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Original Article

Circ_0000190 inhibits the progression of triple negative breast cancer by regulating miR-301a/MEOX2 pathway

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Abstract: Circular RNA (circRNA) and microRNA (miRNA) play critical roles in regulating proliferation, apoptosis, and invasion in triple-negative breast cancer (TNBC) cells. To investigate their functional significance, we employed quantitative real-time PCR (qRT-PCR) to assess the differential expression of circ_0000190, miR-301a, and mesenchyme homeobox 2 (MEOX2) between TNBC cell lines and normal breast epithelial cells. Subsequently, we established overexpression and knockdown systems for these molecules to examine their effects on TNBC cell proliferation, apoptosis, migration, invasion, and epithelial-mesenchymal transition (EMT). Additionally, we evaluated the impact of circ_0000190 overexpression on tumor growth using a mouse xenograft model, measuring tumor volume and weight. Our findings revealed that circ_0000190 and MEOX2 expression were significantly downregulated ($P<0.05$) in TNBC cells compared to normal breast epithelial cells, whereas miR-301a was upregulated ($P<0.05$). Knockdown of circ_0000190 promoted TNBC cell proliferation, migration, invasion, and EMT, while suppressing apoptosis. Mechanistically, circ_0000190 functioned as a molecular sponge for miR-301a, and its overexpression significantly inhibited miR-301a expression ($P<0.001$). Notably, miR-301a mimics partially reversed the suppressive effects of circ_0000190 overexpression on proliferation, migration, invasion, and EMT, as well as its pro-apoptotic effects ($P<0.001$). Furthermore, we identified MEOX2 as a direct target of miR-301a. MEOX2 knockdown attenuated the inhibitory effects of miR-301a silencing on proliferation, migration, invasion, and EMT, while also counteracting its pro-apoptotic function. In vivo experiments demonstrated that circ_0000190 overexpression significantly reduced tumor volume and weight ($P<0.001$), concomitant with elevated MEOX2 mRNA and protein levels ($P<0.001$) and decreased miR-301a expression ($P<0.001$). In conclusion, our study elucidates that circ_0000190 suppresses TNBC progression by downregulating miR-301a and upregulating MEOX2, forming a competitive endogenous RNA (ceRNA) network of circRNA-miRNA-mRNA.

Keywords: Triple-negative breast cancer, circ_0000190, miR-301a, MEOX2

Introduction

Breast cancer remains the most prevalent malignancy among women worldwide, with approximately 2.9 million new cases reported in 2022, accounting for 31% of all female cancers [1]. Triple-negative breast cancer (TNBC), which constitutes 10-20% of breast cancer cases, is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) overexpression [2]. Clinically, TNBC exhibits higher recurrence rates, greater metastatic potential, and poorer prognosis compared to other subtypes [3]. Due to the lack of effective targeted or endocrine therapies, TNBC patients face limited treatment options, underscoring the urgent need to identify novel therapeutic targets [4].

Emerging biomarkers, including tumor-associated macrophages (TAMs), microRNAs (miRNAs), and long non-coding RNAs (lncRNAs), have demonstrated diagnostic, prognostic, and therapeutic potential in cancer research [5]. Among these, miRNAs-small (~19-25 nt) non-coding RNAs - play pivotal roles in post-transcriptional gene regulation, influencing tumorigenesis by modulating apoptosis, proliferation, and metastasis [6]. Dysregulated miRNAs contribute to TNBC progression by altering key signaling pathways. For instance, miR-29b-3p is significantly upregulated in MDA-MB-231 cells compared to MCF-10A, and its inhibition reduc-

es cell viability, migration, and invasion by targeting TRAF3 and suppressing NF-κB signaling [7].

In recent years, circular RNAs (circRNAs) have gained attention as stable, abundant, and conserved non-coding RNAs with diverse regulatory functions in cancer, including miRNA sponging, RNA-binding protein (RBP) interactions, and transcriptional regulation [8-10]. Many circRNAs contain multiple miRNA-binding sites, enabling them to competitively sequester miRNAs and relieve their repression on target genes [11, 12]. For example, circNR3C2 (hsa_circ_0071127) is downregulated in TNBC and suppresses tumor progression by sponging miR-513a-3p, thereby stabilizing the E3 ubiquitin ligase HRD1 (SYVN1) [13].

Although circ_0000190 has been implicated in various cancers, its role in TNBC remains unexplored [14, 15]. Our preliminary data revealed low circ_0000190 expression in TNBC cells and predicted its interaction with miR-301a. This study investigates the mechanistic roles of circ_0000190 and miR-301a in TNBC pathogenesis, providing novel insights for potential therapeutic strategies.

Materials and methods

Cell culture

MCF-10A (human normal mammary cell line), BT549, SUM-159, MDA-MB-231, MDA-MB-453 and MDA-MB-468 were purchased from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin streptomycin (FBS, Gibco) added in a constant temperature incubator at 37°C and 5% CO_2.

Cell transfection

Potential targets for circ_0000190 were predicted by circbank (http://www.circbank.cn/) and starbase. The potential binding targets of miR-301a were predicted by targetscan and miRDB. TNBC cells were transfected with circ_0000190 overexpressed plasmid targeting circ_0000190 (circRNA sh#1 and/circRNA sh#2). The pcDNA3.1 (+) Vector (circRNA) and/or negative control (Vector) for overexpressing circ_0000190 were purchased from Sigma-Aldrich (St Louis, MO, USA). Set the group into no-load group (Vector), circ_0000190 overexpressed group, sh-circ_0000190 group, and Scramble group. circ_0000190 siRNA1: 5'-ACCAAAGCAUCUAGUGUUU-3', circ_0000190 siRNA2: 5'-UUCUCCGAACGUGUCACGU-3'; NC: 5'-UUCUCCGAACGUGUCACGUTT-3'. And short hairpin RNAs (shRNA) for circ_0000190 were synthesized by GenePharma Co., Ltd. (Shanghai, China).

Interference sequences targeting MEOX2 (MEOX2 sh#1 and/MEOX2 sh#2) and their corresponding negative control (Scramble) plasmids were transfected into breast cancer cells. Human breast cancer cells were inoculated into a 6-well plate. When the cell synthesis rate was about 70%-80%, the plasmid was transfected with lipo3000. Opti MEM, Lipo3000 and the plasmid were evenly mixed and placed at room temperature for 10-20 minutes. Plasmids were evenly added to the cells according to the experimental groups, and incubated at 37°C and 5% CO_2 for 48 hours. MEOX2 siRNA1: 5'-AAGGUAGGACAUGUGGUCAGAUCUU-3', MEOX2 siRNA2: 5'-GCUCUGAGCAUGCACACUU-3'; NC: 5'-AAUUAGCGGAGGCUAUAAGAUGUUC-3'. And short hairpin RNAs (shRNA) for MEOX2 were synthesized by GenePharma Co., Ltd. (Shanghai, China).

Transient transfection of miR-301a mimics and inhibitors into TNBC cells was performed, and the cells were divided into miR-NC group, miR-301a mimics group, inhibitor NC group, and miR-301a inhibitor group. miR-301a mimics: 5'-GCUCUGACUUUAUUGCACUACU-3'; mimic NC: 5'-UCACAACCUCCUAGAAAGAGUAGA-3'; miR-301a inhibitor: 5'-AGUAGUGCAAUAAAGUCAGAGC-3'; inhibitor NC: 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'. The miR-301a mimics, miR-301a inhibitor, NC inhibitor and NC mimics were procured from GenePharma (Shanghai, China). The cells were inoculated on 6-well plates and transfected with miR-301a mimics or miR-301a inhibitors using lipo3000 when the cell combination rate was 70%-80%. The dosage of each component of transfection was as follows: Opti-MEM 250 μL, lipo3000 15 μL, miRNA (20 μM) 10 μL, which was placed at room temperature for 15 min, mixed and added into cells, and incubated in a constant temperature incubator for 48 h.

Table 1. Primer sequences

Primer name	Primer sequence
circ_0000190 upstream primer	5'-GCCGAGTGGTAACATGGGAG-3'
circ_0000190 downstream primer	5'-AGCAGAGCAAGTGGAAACCA-3'
miR-301a upstream primer	5'-CGTGCGAAGCTCAGGAGGG-3'
miR-301a downstream primer	5'-TGGCTGTCGTGGACTGCG-3'
MEOX2 upstream primer	5'-TCAGAAGTCAACAGCAAACCCAG-3'
MEOX2 downstream primer	5'-TTCACCAGTTCCTTTTCCCGAG-3'
E-cadherin upstream primer	5'-CTGTTTCTGGTTTCTGTTGG-3'
E-cadherin downstream primer	5'-CCTTCTCCGTAT TCTCCTCCCT-3'
N-cadherin upstream primer	5'-CTATGAGTGGAACAGGAACG-3'
N-cadherin downstream primer	5'-CAATGTCATAATCAAGTGCTGTA-3'
Vimentin upstream primer	5'-AGGCAAAGCAGGAGTCCACTGA-3'
Vimentin downstream primer	5'-ATCTGGGCGTTCCAGGGACTCAT-3'
GAPDH upstream primer	5'-TGTTCGTCATGGGTGTGAAC-3'
GAPDH downstream primer	5'-ATGGCATGGACTGTGGTCAT-3'
U6 upstream primer	5'-CTCGCTTCGGCAGCACA-3'
U6 downstream primer	5'-AACGCTTCACGAATTTGCGT-3'

MEOX2: mesenchyme homeobox 2. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

RNA pull down

Biotinylation probes for circ_0000190 (Bio-circ_00190) and Bio-NC were constructed by GenePharma. TNBC cells (1×10^7) were lysed, and cell lysates were co-cultured with M-280 streptavidin beads and biotinated probes. The relative enrichment of miRNA was detected by qRT-PCR.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA samples were extracted from tissues and cells with the TRIzol reagent (Invitrogen). The total RNA was added to RNase-free water until completely dissolved, and the concentration and purity of total RNA were detected by Nanodrop 2000 spectrophotometer. Complementary DNA (cDNA) of circ_0000190 and MEOX2 was synthesized from 1 μg of RNA using the Bio-Rad iScript kit (Bio-Rad), and PCR was carried out with iQSYBR Green SuperMix (Bio-Rad, USA) using cDNA as template DNA. It was prepared according to the following system: SYBR premix ex taq 6.0 μL, primer mix (5 μM) 0.3 μL, template 0.6 μL, and RNase-Free H_2O supplemented to 10 μL. Amplification program: Pre denature at 94°C for 5 minutes, denature at 94°C for 15 seconds, anneal at 60°C for 30 seconds, extend at 68°C for 20 seconds, perform 35 cycles, and set up 3 wells for each experiment. After the reaction, the relative expression levels of circ_0000190, E-cadherin, N-cadherin, and Vimentin were calculated using $2^{-\Delta\Delta Ct}$ based on the internal reference GAPDH, and the relative expression level of miR-301a was calculated based on the internal reference U6. The primers are shown in **Table 1**.

Cell counting kit (CCK)-8 assay

Breast cancer cells were inoculated into 96-well plates at a density of 2×10^4 cells/mL, each well was 200 μL, and each group of cells was provided with 6 multiple pores. After 10 μL CCK-8 reagent was added and the transfected breast cancer cells were incubated in a constant temperature incubator containing 5% CO_2 at 37°C for 1.5 h, the absorbance of each hole at 450 nm was detected on an enzyme marker, and the proliferation curve was calculated.

Ethynyl-2'-deoxyuridine (EdU) staining

Different group of TNBC cells, at a density of 1×10^5 cells/well, were washed and then fixed with 4% paraformaldehyde for 10 min. Afterwards, 20 μM EdU reagent was added to the culture medium for treating TNBC cells. Then, cells were kept from lights at room temperature. Add 300 μL Triton X-100/PBS permeable membrane and react at room temperature for 20 min. After washing with BSA/PBS for 2 times, 4', 6-diamino-2-phenylindole (DAPI) was added to the cell nucleus and the reaction was carried out at room temperature for 30 min.

Flow cytometry

The apoptosis of different groups of TNBC cells was detected by flow cytometry analysis. Briefly, at room temperature, after suspension in 500 μl of flow cytometry binding buffer, transfected TNBC cells were labeled with the same volume of annexin V/fluorescein isothiocyanate (FITC) or propidium iodide (PI) in the dark for 20 min. Signals were detected and then analyzed.

Cell migration measurement

The migration ability of TNBC cells in different groups was detected by cell scratch test. Markers were drawn at the bottom of the 6-well plate using a marker and a ruler, parallel to the long axis. The breast cancer cells of each group in logarithmic stage were inoculated into a 6-well plate according to 5×10^5, and 3 multiple Wells were set in each group. After incubating for 24 hours, use the gun head to create a scratch in a direction perpendicular to the drawn horizontal line. The culture was continued in serum-free medium for 24 h, washed twice with PBS, and observed under an inverted microscope. The scratch width was calculated using Image J software.

Transwell-invasion assay

Cell invasion ability was tested by transwell assays with the application of transwell chambers (Corning, NY, USA). TNBC cells were plated into the top compartment with free serum medium, while the medium blended with 10% FBS was put in the lower compartment. Moreover, Matrigel (Millipore, Billerica, MA, USA) was pre-coated on the upper chamber before the plating of cells. After 48-h incubation, cells were fixed by methanol and then dyed with crystal violet. Cells invaded to the bottom chamber were observed via a microscope (Olympus, Japan).

Western blotting

Transfected TNBC cells were lysed in 100 μl of lysis buffer for 20 min on ice. After concentration detection, protein was loaded onto 10-15% sodium dodecyl sulfate gel and fractionated onto polyvinylidene difluoride membranes (Burlington, MA, USA). The membranes were then washed with Tris-buffered saline-Tween 20 and, after blocking with skim milk, the membranes were incubated with primary antibodies. The primary antibody used was rabbit monoclonal antibody (Abcam, Cambridge, UK) and the membrane was transferred to the E-cadherin (1:300), N-cadherin (1:300), Vimentin (1:300), MEOX2 (1:300), GAPDH (1:500) primary antibody working solution and incubated at room temperature for 2 hours. The secondary antibody was a sheep anti rabbit IgG antibody (1:2000) labeled with horseradish peroxidase (HRP) and incubated at room temperature for 1 hour. After removing the membrane, rinse it with TBST, add ECL color solution, and place it in an ECL luminescent instrument for gray-scale analysis using the Image J system (NIH, Bethesda, MD, USA).

Dual-luciferase reporter assay

The fragment of circ_0000190 or MEOX2 3'UTR harboring the wild-type binding sites with miR-301a was amplified and cloned to pmiR-GLO vector (Promega, Wisconsin, USA), termed as circ_0000190-WT or MEOX2-WT. The segments were constructed into pmiRGLO vectors and named circ_00000190-WT and Circ_00-000190-MUT, MEOX2-WT and MEOX2-MUT, respectively. The plasmid (50 ng) was transfected into breast cancer cells using lipo3000, and then co-transfected with miR-301a mimics (20 nM) or miR-NC. After transfection for 48 h, the signal was detected, and the luciferase activity of different groups was detected by dual luciferase reporter gene assay system.

Xenograft tumor assay

BALB/c nude mice were purchased from Orient Bio company, and 12 BALB/c nude mice aged 8-10 weeks were randomly divided into two groups (n=6/group): (1) Circ_0000190 overexpression group: Mice received subcutaneous injections of MDA-MB-468 cells stably transfected with circ_0000190 overexpression vector (200 μL PBS suspension); (2) Empty vector control group: Mice received equivalent injections of MDA-MB-468 cells transfected with empty vector.

Record tumor volume starting one week after injection, with measurements taken every three days for a total observation period of 28 days. 28 days after injection, animals were euthanized by cervical dislocation method, tumor specimens were collected, and the size of transplanted tumors was compared and photographed. qRT-PCR was used to detect the expression of circ_0000190, miR-301a and MEOX2 mRNA, and the protein expression of MEOX2 was detected by Western blot. All behaviors comply with the International Convention on the Ethics of Laboratory Animals, relevant national regulations, animals comply with Chinese animal welfare legislation, and animal research complies with ARRIVE guidelines.

Statistical analysis

The data in this research were analyzed via GraphPad Prism 8.0 (La Jolla, CA, USA) and manifested as mean ± standard deviation (SD). Parametric statistics was done via utilizing student's t-test or one-way/two-way analysis of variance (ANOVA). Above experiments were conducted at least three times. $P<0.05$ was set as the threshold for statistical significance.

Results

Circ_0000190 inhibits the proliferation of TNBC cells and induces cell apoptosis

The level of hsa_circ_0000190 in breast cancer cell lines (BT-549, SUM-159, MDA-MB-231, MDA-MB-453, MDA-MB-468) was significantly lower than that in normal breast cell lines ($P<0.05$) (**Figure 1A**). We selected SUM-159 and MDA-MB-468 cells for further experiments. Circ_0000190 was knocked down and overexpressed in SUM-159 and MDA-MB-468 cell lines, respectively, and the knockdown effect of circRNAsh2# was better in SUM-159 cells (**Figure 1B**), so circRNAsh2# was selected for subsequent experiments. Knockdown of circ_0000190 promoted the proliferation of TNBC cells, while overexpression of circ_0000190 inhibited the proliferation of TNBC cells (**Figure 1C**, **1D**). The apoptosis rate of TNBC cells decreased after knockdown of circ_0000190. However, overexpression of circ_0000190 increased the apoptosis rate of TNBC cells ($P<0.05$) (**Figure 1E**).

Circ_0000190 inhibits TNBC cell migration, invasion and epithelial mesenchymal transformation (EMT)

Knockdown of circ_0000190 promoted the migration ability of TNBC cells, while overexpression of circ_0000190 inhibited the migration ability (**Figure 2A**). Knockdown of circ_0000190 promoted the invasion ability of TNBC cells, while overexpression of circ_0000190 inhibited the invasion ability of TNBC cells (**Figure 2B**). After knockdown circ_0000190, the expression of E-cadherin mRNA and protein in TNBC cells was significantly decreased, while the expression of N-cadherin and Vimentin mRNA and protein was significantly increased ($P<0.05$) (**Figure 2C**, **2D**). The results showed that knockdown of circ_0000190 promoted EMT in TNBC cells, while overexpression of circ_0000190 inhibited EMT in TNBC cells.

Circ_0000190 targets miR-301a and negatively regulates miR-301a expression

circbank and starbase were used to predict the miRNAs that could be targeted by circ_0000190. The results showed that circbank and starbase predicted a total of 12 miRNAs that could be targeted by circ_0000190. They are miR-1252-5p, miR-130a-3p, miR-130b-3p, miR-135a-5p, miR-135b-5p, miR-148a-3p, miR-148b-3p, miR-152-3p, miR-301a, miR-301b, and miR-4295, miR-6820-3p (**Figure 3A**). The abundance of circ_0000190 targeted miRNA in SUM-159 and MDA-MB-468 cell lines was investigated by RNA pull down assay. The results showed that the abundance of miR-301a was highest in the RNA complex pulled down by the circ_0000190 probe ($P<0.001$) (**Figure 3B**). Luciferase reporter gene assay confirmed the validity of circ_0000190 binding site to miR-301a. miR-301a mimics significantly reduced the luciferase activity of circ_0000190-WT ($P<0.001$), but had little effect on the luciferase activity of circ_0000190-MUT (**Figure 3C**). TCGA data showed that miR-301a was highly expressed in breast cancer susceptibility gene (BRCA). The qRT-PCR results showed that compared with the MCF-10A cell line, miR-301a was upregulated in the TNBC cell line ($P<0.05$) (**Figure 3D**, **3E**). Knockdown of circ_0000190 promoted the expression of miR-301a in TNBC cells, while overexpression of circ_0000190 inhibited the expression of miR-301a in TNBC cells (**Figure 3F**, **3G**).

Circ_0000190 inhibits TNBC cell proliferation by down-regulating miR-301a

Overexpression of circ_0000190 inhibited the expression of miR-301a, while miR-301a mimics promoted the expression of miR-301a (**Figure 4A**). Moreover, the overexpression of miR-301a partially reversed the inhibitory effect of circ_0000190 overexpression on cell proliferation ($P<0.01$) (**Figure 4B**, **4C**). In addition, overexpression of miR-301a partially reversed the promoting effect of circ_0000190 overexpression on apoptosis of TNBC cells ($P<0.01$) (**Figure 4D**).

Circ_0000190 inhibits TNBC migration, invasion and EMT process by down-regulating miR-301a

Cell scratch experiments showed that miR-301a overexpression partially reversed the in-

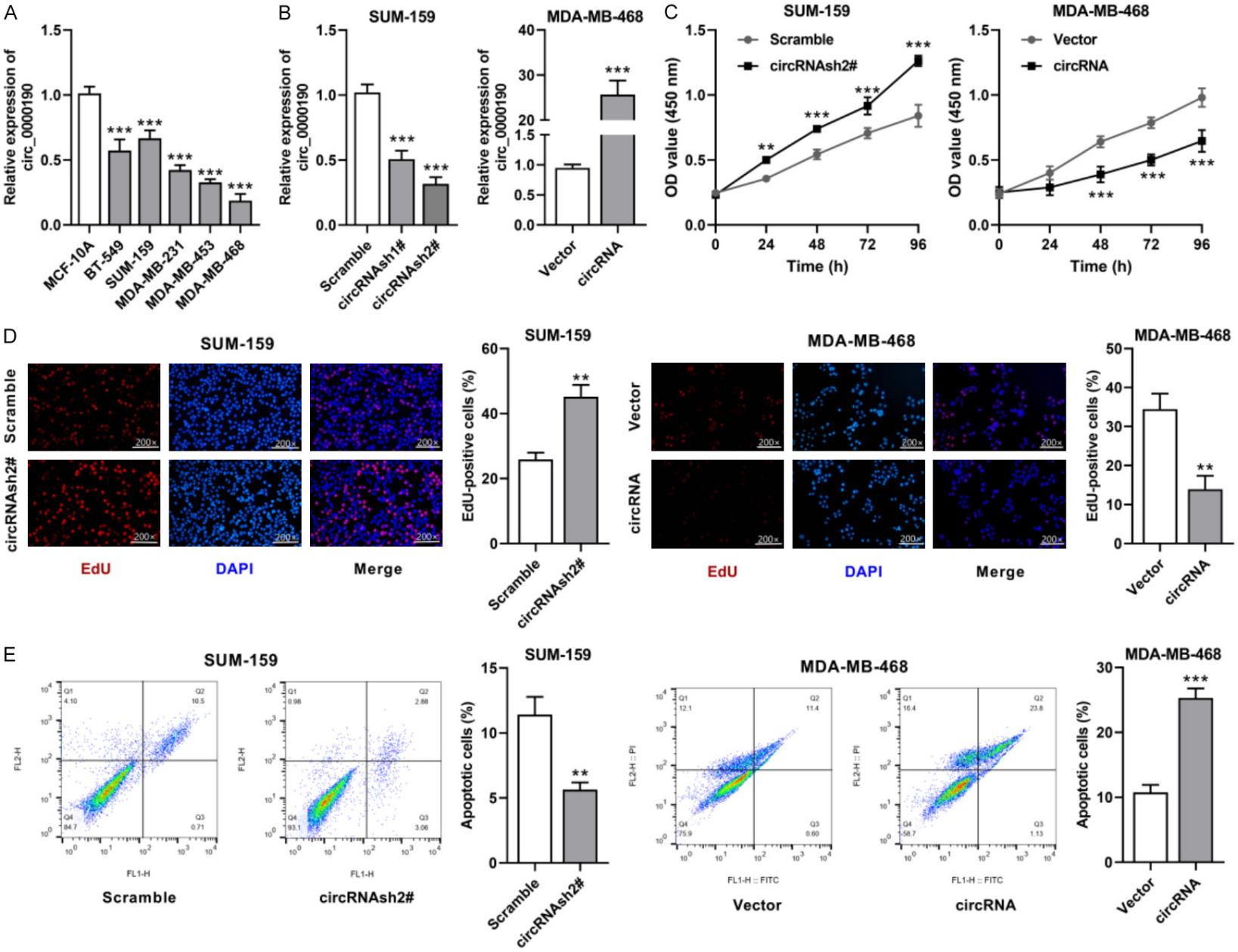
A
Relative expression of circ_0000190
MCF-10A
BT-549
SUM-159
MDA-MB-231
MDA-MB-453
MDA-MB-468
B
SUM-159
Scramble
circRNAsh1#
circRNAsh2#
MDA-MB-468
Vector
circRNA
C
SUM-159
Scramble
circRNAsh2#
OD value (450 nm)
Time (h)
MDA-MB-468
Vector
circRNA
D
SUM-159
Scramble
circRNAsh2#
EdU
DAPI
Merge
EdU-positive cells (%)
MDA-MB-468
Vector
circRNA
E
SUM-159
Scramble
circRNAsh2#
Apoptotic cells (%)
MDA-MB-468
Vector
circRNA

Figure 1. Expression of circ_0000190 in TNBC and its effect on TNBC cell proliferation and apoptosis. A: Expression of circ_0000190 in TNBC cell line and normal breast epithelial cell line. MCF-10A is a normal breast epithelial cell, BT-549, SUM-159, MDA-MB-231, MDA-MB-453, and MDA-MB-468 are TNBC cell lines. B: circ_0000190 knockdown and overexpression results. circRNAsh was knockdown for circ_0000190, vector was empty, and circRNA was overexpressed for circ_0000190. C, D: Effect of circ_0000190 on cell proliferation. circRNAsh#2 is the circ_0000190 knockdown group, vector is the no-load group, and circRNA is the circ_0000190 overexpression group. E: Effect of circ_0000190 on apoptosis. circRNAsh#2 is the circ_0000190 knockdown group, vector is the no-load group, and circRNA is the circ_0000190 overexpression group. *P<0.05, **P<0.01, ***P<0.001. TNBC: triple negative breast cancer.

hibitory effect of circ_0000190 overexpression on cell migration (**Figure 5A**). Transwell experiment showed that circ_0000190 could inhibit cell invasion in SUM-159 and MDA-MB-468 cells (P<0.001). However, miR-301a mimics partially reversed the inhibitory effect of circ_0000190 overexpression on cell invasion (P<0.001) (**Figure 5B**). Overexpression of circ_0000190 promoted the expression of EMT-related molecules E-cadherin mRNA and protein, and inhibited the expression of N-cadherin and Vimentin mRNA and protein (P<0.001). However, miR-301a mimics partially reversed the inhibitory effect of circ_0000190 overexpression on EMT (P<0.001) (**Figure 5C**, **5D**).

MEOX2 acts as an endogenous competitive RNA of miR-301a

MEOX2 was predicted as a possible target of miR-301a by targetscan and miRDB, and the validity of the binding site was verified by double luciferase reporter gene assay. miR-301a mimics significantly decreased the luciferase activity of MEOX2-WT in SUM-159 and MDA-MB-468 cells (P<0.001), but had little effect on the luciferase activity of MEOX2-MUT (**Figure 6A**). TCGA data showed that MEOX2 expression was decreased in BRCA. In addition, the expression of MEOX2 mRNA and protein was decreased in TNBC cell line compared with MCF-10A cell line (**Figure 6B**, **6C**). In SUM-159 and MDA-MB-468 cells, the expression of MEOX2 mRNA and protein in miR-301a mimics group was significantly decreased compared with that in miR-NC group (P<0.01). Moreover, MEOX2 mRNA and protein expressions of miR-301a inhibitor group were significantly increased compared with inhibitor NC group (P<0.01) (**Figure 6D**, **6E**). In addition, circ_0000190 overexpression promoted up-regulation of ME-0X2 mRNA and protein expression in SUM-159 and MDA-MB-468 cells (P<0.001). Howev-er, miR-301a mimics transfected cells could partially inhibit the promotion effect of circ_0000190 overexpression on MEOX2 expression (P<0.001) (**Figure 6F**, **6G**).

Knockdown of miR-301a inhibits the proliferation of TNBC cells by up-regulating MEOX2 expression

In the SUM-159 and MDA-MB-468 cell lines, the MEOX2 mRNA and protein expressions in the MEOX2 sh1# and MEOX2 sh2# groups were significantly down-regulated compared with the Scramble group (P<0.01), and the knock-out effect of MEOX2sh1# was better. Therefore, MEOX2sh1# was selected for the follow-up experiment (**Figure 7A**, **7B**). In TNBC cell line, compared with control group, MEOX2 mRNA and protein expression of miR-301a inhibitor group was significantly up-regulated (P<0.001). After further transfection with MEOX2sh1#, MEOX2 expression was inhibited (P<0.001) (**Figure 7C**, **7D**). Compared with the control group, the cell proliferation rate of miR-301a inhibitor group was significantly decreased (P< 0.001). However, compared with miR-301a inhibitor group, the cell proliferation rate of miR-301a inhibitor + MEOX2 sh1# group was significantly increased. Therefore, MEOX2 knockdown partially reversed the inhibitory effect of miR-301a knockdown on cell proliferation (**Figure 7E**, **7F**). Compared with the control group, the apoptosis rate of miR-301a inhibitor group was significantly increased (P<0.001). However, compared with miR-301a inhibitor group, the apoptosis rate of miR-301a inhibitor + MEOX2 sh1# group was significantly decreased (P< 0.01). Therefore, MEOX2 knockdown partially reversed the promoting effect of miR-301a knockdown on apoptosis (**Figure 7G**).

