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RESEARCH PAPER

Long noncoding RNA *LINC00963* promotes breast cancer progression by functioning as a molecular sponge for microRNA-625 and thereby upregulating HMGA1

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ABSTRACT

Extensive research has shown that *LINC00963* is aberrantly expressed in human cancers, and that dysregulation of *LINC00963* is implicated in the initiation and progression of human cancers. The expression and functions of *LINC00963* in breast cancer are still unclear. Our aims were to measure the expression of *LINC00963* in breast cancer, determine its effects on malignant behaviors of tumor cells, and uncover the molecular events underlying the actions of *LINC00963* in breast cancer. Herein, *LINC00963* was found to be overexpressed in breast cancer samples, and its overexpression was correlated with lymph node metastasis, TNM stage and differentiation grade. Patients with breast cancer harboring higher *LINC00963* expression showed shorter overall survival than did the patients with lower *LINC00963* expression. Functional experiments revealed that depletion of *LINC00963* inhibited breast cancer cell proliferation, migration, and invasion and facilitated apoptosis *in vitro* and impaired tumor growth *in vivo*. Mechanism investigation revealed that *LINC00963* can interact with microRNA-625 (miR-625). *LINC00963* worked as a competitive endogenous RNA for miR-625 to weaken the suppressive effect of miR-625 on high mobility group AT-hook 1 (HMGA1) in breast cancer cells. Furthermore, miR-625 inhibition and HMGA1 restoration both abrogated the effects of *LINC00963* silencing on breast cancer cells. Our findings indicate that the *LINC00963*–miR-625–HMGA1 pathway plays an important role in the malignancy of breast cancer *in vitro* and *in vivo*. Hence, targeting this pathway may be a novel strategy against breast cancer.

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Introduction

Breast cancer is the most frequently diagnosed human cancer and the second leading cause of cancer-associated deaths among women globally [1]. In China, breast cancer accounts for 12.2% of new cancer cases and 9.6% of all cancer-related deaths [2]. Currently, the first-line therapeutic strategies include surgical resection, adjuvant radiotherapy, hormone therapy, chemotherapy, and targeted biotherapy [3]. In spite of the noticeable progress in the diagnosis and treatment in the past decades, the clinical outcomes for patients with breast cancer are still devastating, especially for the patients presenting with recurrence and metastasis [4,5]. Thus, it is worthwhile to thoroughly characterize the mechanisms underlying the aggressive characteristics of breast cancer for developing novel targets for the diagnosis and treatment.

MicroRNAs (miRNAs or miRs) are a class of noncoding short RNA molecules that consist of ~22 nucleotides [6]. miRNAs have been proven to downregulate gene expression through partial or complete base pairing with the 3′ untranslated region (3'- UTRs) of their target mRNAs, thereby leading to translation inhibition and/or mRNA degradation [7]. Some studies on miRNA expression indicate that various miRNAs are dysregulated during breast carcinogenesis and progression of this cancer [8–10]. In breast cancer, miRNAs can be subdivided into two groups: oncogenic miRNAs and tumor-suppressive miRNAs. The functions of oncogenic miRNAs increase the malignancy of breast cancer, while tumor-suppressive miRNAs play the opposite roles [11–13].

Long noncoding RNAs (lncRNAs) are another group of transcripts; they are over 200 nucleotides long but lack the protein-coding ability [14]. LncRNAs modulate the expression of tumor suppressors or oncogenes through multiple mechanisms, e.g., by acting as an RNA decoy, via alternative splicing, and through epigenetic,

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Table 1. Correlation of clinicopathological parameters with LINC00963 expression in patients with breast cancer.

	LINC00963 expression		
Clinicopathological parameters	High	Low	P
Age (years)	27	26	0.569
< 50	11 (41%)	8 (31%)	
≥50	16 (59%)	18 (69%)	
Tumor size (mm)			0.583
< 2	17 (63%)	14 (54%)	
≥ 2	10 (37%)	12 (46%)	
ER status			0.412
Negative	14 (52%)	10 (39%)	
Positive	13 (48%)	16 (61%)	
PR status			0.170
Negative	12 (44%)	17 (65%)	
Positive	15 (56%)	9 (35%)	
Her-2 status			0.785
Negative	16 (59%)	14 (54%)	
Positive	11 (41%)	12 (46%)	
Lymph node metastasis			0.002
Negative	14 (52%)	21 (81%)	
Positive	13 (48%)	5 (19%)	
TNM stage			0.042
I–II	18 (67%)	24 (92%)	
III	9 (33%)	2 (8%)	
Differentiation grade			0.024
Well and moderately	12 (44%)	20 (77%)	
Poorly and undifferentiated	15 (56%)	6 (23%)	

chi-squared test.

transcriptional, and post-transcriptional modifications [15,16]. Accumulating evidence has revealed that lncRNAs play crucial roles in various fundamental biological processes and human diseases, including cancer [17–19]. The aberrant expression of lncRNAs has been reported to be widespread among various types of human cancer, suggesting that aberrantly expressed lncRNAs may be involved in carcinogenesis and cancer progression [20,21]. A number of lncRNAs are dysregulated in breast cancer and exert oncogenic or tumor-suppressive actions [22–24]. For instance, *LINC01355* [25], *PANDAR* [26], and *MIR503HG* [27] are downregulated in breast cancer and inhibit cancer progression; on the contrary, *SNHG1* [28], *NEAT1* [29], and *ADPGK-AS1* [30] are overexpressed in this cancer and increase the malignancy of breast cancer. Therefore, identification of cancer-related lncRNAs and miRNAs in breast cancer may allow for the identification of potential therapeutic targets to improve the prognosis for patients.

Extensive research suggests that *LINC00963* is aberrantly expressed in human cancers, and that dysregulation of *LINC00963* is implicated in the initiation and progression of human cancers [31–34]. Unfortunately, the expression and functions of *LINC00963* in breast cancer remain largely unexplored. Hence, in this study, we attempted to characterize the expression profile of *LINC00963* in breast cancer and determine the effects of *LINC00963* on the malignant behaviors of breast cancer cells. In addition, a systematic approach was employed to illustrate the molecular events underlying the activities of *LINC00963* in breast cancer.

Materials and methods

Clinical samples

In total, 53 patients with a diagnosis of breast cancer received in Liaocheng People's Hospital were enrolled in this study, and none of these patients had been treated with preoperative radiotherapy, chemotherapy, or other relevant modalities. Breast cancer tissue samples and matched adjacent normal tissues were collected, immediately preserved in liquid nitrogen, and then stored at −80°C until analysis. Written informed consent was provided by all the patients. The work with human subjects was carried out with the approval of the Ethics Committee of Liaocheng People's Hospital and in compliance with the principles of the Declaration of Helsinki.

Cell lines

Breast cancer cell lines (BT-474, MCF-7, MDA-MB-231, and SKBR3) and human normal breast epithelial cell line MCF-10A were bought from the Chinese Academy of Sciences (Shanghai, China) and cultured in accordance with the vendor's instructions. In brief, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% of a penicillin/streptomycin mixture (Gibco, Grand Island, NY, USA) at 37°C in a humidified chamber supplied with 5% of CO_2.

Cell transfection

Small interfering RNA downregulating lncRNA *LINC00963* (si-LINC00963) and negative control (NC) small interfering RNA (si-NC) were acquired

from Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-625 mimics, NC miRNA mimics (miR-NC), miR-625 inhibitor, and NC inhibitor were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, China). The full-length sequence of HMGA1 lacking the 3'-UTR was synthesized by Shanghai GenePharma Co., Ltd., and inserted into the pcDNA3.1 vector to produce the pcDNA3.1-HMGA1 plasmid (pc-HMGA1). The oligonucleotide and plasmid transfection was carried out using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

Reverse-transcription quantitative PCR (RT-qPCR)

The TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for total-RNA isolation. The quality and concentration of total RNA were evaluated on a Nanodrop device (Invitrogen; Thermo Fisher Scientific, Inc.). To quantify *LINC00963* expression, cDNA was prepared from total RNA by means of the PrimeScript RT-Reagent Kit (Takara Bio, Kusatsu, Japan), and then cDNA was subjected to qPCR with the SYBR Premix Ex Taq™ Kit (Takara Bio, Kusatsu, Japan). For miR-625 expression quantitation, total RNA was reverse-transcribed into cDNA using the miScript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). After that, qPCR was carried out with the miScript SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany). *LINC00963* and miR-625 expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and U6, respectively. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay

After incubation for 24 h, transfected cells were collected to prepare a cell suspension. Then, 100 µl of the cell suspension (2×10^4 cells/ml) was seeded in each well of 96-well plates. The cells were kept at 37°C in a humidified chamber with 5% CO_2, and the CCK-8 assay was performed 0, 24, 48, or 72 h later. At every time point, 10 µl of the CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into each well prior to incubation at 37°C for 2 h, followed by measurement of absorbance at 450 nm wavelength on a microplate reader (Bio-Rad, Hercules, CA, USA).

Apoptosis detection by flow cytometry

After 48 h culture, transfected cells were centrifuged, washed twice with ice-cold PBS (Gibco, Grand Island, NY, USA), and subjected to the measurement of the cell apoptosis rate using the Annexin V–Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Biolegend, San Diego, CA, USA). Briefly, the cells were resuspended in 500 µl of 1× binding buffer and then stained with 5 µl of Annexin V-FITC and 5 µl of a propidium iodide solution that came with the kit. After 15 min incubation in the dark at room temperature, the proportion of apoptotic cells was determined on a FACScan flow cytometer (BD Biosciences).

Transwell migration and invasion assays

Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) with membranes of 8 µm pore size were employed for the migration and invasion assays. For the invasion assay, the membranes were precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), whereas no Matrigel was applied in the migration assay. Transfected cells were harvested after 48 h incubation and were used to prepare cell suspension. The cells were resuspended in FBS-free DMEM, and the cell concentration was adjusted to 5×10^6 cells/ml. The upper compartments were covered with 200 µl of the cell suspension, while 500 µl of DMEM supplemented with 20% of FBS was added into the bottom compartments. At 24 h later, we gently removed nonmigratory and noninvasive cells by wiping the membranes with cotton swabs. Cells that passed through the pores to the underside of the membranes were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The stained cells were subsequently counted in five randomly selected visual fields under an inverted microscope (Olympus Corp., Tokyo, Japan).

Tumor xenograft experiment

The procedures for the animal experiments were approved by the Animal Care and Use Committee of Liaocheng People's Hospital and were compliant with the guidelines of the Animal Protection Law of the People's Republic of China-2009 for

experimental animals. MCF-7 cells transfected with either si-LINC00963 or si-NC were harvested at 24 h post-transfection, resuspended in 100 µl of PBS and injected subcutaneously into the flank of 4- to 6-week-old male BALB/c nude mice (Shanghai Experimental Animal Center of Chinese Academy of Sciences; Shanghai, China). The diameters of the tumor xenografts were measured using calipers once every 3 days. All the mice were euthanized after the last measurement. The tumor xenografts were excised, weighed, and subjected to RT-qPCR analysis and western blotting. Tumor volume was calculated via the following formula: tumor volume = 1/2 × tumor length × tumor width^2.

Subcellular fractionation and RNA isolation

The cytoplasmic and nuclear fractions of breast cancer cells were isolated and purified by means of the PARIS Kit (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was extracted separately from cytoplasmic and nuclear fractions and then subjected to RT-qPCR.

RNA immunoprecipitation (RIP) assay

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore Inc., Billerica, MA, USA) was employed for the RIP assay. Cells were lysed in complete RIP lysis buffer, and the cell extract was collected and incubated with magnetic beads conjugated with an anti-Argonaute 2 (AGO2) or anti–immunoglobulin G (IgG) antibody (Millipore Inc., Billerica, MA, USA) at 4 °C for 6 h. Finally, RNA was isolated, and RT-qPCR analysis was performed to assess *LINC00963* and miR-625 expression.

Bioinformatic analysis and luciferase reporter assay

The interaction between *LINC00963* and miRNAs was predicted in starBase 3.0 (http://starbase.sysu.edu.cn/). The fragments of *LINC00963* containing the predicted wild-type (WT) and mutant (MUT) miR-625–binding site were amplified by Shanghai GenePharma Co., Ltd., and separately cloned into the pmirGLO luciferase reporter vector (Promega Corporation, Madison, WI, USA), thus resulting in plasmids called LINC00963-WT and LINC00963-MUT, respectively. Cells were seeded in 24-well plates and allowed to attach for 24 h before transfection. Cotransfection of the constructed plasmids and either the miR-625 mimics or miR-NC was conducted with the Lipofectamine® 2000 reagent. At 48 h post-transfection, luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). Firefly luciferase activity was normalized to that of *Renilla* luciferase.

Western blot analysis

RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was utilized for total-protein isolation. The concentration of total protein was measured by a bicinchoninic acid assay (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 10% gel and were electroblotted to polyvinylidene difluoride membranes. Blocking was conducted in 5% fat-free milk for 2 h at room temperature. The membranes were probed with primary antibodies against HMGA1 (cat. No. ab129153; dilution 1:1,000; Abcam, Cambridge, UK) or GAPDH (cat. No. ab128915; dilution 1:1,000; Abcam) at 4°C overnight, and next incubated with a goat anti-rabbit immunoglobulin G horseradish peroxidase–conjugated secondary antibody (cat. No. ab205718; dilution 1:5,000; Abcam). Finally, the Pierce™ ECL Western Blotting Substrate Kit (Thermo Fisher Scientific, Inc.) was applied to develop the protein signals.

Statistical analysis

All the data were analyzed in the SPSS software (version 19.0; IBM Corp.). Student's *t* test was performed to assess the differences between two groups, whereas one-way analysis of variance followed by Tukey's *post hoc* test was carried out to evaluate the differences among multiple groups. The correlation between *LINC00963* levels and clinical features of the patients with breast cancer was measured by the chi-squared test. The overall-survival curve was analyzed by the Kaplan–Meier

method, and the log-rank test was conducted to compare the survival data. Spearman's correlation analysis was performed to assess the expression correlation between *LINC00963* and miR-625 among the breast cancer tissue samples. All results are presented as the mean ± SD. Data with $P < 0.05$ were considered statistically significant.

Results

Overexpression of *LINC00963* correlates with poor prognosis in breast cancer

To elucidate the expression profile of *LINC00963* in breast cancer, we first measured the expression of this lncRNA in 53 pairs of breast cancer tissue samples and matched adjacent normal tissues. The results of RT-qPCR analysis suggested that the expression of *LINC00963* was higher in breast tumors than in matched adjacent normal tissue samples (Figure 1(a), $^{**}P < 0.01$). To confirm our finding, *LINC00963* expression was also determined in breast cancer cell lines, including BT-474, MCF-7, MDA-MB-231, and SKBR3. In comparison with human normal breast epithelial cell line MCF-10A, *LINC00963* was weakly expressed in all the four tested breast cancer cell lines, as evidenced by RT-qPCR analysis (Figure 1(b), $^{*}P < 0.05$, $^{**}P < 0.01$).

To assess the clinical value of LINC00963 in breast cancer, all the patients were assigned to either a high *LINC00963* expression group (n = 27) or low *LINC00963* expression group (n = 26) according to the median value of *LINC00963* expression among the 53 breast cancer tissue samples. The correlation of *LINC00963* expression and clinical parameters was analyzed by the chi-squared test. The analysis revealed that upregulation of *LINC00963* correlated with lymph node metastasis (P = 0.002), TNM stage (P = 0.042) and differentiation grade (P = 0.024) among our 53 patients with breast cancer (Table 1). In addition, patients with breast cancer harboring high *LINC00963* expression showed shorter overall survival than patients with low *LINC00963* expression (Figure 1(c), p = 0.040). The above results suggested that *LINC00963* was overexpressed in breast cancer and may be associated with cancer progression.

Disruption of the expression of *LINC00963* decreases breast cancer cell growth and metastasis *in vitro*

Cell lines MCF-7 and MDA-MB-231 featured relatively higher *LINC00963* expression among the four tested breast cancer cell lines; therefore, these two cell lines were chosen for the following

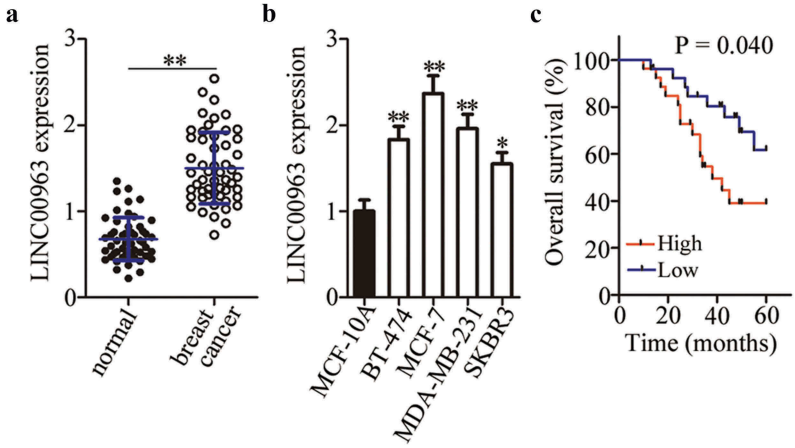

Figure 1. *LINC00963* expression is excessive in breast cancer and predicts poorer prognosis.

(a) RT-qPCR analysis was performed to determine *LINC00963* expression in 53 pairs of breast cancer tissue samples and matched adjacent normal tissues. Paired student's t-test is utilized for statistical analysis. $^{**}P < 0.01$ vs. adjacent normal tissues. (b) Expression of *LINC00963* in four breast cancer cell lines (BT-474, MCF-7, MDA-MB-231, and SKBR3) and in human normal breast epithelial cell line MCF-10A was tested via RT-qPCR. Unpaired student's t-test is utilized for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MCF-10A. (c) The Kaplan–Meier method and logrank test were utilized to analyze the correlation between *LINC00963* expression and the overall survival among patients with breast cancer. The overall-survival curve was analyzed by the Kaplan–Meier method, and the log-rank test was conducted to compare the survival data. P = 0.040.

experiments. MCF-7 and MDA-MB-231 cells were transfected with si-LINC00963 to silence *LINC00963* expression. The decreased expression of *LINC00963* in MCF-7 and MDA-MB-231 cells after si-LINC00963 transfection was confirmed via RT-qPCR analysis (Figure 2(a), ***P < 0.001). The influence of *LINC00963* silencing on breast cancer cell proliferation was tested by the CCK-8 assay. Transfection with si-LINC00963 resulted in significant inhibition of MCF-7 and MDA-MB-231 cell proliferation (Figure 2b, **P < 0.01). Then, flow-cytometric analysis was conducted to examine the impact of the *LINC00963* knockdown on the apoptosis of breast cancer cells. *LINC00963* depletion dramatically increased the apoptosis of MCF-7 and MDA-MB-231 cells (Figure 2c, **P < 0.01). The migratory and invasive abilities of tumor cells are required for tumor metastasis; accordingly, the Transwell cell migration and invasion assays were conducted to test the migration and invasiveness of *LINC00963*-depleted breast cancer cells. The results showed that the numbers of migratory (Figure 2d, **P < 0.01) and invasive (Figure 2e, **P < 0.01) MCF-7 and MDA-MB-231 cells were remarkably reduced in the cell groups transfected with si-LINC00963. In a word, these results revealed that a reduction of *LINC00963* expression may reduce the capacity of breast cancer cells for growth and metastasis *in vitro*.

LINC00963 works as a competitive endogenous RNA (cerna) for mir-625 in breast cancer cells

To explore the mechanisms by which *LINC00963* exerted the oncogenic actions in breast cancer cells, we first investigated the subcellular localization of *LINC00963* in MCF-7 and MDA-MB-231 cells. As depicted in Figure 3a, *LINC00963* is mainly present in the cytoplasm of MCF-7 and MDA-MB-231 cells, suggesting that *LINC00963* may function as a ceRNA for some miRNA in breast cancer cells. We, therefore, searched starBase 3.0 for the potential miRNAs that could be sponged by *LINC00963*. MiR-625 was found to have a high probability of binding to *LINC00963* (Figure 3b). In addition, another study has revealed that miR-625 performs tumor-suppressive functions in breast cancer [35]. Hence, miR-625 was selected for further experiments. The luciferase reporter assay was conducted to dissect the direct interaction between *LINC00963* and miR-625 in breast cancer cells. First, the transfection efficiency of the miR-625 mimics was validated by RT-qPCR; it was observed that the expression of miR-625 was markedly higher in miR-625 mimics–transfected MCF-7 and MDA-MB-231 cells (Figure 3c, ***P < 0.001). The luciferase activity of MCF-7 and MDA-MB-231 cells transfected with LINC00963-WT was decreased by miR-625 overexpression (*P < 0.05, **P < 0.01). By contrast, the miR-625 mimics transfection did not affect the luciferase activity in the cells cotransfected with LINC00963-MUT (Figure 3d). In addition, the RIP assay indicated that *LINC00963* and miR-625 were preferentially enriched by the anti-AGO2 antibody after the immunoprecipitation in the lysates of MCF-7 and MDA-MB-231 cells, suggesting that miR-625 is a target of *LINC00963* in breast cancer cells (Figure 3e, **P < 0.01, ***P < 0.001).

