferation of breast cancer cell lines and induced apoptosis in vitro.

Apoptosis is regulated by many gene products that promote or block cell death at different stages. Based on accumulating evidence, the ratios of pro- (Bax and Bid) and anti-apoptotic (Bcl-2 and Bcl-xL) Bcl family proteins, including the Bcl-2/Bax ratio, rather than Bcl-2 expression alone determines the susceptibility of a cell to apoptosis [29, 30]. In addition, the Bcl-2 protein family is located upstream of Caspase-3 activation in the apoptosis pathway [31]. In the present study, Western blots revealed decreased levels of the anti-apoptotic protein Bcl-2 and increased levels of the pro-apoptotic protein Bax and cleaved-Caspase3. Moreover, rescue experiments showed that cell proliferation, which was inhibited by uc.38 over-expression, was rescued when PBX1 was over-expressed. However, cell apoptosis, which was induced by uc.38 over-expression, was abrogated when PBX1 was over-expressed. Western blots revealed a partial abrogation of uc.38-induced Bcl-2 expression in cells over-expressing PBX1. In addition, the over-expression of Bax and cleaved-Caspase-3 observed in response to uc.38 over-expression was also partially abrogated by PBX1 over-expression. Thus, uc.38 directly regulated the expression of PBX1; uc.38 over-expression decreased PBX1 expression and then subsequently decreased the expression of the anti-apoptotic protein Bcl-2 and increased the expression of pro-apoptotic protein Bax to ultimately induce breast cancer cell apoptosis.

In summary, based on in vitro and in vivo evidence, uc.38 suppressed breast cancer cell growth by repressing PBX1 expression. Furthermore, down-regulation of PBX1 expression decreased the expression of the anti-apoptotic protein Bcl-2 and increased the expression of the pro-apoptotic protein Bax, which induced breast cancer cell apoptosis. Our findings therefore provide new insights into the effects of uc.38 and PBX1 on the progression of breast cancer, and the uc.38/PBX1 axis may potentially serve as a therapeutic target for breast cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Li-Jun Ling, Breast Disease Division, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu Province, China. Tel: 86-25-83724440; Fax: 86-25-83724440; E-mail: linglijun@njmu.edu.cn

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The role of lncRNAs in breast cancer

Supplementary Table 1. Sequence of primers and siRNA

Gene Name	Forward (5'-3')	Reverse (5'-3')
PBX1	CGGAGCTGGAGAAATACGAG	CTGTCGCTTGCTTGTGAAA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
uc.38	CCTTGAACCTGCTGGAAGAG	AACAGAGGGATGCTTTATTGC
GAPDH	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC
si-PBX1-1	CCAUCCAGAUGCAGCUCAATT	UUGAGCUGCAUCUGGAUGGTT
si-PBX1-2	GGAAGAGACGGAAUUUCAATT	UUGAAAUUCCGUCUCUUCCTT
si-NEGATIVE CONTROL	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT