

# MicroRNA-301a-3p promotes triple-negative breast cancer progression through downregulating MEOX2

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**Abstract.** Breast cancer is one of the most frequently diagnosed malignancies among women. Triple-negative breast cancer (TNBC) represents a significant challenge for breast oncologists, as the availability of effective therapies for this aggressive disease is limited. The molecular mechanisms underlying TNBC development are not fully understood. Previous studies have demonstrated that microRNAs (miRNAs/miRs) play important roles in the development of various types of cancer, including breast cancer; however, the role of miRNAs in TNBC remains undetermined. The results of the present study revealed that miR-301a-3p may function as an oncogenic miRNA in TNBC. Based on The Cancer Genome Atlas data, miR-301a-3p expression levels were found to be upregulated in breast cancer tissues. Reverse transcription-quantitative PCR analysis demonstrated that the expression levels of miR-301a-3p were upregulated in TNBC tissues compared with non-TNBC tissues, and in MDA-MB-231 cells compared with normal MCF-10A breast cells. miR-301a-3p mimics and inhibitors were subsequently used to overexpress and knock down miR-301a-3p expression, respectively, in MDA-MB-231 cells. Biological functional experiments demonstrated that miR-301a-3p overexpression increased the viability, and the migratory and invasive abilities of MDA-MB-231 cells. By contrast, miR-301a-3p knockdown exerted the opposite effects on MDA-MB-231 cells. Cell apoptosis was negatively regulated by miR-301a-3p. Moreover, overexpression of miR-301a-3p was found to downregulate the expression levels of mesenchyme homeobox 2 (MEOX2). The expression levels of miR-301a-3p were negatively correlated with the expression levels of MEOX2 in clinical tissue specimens from patients with TNBC. Subsequently, the knockdown of MEOX2 expression promoted the viability of

MDA-MB-231 cells. In conclusion, the results of the present study suggested that miR-301a-3p may serve as an oncogenic miRNA in TNBC by regulating MEOX2 expression.

## Introduction

Breast cancer is one of the most frequently diagnosed malignancies among women (1). During the past few decades, the incidence of breast cancer has continued to increase, and a total of 2,088,849 newly diagnosed cases of breast cancer were reported in 2018 worldwide (2,3). The majority of patients with breast cancer are curable at the non-metastatic stage; however, current therapeutics exhibit minimal efficacy against advanced-stage metastatic disease. Based on transcriptional profiling studies, breast cancer has been established as a heterogeneous malignancy comprising different subtypes, including ductal carcinoma, lobular carcinoma, fibroadenoma and ductal carcinoma *in situ* (4,5). Based on the molecular characteristics, breast cancer may be divided into hormone receptor-positive breast cancer, in which breast cancer cells express estrogen receptor (ER) or progesterone receptor (PR), or triple-negative breast cancer (TNBC), in which breast cancer cells are negative for ER, PR and human epidermal growth factor receptor 2 (HER2) expression. Although TNBC only accounts for 15-20% of all breast cancer cases, its aggressive nature and the current lack of effective therapies make it one of the most lethal malignancies. Therefore, further understanding the underlying mechanisms involved in TNBC may help with the development of effective therapeutic strategies.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs, 19-26 nucleotides in length, that have no protein-coding ability. miRNAs bind to target mRNAs to suppress their translation (6). Accumulating evidence indicates that miRNAs serve an important role in tumorigenesis, as they are commonly found to be aberrantly expressed in cancer tissues. The dysregulated expression of miRNAs results in uncontrolled cell proliferation, growth and migration in numerous different types of cancer through regulating the expression of downstream tumor suppressor genes or oncogenes. miR-301a-3p has been recognized as a regulator of the T-helper 17 cell immune response in autoimmune demyelinating diseases (7). In addition, miR-301a-3p was previously demonstrated to play an oncogenic role in various types of cancer, including breast cancer (8-13). However, to the best of our knowledge, the

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downstream target genes of miR-301a-3p in TNBC have not been identified to date.

The present study was undertaken to determine the significance of miR-301a-3p in TNBC. The expression levels of miR-301a-3p were analyzed in normal breast MCF-10A cells and in MDA-MB-231 TNBC cells. The effects of miR-301a-3p on the viability and migratory ability of MDA-MB-231 cells were then examined, and the role of mesenchyme homeobox 2 (MEOX2) in this process was also investigated. The aim was to determine whether miR-301a-3p acts as an oncogenic miRNA in TNBC and whether its effects are mediated by regulating MEOX2 expression.

## Materials and methods

**Patient studies.** Human breast cancer tissues were collected from patients who were diagnosed with TNBC and non-TNBC by three independent pathologists. The patients were enrolled between March 2016 and May 2020 at the Beijing Obstetrics and Gynecology Hospital (Beijing, China). The protocol of the present study was approved by the Clinical Research Ethics Committee of Beijing Obstetrics and Gynecology Hospital, and written, informed consent was obtained from each patient for the use of their tissue prior to participation.

**The Cancer Genome Atlas (TCGA) data analysis.** Normalized transcriptome expression datasets for breast cancer from the TCGA were analyzed using the ENCORI Pan-Cancer Analysis Platform (<http://starbase.sysu.edu.cn/index.php>). Briefly, a total of 1,085 breast cancer tissues and 104 normal tissues were available for miR-301a-3p expression level analysis.

**Cell lines and culture.** MCF-10A normal human breast epithelial cells and MDA-MB-231 TNBC cells were purchased from the American Type Culture Collection. MDA-MB-231 and MCF-10A cells were cultured in RPMI-1640 and DMEM/F12 medium (HyClone; Cytiva), respectively, supplemented with FBS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin-streptomycin solution (Corning, Inc.). The cells were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**Cell transfection.** MDA-MB-231 cells were plated into 6-well plates and, upon reaching 70–80% confluence, the cells were transfected with 20 μM miR-301a-3p mimic, mimic negative control (NC), miR-301a-3p inhibitor and inhibitor NC (all purchased from Guangzhou RiboBio Co. Ltd.) using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of transfection, cells were collected for use in further experiments. The sequences of the oligonucleotides were as follows: miR-301a-3p mimic, 5'-GCU CUGACUUUAUUGCACUACU-3'; mimic NC, 5'-UCACAA CCUCCUAGA AAGAGUAGA-3'; miR-301a-3p inhibitor, 5'-AGUAGUCAUAUAGGACAGAGC-3'; and inhibitor NC, 5'-UCUACUCUUUCUAGGUUGUGA-3'. For MEOX2 knockdown, cells were transfected with 40 nM small interfering (si)RNA targeted to MEOX2 (5'-AAGGUAGGACAUGUG GUCAGAUCUU-3') and NC (5'-AAUUAGCGGAGGCUA UAAGAUGUUC-3'), all purchased from Guangzhou RiboBio Co., Ltd. using RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The full-length open reading frame of the human

MEOX2 gene (NM\_005924.5) was synthesized and cloned into pcDNA3.1 plasmid. pcDNA3.1 MEOX2 or empty vector (3 μg for 6-well plates) was transfected into MDA-MB-231 cells using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of transfection, cells were collected for use in further experiments.