After that, the expression of miR-625 was measured in the 53 pairs of breast cancer tissue samples and matched adjacent normal tissue samples via RT-qPCR analysis. MiR-625 turned out to be significantly downregulated in breast cancer tissue samples relative to the adjacent normal tissues (figure 3f, $p < 0.05$). Furthermore, an inverse correlation between *LINC00963* and miR-625 levels was identified in breast cancer tissue samples (Figure 3g, $R^2 = 0.3783$, $P < 0.0001$), as revealed by Spearman's correlation analysis. Moreover, the regulatory influence of *LINC00963* silencing on endogenous miR-625 expression was examined via RT-qPCR. Downregulation of *LINC00963* caused a significant increase of miR-625 expression in both MCF-7 and MDA-MB-231 cells (Figure 3h, **P < 0.01). *HMGA1* has been previously validated as a direct target gene of miR-625 in breast cancer cells [35]. Hence, we next tested whether *LINC00963* participates in the regulation of *HMGA1* expression in breast cancer cells. The *LINC00963* knockdown notably decreased the HMGA1 protein expression in MCF-7 and MDA-MB-231 cells (Figure 3i, ***P < 0.001). Taken together, these findings suggested that *LINC00963* functioned as a ceRNA for miR-625 in breast cancer cells and thereby upregulated HMGA1.

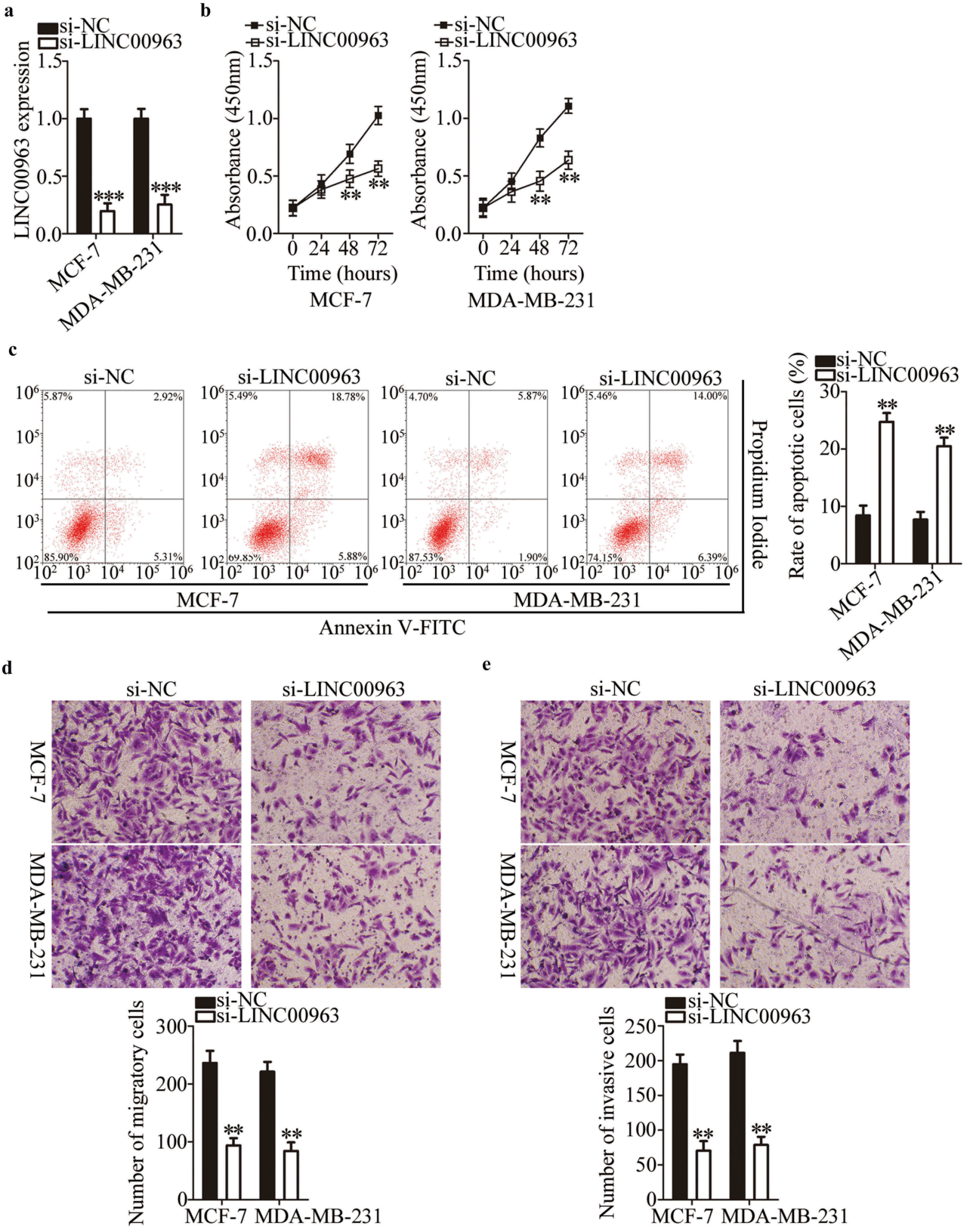

Figure 2. *LINC00963* knockdown attenuates proliferation, migration, and invasiveness and increases apoptosis of breast cancer cells. (a) Si-LINC00963 was designed to successfully knock down endogenous *LINC00963* in MCF-7 and MDA-MB-231 cells. ***P < 0.001 vs. si-NC. (b) The CCK-8 assay shows the impact of the *LINC00963* knockdown on the proliferation of MCF-7 and MDA-MB-231 cells. **P < 0.05 vs. si-NC. (c) Investigation of the apoptosis rate of *LINC00963*-depleted MCF-7 and MDA-MB-231 cells was performed by flow cytometry. **P < 0.05 vs. si-NC. (d, e) Influences of *LINC00963* silencing on the migratory and invasive abilities of MCF-7 and MDA-MB-231 cells were evaluated by Transwell migration and invasion assays. **P < 0.01 vs. si-NC. Unpaired student's t-test is utilized for statistical analysis.

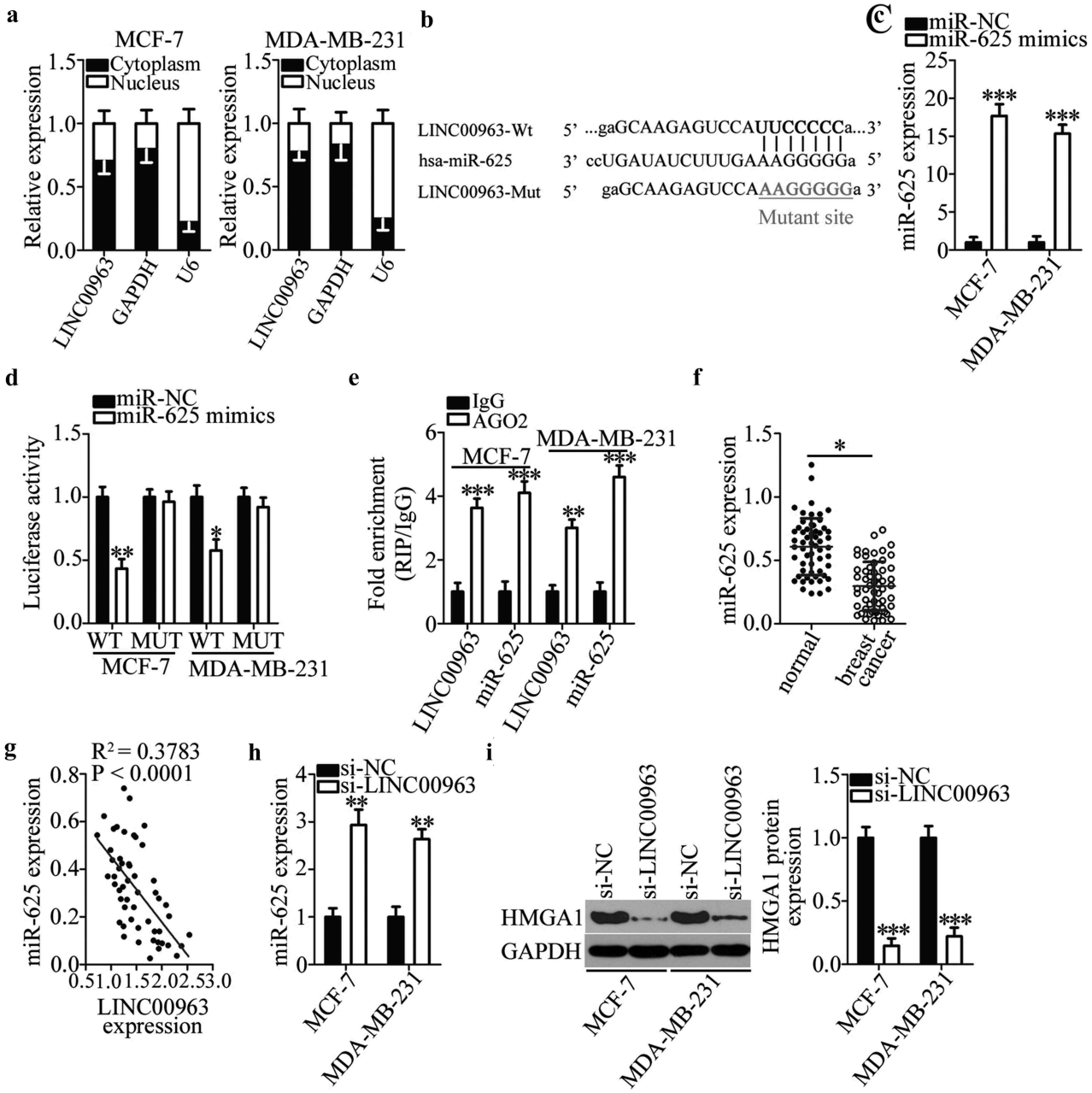

Figure 3. *LINC00963* functions as a sponge for miR-625 in breast cancer cells.

(a) *LINC00963* is mainly expressed in the cytoplasm of MCF-7 and MDA-MB-231 cells. (b) The wild-type miR-625–binding sequence in *LINC00963* was predicted by starBase 3.0. The mutant miR-625–binding sequences are shown too. (c) The miR-625 mimics was introduced into MCF-7 and MDA-MB-231 cells to elevate the endogenous miR-625 expression. Unpaired student's t-test is utilized for statistical analysis. ***P < 0.001 vs. miR-NC. (d) The luciferase reporter assay was conducted to confirm the interaction between miR-625 and *LINC00963* in breast cancer cells. Luciferase activities were measured in MCF-7 and MDA-MB-231 cells after cotransfection with either the miR-625 mimics or miR-NC and either LINC00963-WT or LINC00963-MUT. Unpaired student's t-test is utilized for statistical analysis. *P < 0.05, **P < 0.01 vs. miR-NC. (e) The RIP assay indicates that *LINC00963* and miR-625 are preferentially enriched by the anti-AGO2 antibody after immunoprecipitation in the lysates of MCF-7 and MDA-MB-231 cells. Unpaired student's t-test is utilized for statistical analysis. **P < 0.01, ***P < 0.001 vs. IgG. (f) MiR-625 expression in 53 pairs of breast cancer tissue samples and matched adjacent normal tissue samples, as determined by RT-qPCR. *P < 0.05 vs. adjacent normal tissues. Paired student's t-test is utilized for statistical analysis. (g) Spearman's correlation analysis was carried out to test the expression correlations between *LINC00963* and miR-625 in breast cancer tissues. $R^2 = 0.3783$, P < 0.0001. (h) The impact of the *LINC00963* knockdown on miR-625 expression was examined in MCF-7 and MDA-MB-231 cells after either si-LINC00963 or si-NC transfection. Unpaired student's t-test is utilized for statistical analysis. **P < 0.01 vs.si-NC. (i) Western blotting revealed that HMGA1 protein expression is suppressed by si-LINC00963 in MCF-7 and MDA-MB-231 cells. Unpaired student's t-test is utilized for statistical analysis. ***P < 0.001 vs. si-NC.

Influence of *LINC00963* knockdown on the characteristics of breast cancer cells is dependent on the mir-625–HMGA1 axis

We demonstrated above that a reduction in *LINC00963* expression attenuated breast cancer progression, and that *LINC00963* positively regulates HMGA1 expression by sponging miR-625 in breast cancer. Accordingly, we next performed rescue experiments to test whether the miR-625–HMGA1 axis is required for the oncogenic activities of *LINC00963* in breast cancer cells. To this end, the *LINC00963*-depleted MCF-7 and MDA-MB-231 cells were next treated with the miR-625 inhibitor. RT-qPCR analysis confirmed that transfection with the miR-625 inhibitor led to a significant knockdown of miR-625 expression in MCF-7 and MDA-MB-231 cells (Figure 4a, $^{**}P < 0.01$, $^{***}P < 0.001$). The *LINC00963* downregulation–mediated increase in miR-625 expression (Figure 4b, $^{**}P < 0.01$, $^{\#\#}P < 0.01$) was reversed in MCF-7 and MDA-MB-231 cells that were cotransfected with the miR-625 inhibitor. Simultaneously, the results of functional experiments showed that silencing of *LINC00963* expression inhibited MCF-7 and MDA-MB-231 cell proliferation (Figure 4c, $^{**}P < 0.01$, $^{\#\#}P < 0.01$), promoted their apoptosis (Figure 4d, $^{**}P < 0.01$, $^{\#\#}P < 0.01$), and decreased their migration (Figure 4e, $^{**}P < 0.01$, $^{\#\#}P < 0.01$) and invasiveness (figure 4f, $^{**}P < 0.01$, $^{\#\#}P < 0.01$), in agreement with what we observed above. By contrast, the influence of *LINC00963* inhibition on breast cancer cells was abrogated by treatment with the miR-625 inhibitor.

To confirm that the downregulation of *LINC00963* has an inhibitory action on the malignancy of breast cancer cells via the miR-625–HMGA1 axis, either the HMGA1-overexpressing plasmid (pc-HMGA1) or the empty vector was cotransfected with si-LINC00963 into MCF-7 and MDA-MB-231 cells. The suppression of HMGA1 protein expression induced by the *LINC00963* knockdown was reversed in MCF-7 and MDA-MB-231 cells after cotransfection with pc-HMGA1 (Figure 5a, $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$). Unsurprisingly, subsequent functional experiments indicated that the effects of *LINC00963* silencing on the proliferation (Figure 5b, $^{**}P < 0.01$, $^{\#\#}P < 0.01$), apoptosis (Figure 5c, $^{**}P < 0.01$, $^{\#\#}P < 0.01$), migration (Figure 5d, $^{**}P < 0.01$, $^{\#\#}P < 0.01$), and invasiveness (Figure 5e, $^{**}P < 0.01$, $^{\#\#}P < 0.01$) of MCF-7 and MDA-MB-231 cells were partly neutralized by the reintroduction of HMGA1 expression. Overall, these data suggested that the *LINC00963*–miR-625–HMGA1 pathway regulates the malignant progression of breast cancer cells *in vitro*.

Inhibition of *LINC00963* restricts breast cancer growth *in vivo*

To further illustrate the effects of *LINC00963* silencing on breast carcinogenesis and on the growth of breast cancer cells *in vivo*, the tumor xenograft experiment was conducted via injection of either si-LINC00963–transfected or si-NC–transfected MCF-7 cells into the flank of nude mice. Four weeks after the inoculation, the tumor growth curves revealed that the downregulation of *LINC00963* significantly slowed the tumor growth of breast cancer cells *in vivo* (Figure 6a,b, $^{*}P < 0.05$, $^{**}P < 0.01$). The weight of tumor xenografts was lower in the si-LINC00963 group than in the si-NC group (Figure 6c, $^{**}P < 0.01$). In addition, the expression of *LINC00963* was still low (Figure 6d, $^{*}P < 0.05$) while miR-625 expression was high (Figure 6e, $^{**}P < 0.01$) in tumor xenografts derived from si-LINC00963–transfected MCF-7 cells. The protein level of HMGA1 was lower in the si-LINC00963 group than in the si-NC group (figure 6f, $^{**}P < 0.01$), as determined by western blotting. These results implied that the *LINC00963* knockdown hindered the tumor growth of breast cancer cells through regulation of the miR-625–HMGA1 axis.

Discussion

In recent years, much attention was given to the expression and functions of lncRNAs in tumorigenesis and during tumor progression [36–38]. The dysregulation of lncRNAs has been confirmed in breast cancer and may be closely related to the formation and progression of breast cancer [39–41]. Therefore, exploring the important roles of lncRNAs in breast carcinogenesis and during breast cancer progression may offer novel insights into breast cancer pathogenesis and may facilitate the identification of potential targets for anticancer therapies. In this study, we first measured

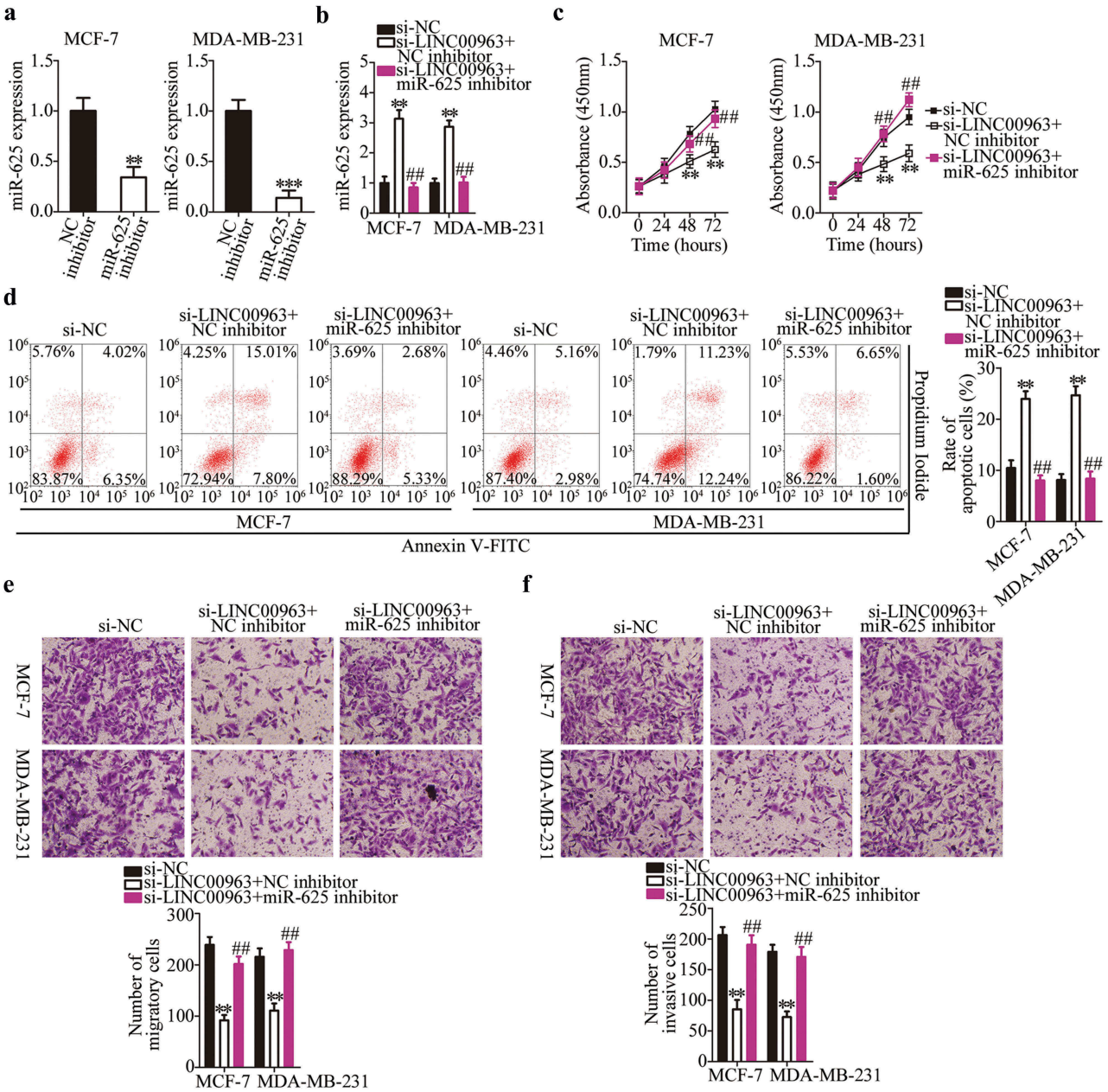

Figure 4. A reduction in *LINC00963* expression suppresses the proliferation, migration, and invasiveness and accelerates the apoptosis of breast cancer cells via direct modulation of miR-625.

(a) MCF-7 and MDA-MB-231 cells transfected with either the miR-625 inhibitor or NC inhibitor were collected and subjected to RT-qPCR analysis for the determination of miR-625 expression. Unpaired student's t-test is utilized for statistical analysis. **P < 0.01, ***P < 0.001 vs. NC inhibitor. (b) Si-LINC00963 and either the miR-625 inhibitor or NC inhibitor were cotransfected into MCF-7 and MDA-MB-231 cells. After 48 h culture, RT-qPCR analysis was conducted to assess miR-625 expression. *P < 0.01 vs. si-NC. ##P < 0.01vs. si-LINC00963+ NC inhibitor. (c, d) The CCK-8 assay and flow-cytometric analysis were performed to prove that the effects of si-LINC00963 on the proliferation and apoptosis of MCF-7 and MDA-MB-231 cells are abrogated by the miR-625 inhibitor. **P < 0.01 vs. si-NC. ##P < 0.01 vs. si-LINC00963+ NC inhibitor. (e, f) Transwell migration and invasion assays were conducted to analyze the migration and invasion status of MCF-7 and MDA-MB-231 cells cotransfected with si-LINC00963 and either the miR-625 inhibitor or NC inhibitor. **P < 0.01 vs. si-NC. ##P < 0.05 vs. si-LINC00963+ NC inhibitor. One-way analysis of variance followed by Tukey's *post hoc* test was utilized for statistical analysis.