Inhibition of miR-301a inhibits TNBC cell migration, invasion and EMT process by upregulating MEOX2 expression

The results of cell scratch healing experiment showed that compared with miR-301a inhibitor group, the cell migration rate of miR-301a

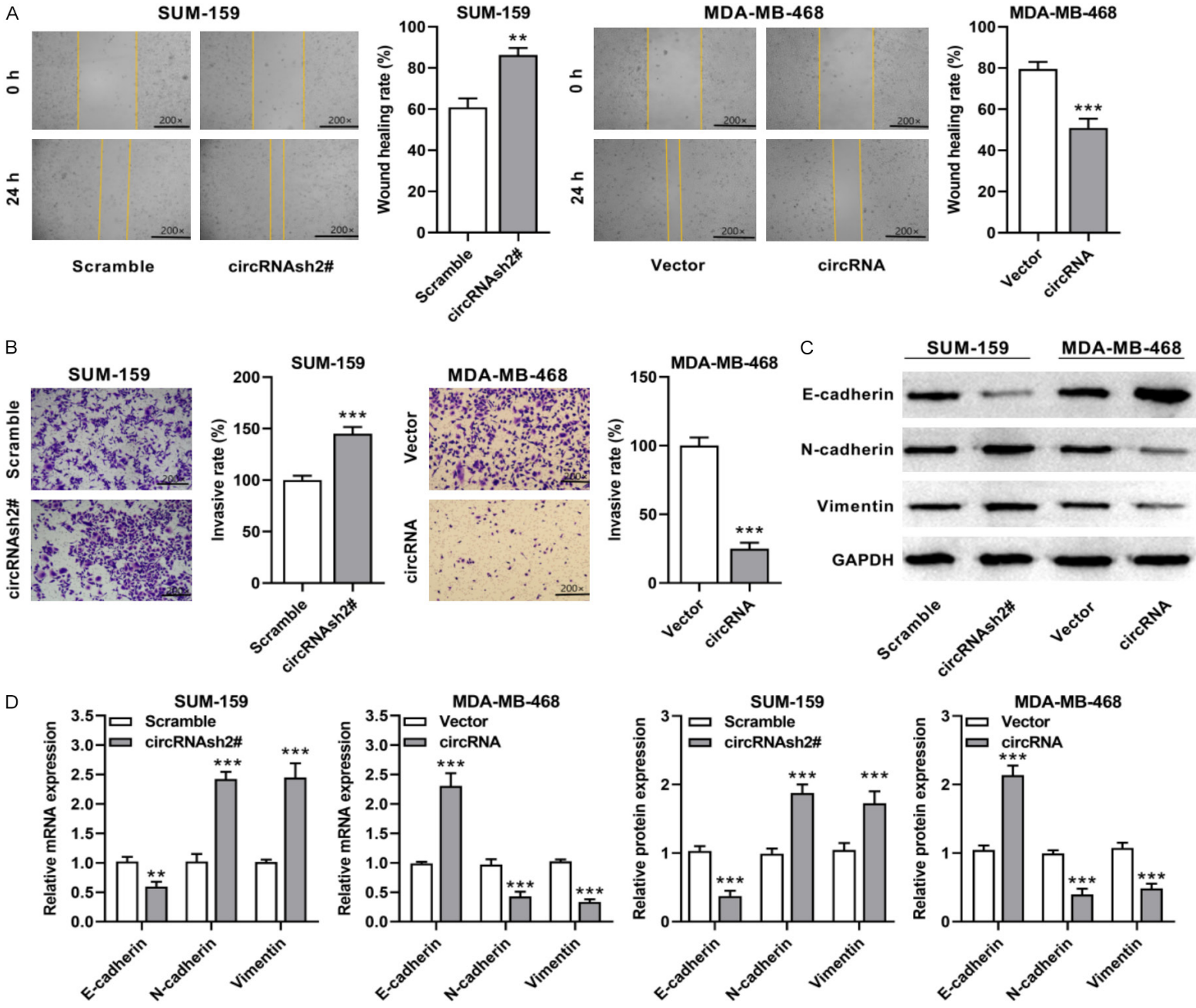
A
SUM-159
0 h
24 h
Scramble
circRNAsh2#
Wound healing rate (%)
MDA-MB-468
Vector
circRNA
B
Invasive rate (%)
C
E-cadherin
N-cadherin
Vimentin
GAPDH
D
Relative mRNA expression
Relative protein expression