**Reverse transcription-quantitative PCR (RT-qPCR) analysis.** MDA-MB-231 cells were washed with PBS and lysed in 0.5 ml RNAiso Plus (Takara Biotechnology Co., Ltd.). Following incubation for 10 min on ice, 0.1 ml trichloromethane was added to the lysates and total RNA was isolated. Total RNA was reverse-transcribed into cDNA, using the PrimeScript RT-PCR kit according to the manufacturer's instructions (Takara Biotechnology Co., Ltd.). qPCR was subsequently performed using a SYBR Green PCR kit (Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 10 min. U6 and β-actin served as internal controls for miR-301a-3p and MEOX2 expression levels, respectively. The following primer sequences were used for the qPCR: MEOX2 forward, 5'-TCA GAAGTCAACAGCAAACCCAG-3' and reverse, 5'-TTCACC AGTTCCTTTTCCCGAG-3'; β-actin forward, 5'-GACCTG ACTGACTACCTCATGAAGAT-3' and reverse, 5'-GTCACA CTTCATGATGGAGTTGAAGG-3'; miR-301a-3p, forward 5'-CGTGCGAAGCTCAGGAGGG-3' and reverse, 5'-TGG CTGTCGTGGACTGCG-3'; and U6 forward 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGCGT-3'. RNA expression levels were normalized to U6 and β-actin. The 2<sup>-ΔΔC<sub>q</sub></sup> method was performed to determine the relative expression (14).

**Western blotting.** Total protein was extracted from MDA-MB-231 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a BCA assay kit, and protein lysates were then suspended in loading buffer and boiled at 98°C for 10 min. Subsequently, 30 μg protein/lane was separated via 10–12% SDS-PAGE and separated proteins were transferred onto PVDF membranes, which were then blocked in 5% skimmed milk at 4°C overnight. After washing with TBS, the membranes were incubated with the following primary antibodies at 4–8°C for ≥12 h: Anti-MEOX2 (1:1,000; cat. no. P50222; RayBiotech, Inc.) and β-actin (1:1,000; cat. no. GTX11003, ProteinTech Group, Inc.). Following primary antibody incubation, the membranes were washed with PBS-Tween-20 (0.1%) thrice and incubated with the corresponding secondary antibodies [HRP-conjugated goat anti-mouse IgG (H + L), 1:2,000; cat. no. AS003; ABclonal Biotech Co., Ltd.] at room temperature for 2 h. Protein bands were visualized using an ECL-Plus kit (Thermo Fisher Scientific, Inc.).

**Cell viability assay.** MDA-MB-231 cell viability was determined using an MTT assay. Briefly, 5 × 10<sup>3</sup> cells/well were seeded into 96-well plates and cultured for 72 h. Subsequently, MTT (5 mg/ml) was added into each well and incubated for a further 3 h. The culture medium was subsequently discarded and 100 μl DMSO was added to each well to dissolve the purple

formazan crystals. The optical density value was measured at a wavelength of 595 nm to analyze cell viability.

**Flow cytometric analysis of apoptosis.** Cell apoptosis was analyzed using a propidium iodide (PI)/Annexin V-FITC kit (Beyotime Institute of Biotechnology). Briefly, MDA-MB-231 cells were digested with EDTA-free trypsin and centrifuged at 157 x g for 5 min at room temperature, then the cell pellet was washed twice with PBS. Subsequently,  $0.5-1 \times 10^5$  cells were dissolved in binding buffer and incubated with 5  $\mu$ l Annexin V-FITC and 10  $\mu$ l PI. Apoptotic cells were analyzed using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences) using BD Accuri™ C6 software (BD Biosciences), after which the flow cytometric data were analyzed using a Guava easy-Cyte flow cytometer (EMD Millipore).

**Cell invasion and migration assays.** Transwell plates with or without Matrigel precoating were used to determine cell invasion and migration, respectively. The upper surface of the chamber was precoated with 15  $\mu$ l of Matrigel (BD Biosciences) at room temperature for the cell invasion assay. A total of  $1.5 \times 10^5$  MDA-MB-231 cells were plated into 8.0- $\mu$ m pore Transwell chambers (BD Biosciences) and cultured at 37°C with 5% CO<sub>2</sub>. The upper chamber was incubated with FBS free RPMI-1640 and DMEM/F12 medium, respectively, whereas a total of 500  $\mu$ l 10% FBS medium was added in the lower chamber. Following 24 h of incubation, cells in the upper chamber were removed by cotton-tipped swabs, while cells in the lower chamber were fixed with 100% methanol for 30 min at room temperature and stained with 0.2% crystal violet solution at room temperature. The chambers were then washed with PBS and dried at room temperature. Migratory or invasive cells were visualized under an inverted microscope (magnification, x200; Olympus Corporation).

**Wound healing assay.** Wound healing assay was performed to analyze the cell migratory ability. Briefly,  $2 \times 10^5$  MDA-MB-231 cells were seeded into 6-cm culture plates and cultured in RPMI-1640 medium supplemented with 10% FBS for 48 h. Upon cells reaching 90% confluence, a 200- $\mu$ l pipette tip was used to create a single linear scratch in the middle of the cell monolayer. The medium was removed, and the cells were incubated with RPMI-1640 medium containing 1% FBS. The wound was visualized using an inverted microscope (magnification, x200; Olympus Corporation) and images were captured at 0 and 24 h.

**Dual luciferase reporter assay.** Wild-type (WT) or mutant (MUT) 3'-untranslated region (UTR) sequences of MEOX2 were cloned into psi-CHECK vectors (Promega Corporation). Cells were subsequently transfected with the aforementioned vectors and co-transfected with miR-301a-3p mimic (5'-GCU CUGACUUUAUUGCACUACU-3') or negative control (5'-UCACAACCUCCUAGA AAGAGUAGA-3') (Beijing SyngenTech Co., Ltd.) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After transfection for 48 h, relative luciferase activity was measured using a Dual-Luciferase Reporter assay system (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software (version 8.0; GraphPad Software, Inc.) and data are presented as the mean  $\pm$  SEM. Statistical differences between two groups were analyzed using unpaired Student's t-test, while statistical differences between multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**miR-301a-3p increases the viability of MDA-MB-231 cells.** The expression levels of miR-301a-3p were upregulated in breast cancer tissues in datasets from the TCGA database (Fig. 1A). In addition, miR-301a-3p expression levels were found to be upregulated in TNBC tissues compared with normal breast tissue and luminal type breast cancer tissue according to the TCGA database (Fig. 1B). No statistically significant differences were observed in miR-301a-3p expression levels between TNBC and HER<sup>+</sup> breast cancer samples (Fig. 1B). The expression levels of miR-301a-3p in samples from patients with and without TNBC were subsequently analyzed. RT-qPCR analysis revealed that the expression levels of miR-301a-3p were upregulated in TNBC tissues compared with adjacent non-TNBC tissues (Fig. 1C). Subsequently, the expression levels of miR-301a-3p in normal MCF-10A breast epithelial cells and MDA-MB-231 TNBC cells were determined. miR-301a-3p expression levels were found to be upregulated in MDA-MB-231 cells compared with those in MCF-10A cells (Fig. 1D). Thus, MDA-MB-231 cells were selected to further investigate the role of miR-301a-3p in TNBC. The cells were divided into five experimental groups: i) Untreated; ii) miR-301a-3p mimic; iii) mimic NC; iv) miR-301a-3p inhibitor; and v) inhibitor NC. The transfection of cells with the miR-301a-3p mimic or inhibitor led to significantly increased and decreased miR-301a-3p expression levels, respectively, compared with the respective NCs (Fig. 2A). No statistically significant differences in cell viability were observed among the untreated, NC mimic-transfected and NC inhibitor-transfected MDA-MB-231 cells (Fig. 2B). However, miR-301a-3p overexpression increased MDA-MB-231 cell viability, whereas miR-301a-3p knockdown exerted the opposite effect on MDA-MB-231 cells (Fig. 2B). These findings suggested that miR-301a-3p may serve as an oncogenic miRNA in TNBC.

**miR-301a-3p suppresses cell apoptosis.** The regulatory role of miR-301a-3p on apoptosis was subsequently determined in MDA-MB-231 cells using PI/Annexin V-FITC double staining and flow cytometric analysis. Compared with the cells transfected with the mimic NC, transfection with the miR-301a-3p mimic significantly inhibited the apoptosis of MDA-MB-231 cells. Conversely, miR-301a-3p knockdown induced MDA-MB-231 cell apoptosis (Fig. 2C and D). These results suggested that miR-301a-3p may suppress apoptosis in TNBC cells.

**miR-301a-3p induces the migration and invasion of MDA-MB-231 cells.** TNBC is a highly metastatic cancer. Thus, transwell plates with or without Matrigel precoating and wound healing assays were performed to analyze cell invasion and

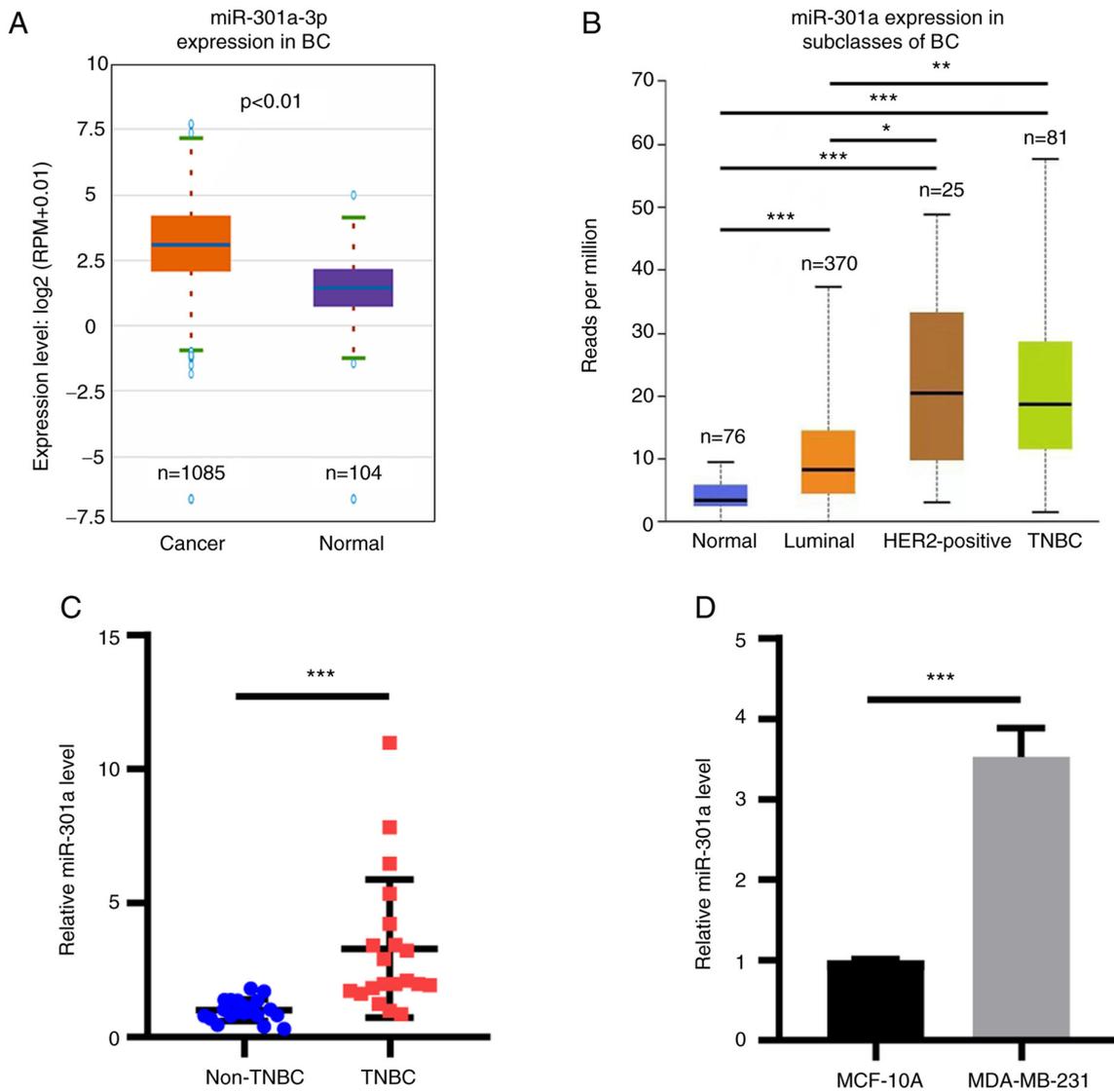


Figure 1. miR-301a-3p expression is upregulated in breast cancer tissues and cells. (A) miR-301a-3p expression was analyzed in breast cancer tissues from TCGA database.  $P < 0.01$ . (B) miR-301a-3p expression in breast cancer subclasses was analyzed from TCGA database. Normal vs. TNBC, luminal and HER2-positive, all  $***P < 0.001$ . Luminal vs. TNBC,  $**P < 0.01$ . HER2-positive vs. TNBC, not significant. Luminal vs. HER2-positive,  $*P < 0.05$ . (C) RT-qPCR analysis of miR-301a-3p in non-TNBC and TNBC tissues.  $***P < 0.001$ . (D) RT-qPCR analysis of miR-301a-3p expression in MCF-10A and MDA-MB-231 cells.  $***P < 0.001$ . TCGA, The Cancer Genome Atlas; miR, microRNA; BC, breast cancer; TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor 2; RT-qPCR, reverse transcription-quantitative PCR.

migration, respectively. No statistically significant differences were observed among the untreated, mimic NC-transfected and inhibitor NC-transfected MDA-MB-241 cells. The transfection of MDA-MB-231 cells with the miR-301a-3p mimic increased the migratory and invasive abilities. By contrast, transfection of cells with the miR-301a-3p inhibitor inhibited cell migration and invasion (Fig. 3). These data indicated that miR-301a-3p may function as an oncomiR in TNBC.

*miR-301a-3p downregulates the expression levels of MEOX2.* Downstream target genes of miR-301a-3p in MDA-MB-231 cells were subsequently identified. RT-qPCR analysis revealed that MEOX2 mRNA expression levels were down-regulated in MDA-MB-231 cells following transfection with the miR-301a-3p mimic. Conversely, transfection with the miR-301a-3p inhibitor upregulated the expression levels of MEOX2 in MDA-MB-231 cells (Fig. 4A). The results of

western blotting demonstrated that MEOX2 protein expression levels were also decreased and increased following the overexpression and knockdown of miR-301a-3p, respectively (Fig. 4B). Analysis of data from TCGA database revealed that miR-301a-3p expression levels were inversely associated with MEOX2 expression levels in breast cancer tissues (Fig. 4C). To investigate whether miR-301a-3p bound to the 3'-UTR sequence of MEOX2, WT and MUT 3'-UTR sequences of MEOX2 were synthesized and cloned into luciferase reporter vectors. The relative luciferase activity was subsequently measured following the overexpression of miR-301a-3p. The results demonstrated that miR-301a-3p overexpression reduced the relative luciferase activity of MDA-MB-231 cells when co-transfected with the WT, but not the MUT, 3'-UTR sequence of MEOX2 (Fig. 4D and E). Collectively, these results suggested that miR-301a-3p may directly target MEOX2 by binding to its 3'-UTR sequence.

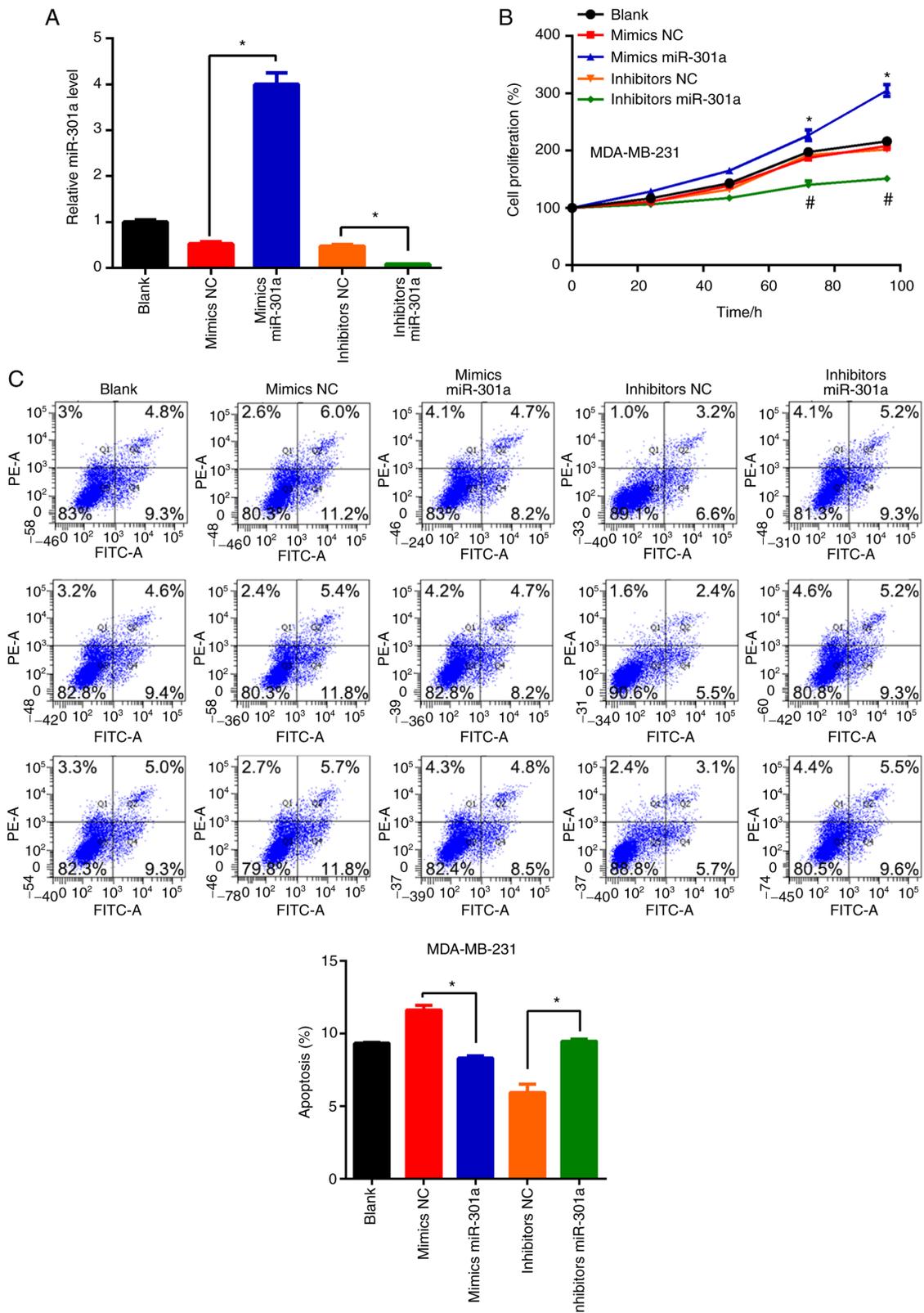


Figure 2. miR-301a-3p regulates the proliferation and apoptosis of MDA-MB-231 cells. (A) MDA-MB-231 cells, untreated (blank) or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p were subjected to reverse transcription-quantitative PCR analysis of miR-301a-3p expression. \* $P < 0.05$  vs. NC. (B) MDA-MB-231 cells, untreated (blank) or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p were subjected to MTT analysis of cell proliferation. \* $P < 0.05$  vs. mimics NC; # $P < 0.05$  vs. inhibitor NC. (C) MDA-MB-231 cells of untreated or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p were subjected to flow cytometry analysis of apoptosis. (D) Quantification of apoptosis as shown in C. \* $P < 0.05$  vs. NC. miR, microRNA; NC, negative control.

*MEOX2* acts as a tumor suppressor gene in TNBC. Finally, the role of *MEOX2* in TNBC cells was investigated.