LINC00963 expression in breast cancer, and then the clinical value of *LINC00963* was examined in patients with breast cancer. In addition, the regulatory effects of *LINC00963* on the aggressive characteristics of breast cancer cells were investigated in detail. The mechanisms underlying the

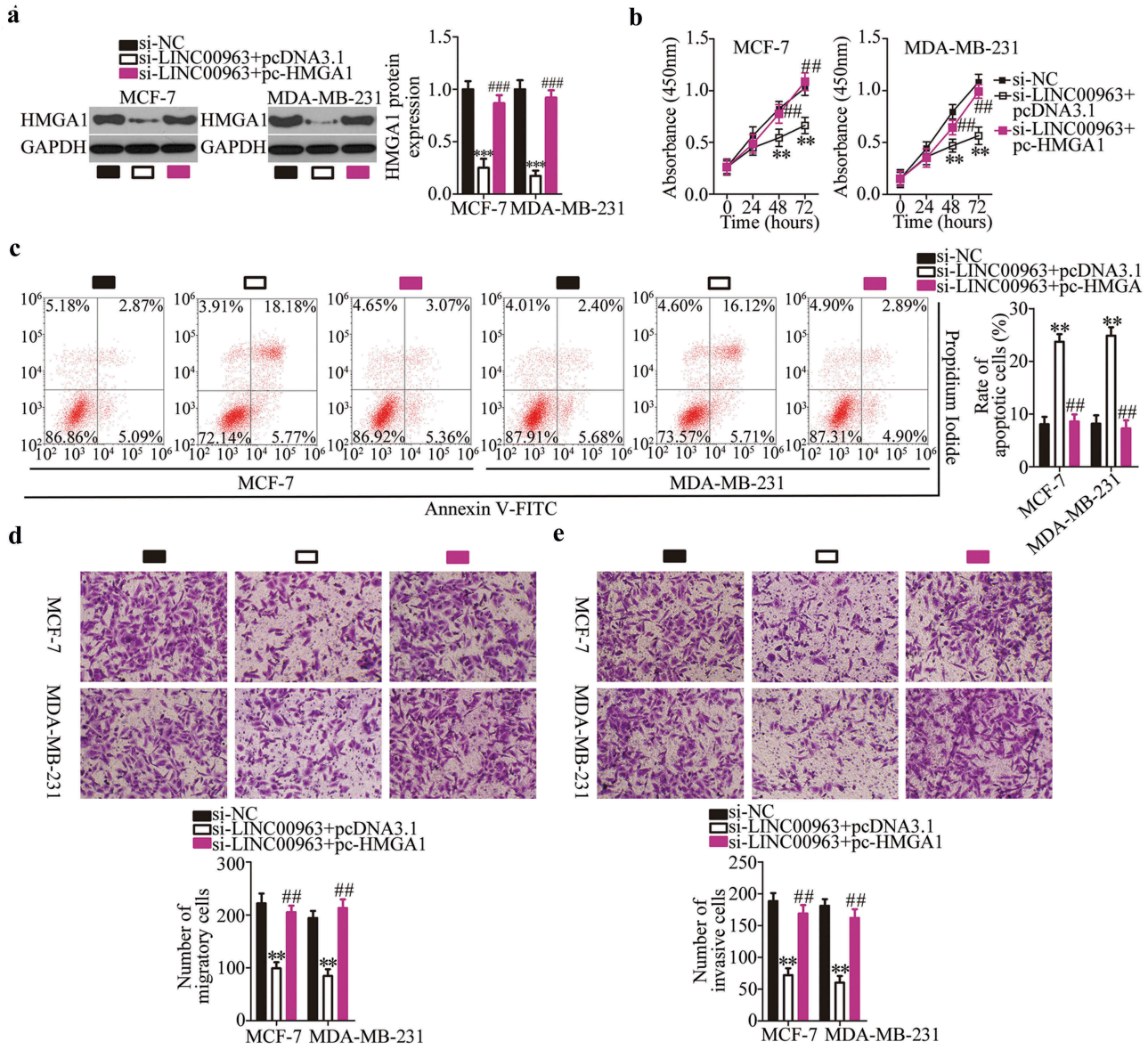

Figure 5. Recovery of HMGA1 expression rescinds the effects of the *LINC00963* knockdown on MCF-7 and MDA-MB-231 cells.
Either the HMGA1-overexpressing plasmid (pc-HMGA1) or the empty pcDNA3.1 vector in combination with si-LINC00963 was introduced into MCF-7 and MDA-MB-231 cells. The transfected cells were studied in the following experiments. (a) The protein expression of HMGA1 in MCF-7 and MDA-MB-231 cells treated as described above was examined by western blotting. ***P < 0.001 vs. si-NC. ###P < 0.001 vs. si-LINC00963+ pcDNA3.1. (b–e) The CCK-8 assay, flow cytometry, and Transwell migration and invasion assays were carried out to determine the proliferation, apoptosis, migration, and invasiveness of MCF-7 and MDA-MB-231 cells that were cotransfected with si-LINC00963 and either pc-HMGA1 or pcDNA3.1. **P < 0.01 vs. si-NC. ##P < 0.01 vs. si-LINC00963 + pcDNA3.1. One-way analysis of variance followed by Tukey's *post hoc* test was utilized for statistical analysis.

activity of *LINC00963* in breast cancer cells were explored at the molecular level.

LINC00963 is upregulated in osteosarcoma, and this upregulation significantly correlates with poor clinical outcomes among patients with osteosarcoma [31]. The expression of *LINC00963* is also high in melanoma. Patients with melanoma featuring high *LINC00963* expression exhibit shorter overall survival than do the patients with low *LINC00963* expression [32]. *LINC00963* is reported to be overexpressed in hepatocellular carcinoma [33] and prostate cancer [34]. *LINC00963* overexpression correlates with tumor size and TNM stage in patients with hepatocellular carcinoma [33]. However, whether *LINC00963* is aberrantly expressed in breast cancer has been