*MEOX2* expression was knocked down by transfection with siRNA and overexpressed by the pcDNA3.1 vector

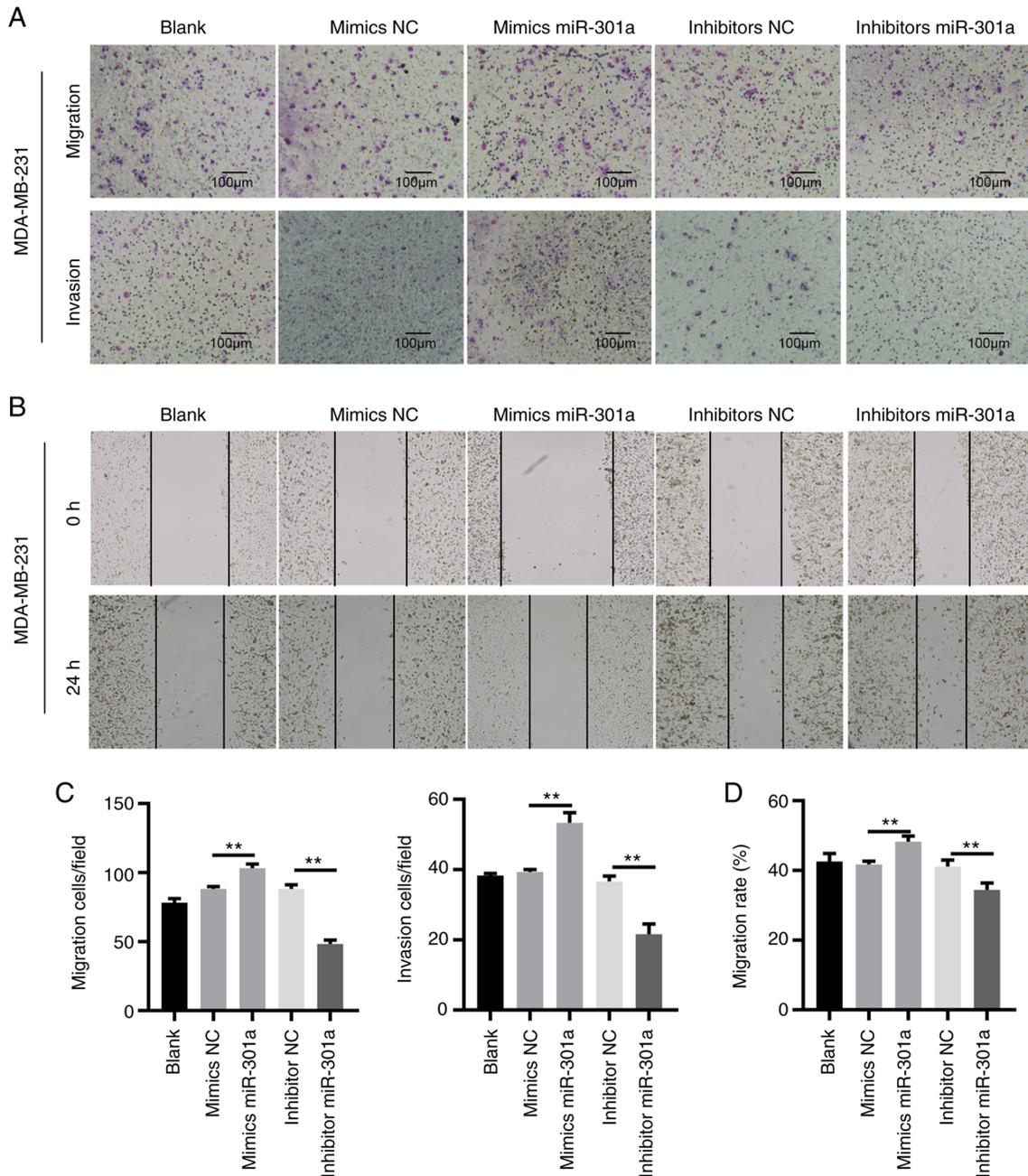


Figure 3. miR-301a-3p promotes the migration and invasion of MDA-MB-231 cells. (A) MDA-MB-231 cells, untreated (blank) or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p were subjected to Transwell migration (upper images) and invasion (lower images) assays (magnification, x200). (B) MDA-MB-231 cells, untreated (blank) or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p were subjected to wound healing assay. Images were captured at 0 h (upper panels) and 24 h (lower panels) (magnification, x200). (C and D) Quantification of cell migration and invasion as shown in A and B. \*\*P<0.01. miR, microRNA; NC, negative control.

RT-qPCR and western blotting confirmed successful MEOX2 knockdown and overexpression in MDA-MB-231 cells (Fig. 5A and B). The results of the MTT assay revealed that MEOX2 knockdown enhanced, while MEOX2 overexpression reduced MDA-MB-231 cell viability (Fig. 5C). Furthermore, MEOX2 was overexpressed in cells also overexpressing miR-301a-3p (Fig. 5D). The overexpression of MEOX2 further suppressed the viability of cells overexpressing miR-301a-3p (Fig. 5E). MEOX2 overexpression also increased the levels of apoptosis in MDA-MB-231 cells (Fig. 5F). These findings suggested that MEOX2 may serve as a tumor suppressor in TNBC.

## Discussion

Breast cancer is the most common malignancy among women, and poses a major threat to women's health in both developed and developing countries. Over the past few decades, researchers have made significant efforts to determine the molecular mechanisms underlying breast cancer tumorigenesis. The expression of the ER has been reported as a major risk factor for breast cancer development. Tamoxifen, which interrupts the interaction between estrogen and the ER, is a drug approved for the treatment of ER<sup>+</sup> breast cancer. The discovery of HER-targeted therapy also represented a

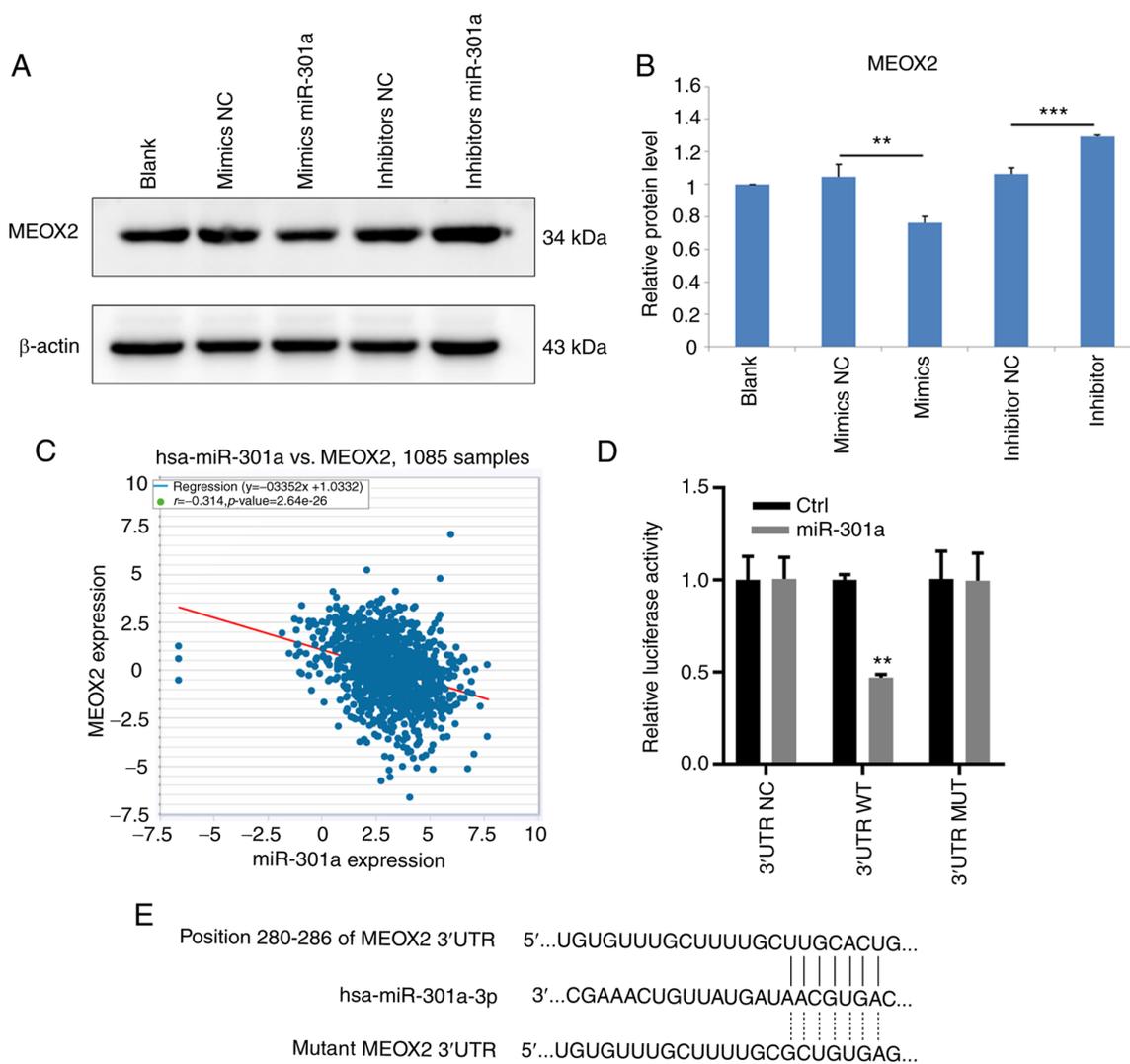


Figure 4. MEOX2 is a downstream target of miR-301a-3p in MDA-MB-231 cells. (A) Western blot result of MEOX2 in MDA-MB-231 cells, untreated (blank) or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p. (B) Relative protein level of MEOX2 in MDA-MB-231 cells, untreated or transfected with mimics NC, mimics miR-301a-3p, inhibitor NC and inhibitor miR-301a-3p. \*\* $P < 0.01$  vs. mimics NC; and \*\*\* $P < 0.001$  vs. inhibitor NC. (C) Analysis of the correlation between miR-301a-3p and MEOX2 in breast cancer tissues from The Cancer Genome Atlas database. (D) WT or MUT sequence of MEOX2 3'-UTR was inserted into luciferase reporter vectors. At 48 h after transfecting MEOX2 overexpression vectors, luciferase activity was examined as indicated. \*\* $P < 0.01$  vs. control. (E) Binding sequence of miR-301a-3p with the 3'-UTR of MEOX2. MEOX2, mesenchyme homeobox 2; miR, microRNA; NC, negative control; WT, wild-type; MUT, mutant; UTR, untranslated region.

significant milestone in breast cancer research. Trastuzumab, which was developed in 1990, inhibits the extracellular segment of HER2 and, combined with chemotherapy, can effectively prolong the overall and progression-free survival of patients with HER<sup>+</sup> breast cancer (15-17). However, 15-20% of patients with breast cancer are diagnosed with TNBC, which remains difficult to treat as the currently available targeted therapies are not applicable, as this type of cancer is negative for ER, PR and HER2 expression. Therefore, there are currently no effective treatment options for patients with TNBC, except for traditional methods, such as surgical resection, radiotherapy and chemotherapy.

The present study aimed to determine the role of miR-301a-3p in TNBC. miR-301a-3p expression levels were upregulated in breast cancer tissues. Notably, the expression levels of miR-301a-3p were upregulated in TNBC samples compared with adjacent non-TNBC samples. The overexpression of miR-301a-3p increased the viability and migratory ability of

MDA-MB-231 cells. By contrast, miR-301a-3p knockdown suppressed the viability and migration of MDA-MB-231 cells. Mechanistically, miR-301a-3p downregulated the expression levels of MEOX2 by directly binding to the 3'-UTR sequence of MEOX2. Further experiments demonstrated that MEOX2 acted as a tumor suppressor gene in TNBC.

miR-301a, which comprises miR-301a-3p and miR-301a-5p, was found to play a crucial role in cancer development, including pancreatic, colorectal and gastric cancer. The expression levels of miR-301a were reported to be upregulated in pancreatic cancer and promoted pancreatic cancer cell proliferation by targeting Bim (18). The knockdown of miR-301a-3p expression in pancreatic cancer cells was also found to increase the sensitivity of cells to gemcitabine treatment (19). In addition, miR-301a promoted the growth and invasion of colorectal cancer by targeting suppressor of cytokine signaling 6 or transforming growth factor  $\beta$  receptor 2 (20,21). In gastric cancer, miR-301a was also discovered to function as an oncogene.