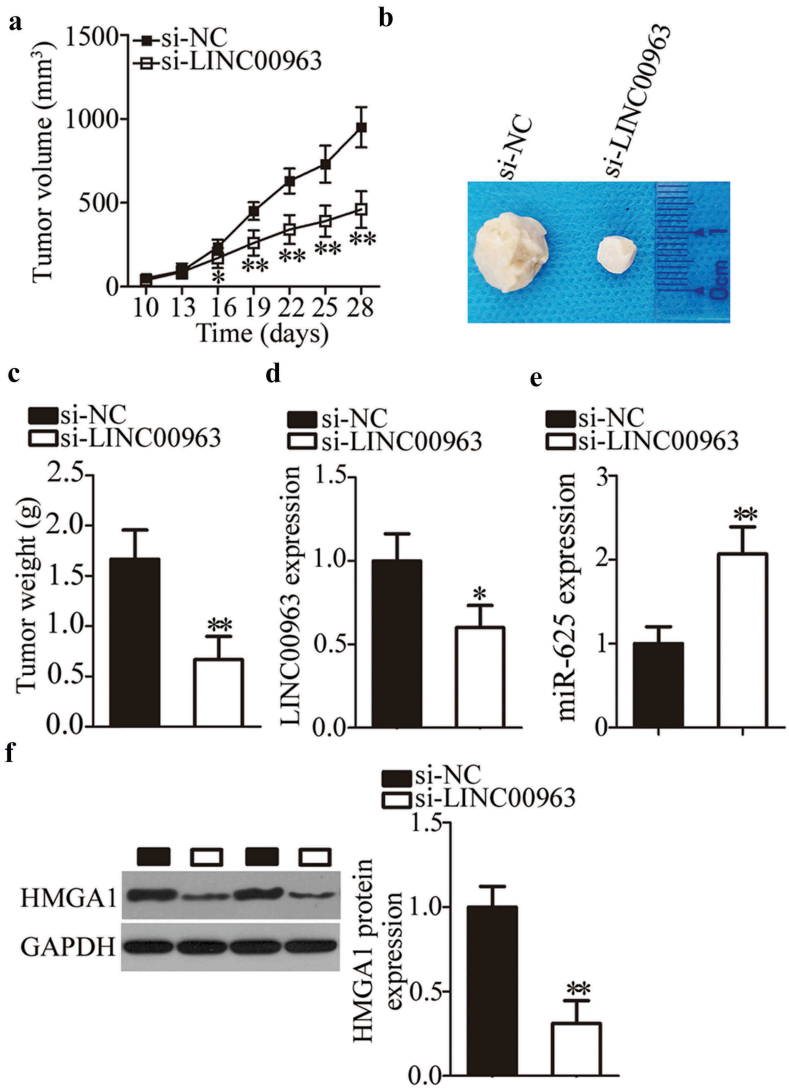

Figure 6. *LINC00963* silencing restrains breast tumor growth *in vivo*.

(a) MCF-7 cells transfected with either si-LINC00963 or si-NC were injected into nude mice. Tumor growth curves revealed that the tumor xenografts grew at a markedly slower rate in the si-LINC00963 group than in the si-NC group. *P < 0.05. **P < 0.01 vs. si-NC. (b) At the end of this experiment, the mice were anesthetized, and tumor xenografts were excised. The representative images of tumor xenografts are presented. (c) The weight of tumor xenografts obtained from groups si-LINC00963 and si-NC was measured. **P < 0.01 vs. si-NC. (d, e) The levels of *LINC00963* and miR-625 in the tumor xenografts were assessed by RT-qPCR. *P < 0.05, **P < 0.01 vs. si-NC. (f) Western blotting was carried out to measure HMGA1 protein expression in the tumor xenografts. **P < 0.01 vs. si-NC. Unpaired student's t-test is used for all above statistical analysis.

unknown until our study. Herein, our results showed that *LINC00963* expression is higher in breast tumors and breast cancer cell lines in comparison with the adjacent normal tissues and human normal breast epithelial cell line MCF-10A, respectively. Besides, *LINC00963* overexpression was found to correlate with the lymph node metastasis, TNM stage and differentiation grade among our 53 patients with breast cancer. Patients with breast cancer featuring high *LINC00963* expression showed shorter overall survival than did the patients with low *LINC00963* expression. These findings suggest that *LINC00963* may serve as a biomarker for the diagnosis and prognosis of breast cancer.

LINC00963 plays oncogenic roles in carcinogenesis and cancer progression. For instance, upregulation of *LINC00963* accelerates osteosarcoma cell growth and metastasis *in vitro* by deactivating the miR-204-3p–FN1 pathway [31]. Inhibition of *LINC00963* restricts

melanoma cell proliferation, migration, and invasion because of weaker sponging of miR-608 and therefore greater inhibition of NACC1 expression [32]. In hepatocellular carcinoma, *LINC00963* overexpression promotes cell proliferation and shortens the cell cycle G0–G1 transition by promoting PI3K–AKT pathway activation [33]. In prostate cancer, silencing of *LINC00963* expression restrains cell proliferation, motility, and invasion and promotes apoptosis *in vitro* [34]. Nevertheless, the functions of *LINC00963*, if any, in the malignancy of breast cancer have not been studied until our work. In this study, functional investigation revealed that the *LINC00963* knockdown represses cell proliferation, migration, and invasion and induces apoptosis *in vitro* as well as attenuates tumor growth *in vivo*.

Next, a systemic approach was employed to elucidate the mechanisms behind the oncogenic activities of *LINC00963* in breast cancer. First, our investigation of the subcellular localization of *LINC00963* in breast cancer revealed that *LINC00963* is mainly present in the cytoplasm of breast cancer cells, suggesting that *LINC00963* may work as a ceRNA for certain miRNA in breast cancer. Second, bioinformatics prediction identified a high-probability binding site for miR-625 in *LINC00963*. Third, luciferase reporter and RIP assays indicated that miR-625 is a target of *LINC00963* in breast cancer cells. Fourth, miR-625 was found to be downregulated in breast cancer, with an inverse correlation with *LINC00963* levels. Fifth, *LINC00963* inhibition increased miR-625 expression and downregulated the HMGA1 protein in breast cancer cells. Finally, the miR-625 knockdown and HMGA1 restoration both attenuated the effects of *LINC00963* downregulation on breast cancer cells. Collectively, these data mean that *LINC00963* functions as a ceRNA for miR-625 and thereby promotes HMGA1 expression in breast cancer.

MiR-625 levels are reported to be low in breast cancer tissues and cell lines [35]. This downregulation is associated with estrogen receptor, human epidermal growth factor receptor 2, and clinical stage among patients with breast cancer [35]. In addition, Kaplan–Meier and multivariate analyzes identified miR-625 as an independent factor predicting the poor clinical outcomes in patients with breast cancer [35]. MiR-625 plays tumor-suppressive roles in the progression of breast cancer by directly targeting *HMGA1* mRNA and downregulating its expression [35]. HMGA1, a member of the HMGA protein family, regulates chromatin structure by directly binding to the A/T-rich DNA sequences located in the promoter and enhancer regions of multiple human genes [42]. HMGA1 exerts a crucial effect on the malignancy of breast cancer and is implicated in the regulation of various malignant characteristics [43–45]. Our current study revealed that the depletion of *LINC00963* restrains the aggressiveness of breast cancer cells *in vitro* and *in vivo*; these influences turned out to be mediated by decreased sponging of miR-625 and consequent HMGA1 upregulation. Hence, targeting of the *LINC00963*–miR-625–HMGA1 pathway might be a promising method for the treatment of breast cancer.

Conclusion

In summary, *LINC00963* serves as a ceRNA for miR-625 thereby weakening the suppressive effect of miR-625 on HMGA1 expression in breast cancer. Thus, *LINC00963* plays oncogenic roles in the progression of breast cancer. Our observations may form the basis for further research into the application of *LINC00963* as a prognostic and diagnostic biomarker as well as a possible therapeutic target in breast cancer.

Disclosure statement

No potential conflict of interest was reported by the authors.

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