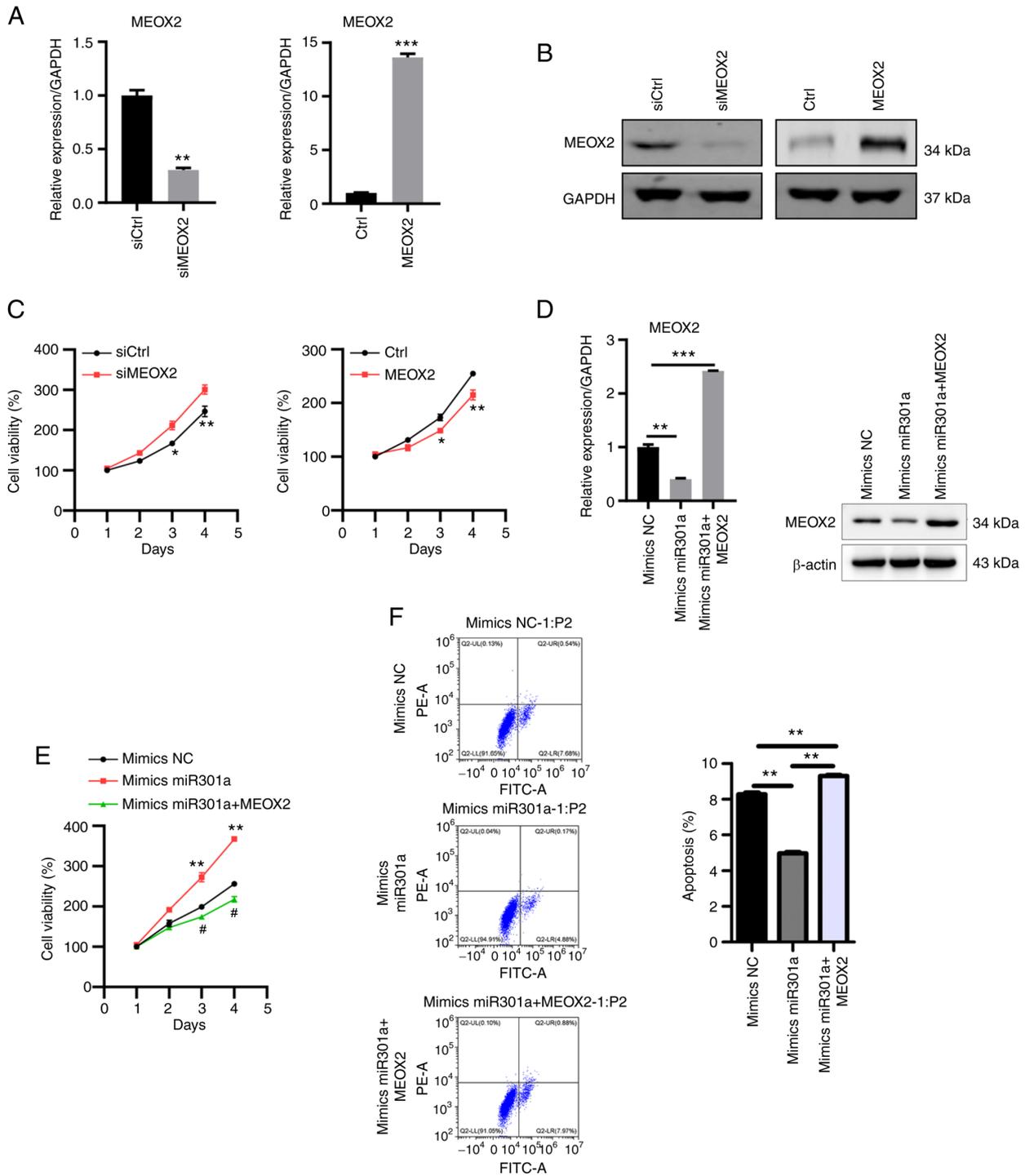


Figure 5. MEOX2 suppresses MDA-MB-231 cell viability. (A) RT-qPCR and (B) western blot analyses of MEOX2 expression in MEOX2-knockdown and -overexpressing MDA-MB-231 cells. <sup>\*\*</sup>P<0.01 vs. siCtrl and <sup>\*\*\*</sup>P<0.001 vs. Ctrl. (C) MTT assay was used to determine the viability of MEOX2-knockdown and -overexpressing MDA-MB-231 cells. <sup>#</sup>P<0.05 vs. mimics, <sup>\*\*</sup>P<0.01 vs. siCtrl or vs. Ctrl, respectively. (D) RT-qPCR and western blot analyses of MEOX2 expression following transfection with mimics NC, mimics miR-301a-3p and mimics miR-301a-3p + MEOX2 in MDA-MB-231 cells. <sup>\*\*</sup>P<0.01 and <sup>\*\*\*</sup>P<0.001 vs. mimics NC. (E) MTT assay was used to determine cell viability following transfection with mimics NC, mimics miR-301a-3p and mimics miR-301a-3p + MEOX2 in MDA-MB-231 cells. <sup>\*\*</sup>P<0.01 vs. mimics NC; and <sup>#</sup>P<0.05 vs. mimics miR301a. (F) MDA-MB-231 cell apoptosis was analyzed following transfection with mimics NC, mimics miR-301a-3p and mimics miR-301a-3p + MEOX2. <sup>\*\*</sup>P<0.01. MEOX2, mesenchyme homeobox 2; miR, microRNA; NC, negative control; si, small interfering RNA; Ctrl, control; RT-qPCR, reverse transcription-quantitative PCR.

The overexpression of miR-301a was associated with a poor prognosis in gastric cancer and was found to downregulate RUNX family transcription factor 3 expression levels to promote disease progression (22,23). The role of miR-301a in breast cancer has also been previously investigated. For

example, upregulated expression levels of miR-301a were found to be inversely associated with the prognosis of breast cancer (8,24). The overexpression of miR-301a also promoted breast cancer cell migration and invasion by downregulating the expression levels of PTEN, a tumor suppressor gene (9).

The results of the present study revealed that the expression levels of miR-301a-3p were upregulated in TNBC tissues. MDA-MB-231 cells transfected with miR-301a-3p mimics exhibited increased viability compared with MDA-MB-231 cells transfected with NC mimics. The opposite effects were observed following miR-301a-3p knockdown. Furthermore, miR-301a-3p overexpression promoted MDA-MB-231 cell migration and invasion. These results suggested that miR-301a-3p may act as an oncogene in TNBC.

MEOX2 has been reported to function both as an oncogene and tumor suppressor, depending on the cancer type. For example, single-nucleotide polymorphism-based sequencing studies reported that MEOX2 served as a tumor suppressor gene in Wilms' tumor (25). In another study, MEOX2 expression was upregulated in laryngeal carcinoma and lung cancer tissues (26,27), and the overexpression of MEOX2 promoted laryngeal cancer growth by activating the PI3K/AKT signaling pathway. The upregulated expression levels of MEOX2 were also found to contribute to chemoresistance in lung cancer. In another study, MEOX2 was found to be downregulated by miR-301a in hepatocellular carcinoma (HCC) (28); miR-301a promoted HCC cell proliferation, migration and invasion by downregulating MEOX2 expression. However, to the best of our knowledge, the association between miR-301a-3p and MEOX2 in TNBC remains to be determined. The findings of the present study revealed that overexpression of miR-301a-3p downregulated MEOX2 expression levels in MDA-MB-231 cells. The results of the dual luciferase reporter assay demonstrated that miR-301a-3p directly bound to the 3'-UTR sequence of MEOX2. In addition, the knockdown of MEOX2 expression promoted TNBC cell viability. Further analysis also identified that the expression levels of miR-301a-3p were inversely correlated with those of MEOX2 in breast cancer samples. Thus, it was suggested that the reduction in MEOX2 expression may promote TNBC cell viability.

In conclusion, the results of the present study indicated that miR-301a-3p may serve as an oncogene in TNBC. The overexpression of miR-301a-3p promoted TNBC cell viability and migration, while the knockdown of miR-301a-3p exerted the opposite effects. Furthermore, miR-301a-3p was found to exert its effects in TNBC by downregulating the expression levels of MEOX2. The findings of the present study highlight the importance of miR-301a-3p in TNBC and may indicate a possible target for the treatment of this disease.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

HL and GW designed the current study. HL performed the experiments. GW supervised the present study. HL and GW confirm the authenticity of all the raw data. HL and GW drafted, reviewed and edited the manuscript. All authors read and approved the final version of manuscript.

#### Ethics approval and consent to participate

The protocol of the present study was approved by the Clinical Research Ethics Committee of Beijing Obstetrics and Gynecology Hospital, and written, informed consent was obtained from each patient for the use of their tissue prior to participation.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

1. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J and Cardoso F: Breast cancer. *Nat Rev Dis Primers* 5: 66, 2019.
2. Waks AG and Winer EP: Breast cancer treatment: A review. *JAMA* 321: 288-300, 2019.
3. Huang J, Chan PS, Lok V, Chen X, Ding H, Jin Y, Yuan J, Lao XQ, Zheng ZJ and Wong MC: Global incidence and mortality of breast cancer: A trend analysis. *Aging (Albany NY)* 13: 5748-5803, 2021.
4. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, *et al*: Molecular portraits of human breast tumours. *Nature* 406: 747-752, 2000.
5. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, *et al*: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 98: 10869-10874, 2001.
6. Gregory RI, Chendrimada TP, Cooch N and Shiekhattar R: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123: 631-640, 2005.
7. Mycko MP, Cichalewska M, Machlanska A, Cwiklinska H, Mariasiewicz M and Selmaj KW: MicroRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. *Proc Natl Acad Sci USA* 109: E1248-E1257, 2012.
8. Yu H, Li H, Qian H, Jiao X, Zhu X, Jiang X, Dai G and Huang J: Upregulation of miR-301a correlates with poor prognosis in triple-negative breast cancer. *Med Oncol* 31: 283, 2014.
9. Ma F, Zhang J, Zhong L, Wang L, Liu Y, Wang Y, Peng L and Guo B: Upregulated microRNA-301a in breast cancer promotes tumor metastasis by targeting PTEN and activating Wnt/ $\beta$ -catenin signaling. *Gene* 535: 191-197, 2014.
10. Li X, Li J, Cai Y, Peng S, Wang J, Xiao Z, Wang Y, Tao Y, Li J, Leng Q, *et al*: Hyperglycaemia-induced miR-301a promotes cell proliferation by repressing p21 and Smad4 in prostate cancer. *Cancer Lett* 418: 211-220, 2018.
11. Wang YG, Wang T, Shi M and Zhai B: Long noncoding RNA EPB41L4A-AS2 inhibits hepatocellular carcinoma development by sponging miR-301a-5p and targeting FOXL1. *J Exp Clin Cancer Res* 38: 153, 2019.
12. Li X, Zhong M, Wang J, Wang L, Lin Z, Cao Z, Huang Z, Zhang F, Li Y, Liu M and Ma X: miR-301a promotes lung tumorigenesis by sponging Runx3. *Mol Cancer* 18: 99, 2019.
13. Hu H, Zhang Q, Chen W, Wu T, Liu S, Li X, Luo B, Zhang T, Yan G, Lu H and Lu Z: MicroRNA-301a promotes pancreatic cancer invasion and metastasis through the JAK/STAT3 signaling pathway by targeting SOCS5. *Carcinogenesis* 41: 502-514, 2020.

14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
15. von Minckwitz G, Huang CS, Mano MS, Loibl S, Mamounas EP, Untch M, Wolmark N, Rastogi P, Schneeweiss A, Redondo A, *et al*: Trastuzumab emtansine for residual invasive HER2-positive breast cancer. *N Engl J Med* 380: 617-628, 2019.
16. von Minckwitz G, Procter M, de Azambuja E, Zardavas D, Benyunes M, Viale G, Suter T, Arahmani A, Rouchet N, Clark E, *et al*: Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer. *N Engl J Med* 377: 122-131, 2017.
17. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, Mackey J, Glaspy J, Chan A, Pawlicki M, *et al*: Adjuvant trastuzumab in HER2-positive breast cancer. *N Engl J Med* 365: 1273-1283, 2011.
18. Chen Z, Chen LY, Dai HY, Wang P, Gao S and Wang K: miR-301a promotes pancreatic cancer cell proliferation by directly inhibiting Bim expression. *J Cell Biochem* 113: 3229-3235, 2012.
19. Xia X, Zhang K, Luo G, Cen G, Cao J, Huang K and Qiu Z: Downregulation of miR-301a-3p sensitizes pancreatic cancer cells to gemcitabine treatment via PTEN. *Am J Transl Res* 9: 1886-1895, 2017.
20. Fang Y, Sun B, Xiang J and Chen Z: MiR-301a promotes colorectal cancer cell growth and invasion by directly targeting SOCS6. *Cell Physiol Biochem* 35: 227-236, 2015.
21. Zhang W, Zhang T, Jin R, Zhao H, Hu J, Feng B, Zang L, Zheng M and Wang M: MicroRNA-301a promotes migration and invasion by targeting TGFBR2 in human colorectal cancer. *J Exp Clin Cancer Res* 33: 113, 2014.
22. Wang M, Li C, Yu B, Su L, Li J, Ju J, Yu Y, Gu Q, Zhu Z and Liu B: Overexpressed miR-301a promotes cell proliferation and invasion by targeting RUNX3 in gastric cancer. *J Gastroenterol* 48: 1023-1033, 2013.
23. Xu XD, He XJ, Tao HQ, Zhang W, Wang YY, Ye ZY and Zhao ZS: Abnormal expression of miR-301a in gastric cancer associated with progression and poor prognosis. *J Surg Oncol* 108: 197-202, 2013.
24. Zheng JZ, Huang YN, Yao L, Liu YR, Liu S, Hu X, Liu ZB and Shao ZM: Elevated miR-301a expression indicates a poor prognosis for breast cancer patients. *Sci Rep* 8: 2225, 2018.
25. Ohshima J, Haruta M, Arai Y, Kasai F, Fujiwara Y, Ariga T, Okita H, Fukuzawa M, Hata J, Horie H and Kaneko Y: Two candidate tumor suppressor genes, MEOX2 and SOSTDC1, identified in a 7p21 homozygous deletion region in a Wilms tumor. *Genes Chromosomes Cancer* 48: 1037-1050, 2009.
26. Tian L, Tao Z, Ye H, Li G, Zhan Z and Tuo H: Over-expression of MEOX2 promotes apoptosis through inhibiting the PI3K/Akt pathway in laryngeal cancer cells. *Neoplasma* 65: 745-752, 2018.
27. Ávila-Moreno F, Armas-López L, Álvarez-Moran A, López-Bujanda Z, Ortiz-Quintero B, Hidalgo-Miranda A, Urrea-Ramírez F, Rivera-Rosales RM, Vázquez-Manríquez E, Peña-Mirabal E, *et al*: Overexpression of MEOX2 and TWIST1 is associated with H3K27me3 levels and determines lung cancer chemoresistance and prognosis. *PLoS One* 9: e114104, 2014.
28. Zhou P, Jiang W, Wu L, Chang R, Wu K and Wang Z: miR-301a is a candidate oncogene that targets the homeobox gene Gax in human hepatocellular carcinoma. *Dig Dis Sci* 57: 1171-1180, 2012.



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