Figure 2. The effect of circ_0000190 on the migration, invasion, and EMT of TNBC cells. A: Effect of circ_0000190 on cell migration. B: Effect of circ_0000190 on cell invasion. C, D: Effect of circ_0000190 on EMT of cells. circRNAsh#2 is the circ_0000190 knockdown group, vector is the no-load group, and circRNA is the circ_0000190 overexpression group. *P<0.05, **P<0.01, ***P<0.001. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

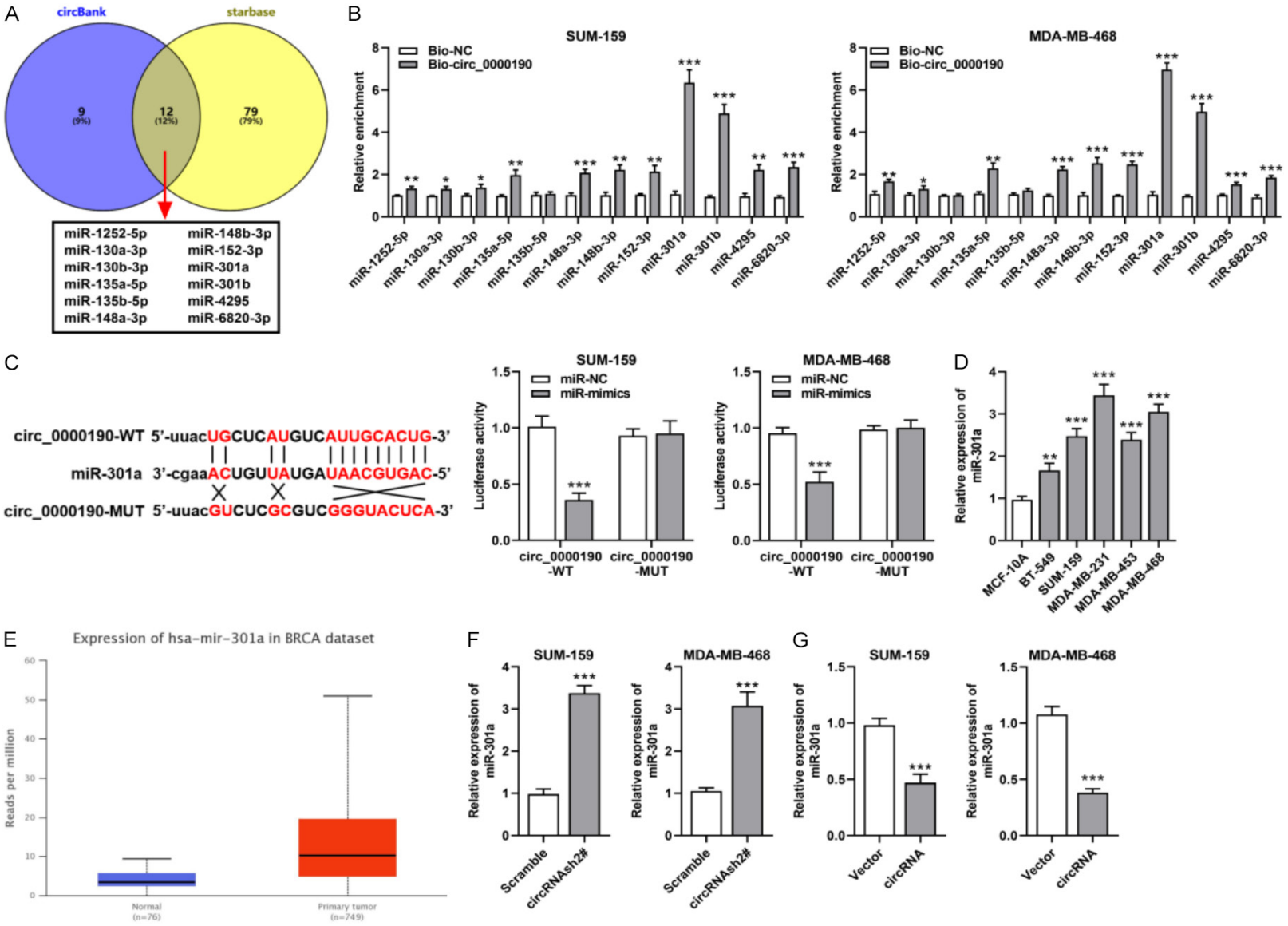

Figure 3. Circ_0000190 binds and negatively regulates the level of miR-301a. A: Prediction of mirnas targeted by circbank and starbase for circ_0000190. B: RNA pull down verification of circ_0000190 target abundance. C: Dual luciferase report assay to verify the binding site of circ_0000190 and miR-301a. D, E: Expression of miR-301a in breast cancer cell lines. F, G: Effect of circ_0000190 on expression of miR-301a in breast cancer cells. circRNAsh#2 is the circ_0000190 knockdown group, vector is the no-load group, and circRNA is the circ_0000190 overexpression group. *P<0.05, **P<0.01, ***P<0.001.

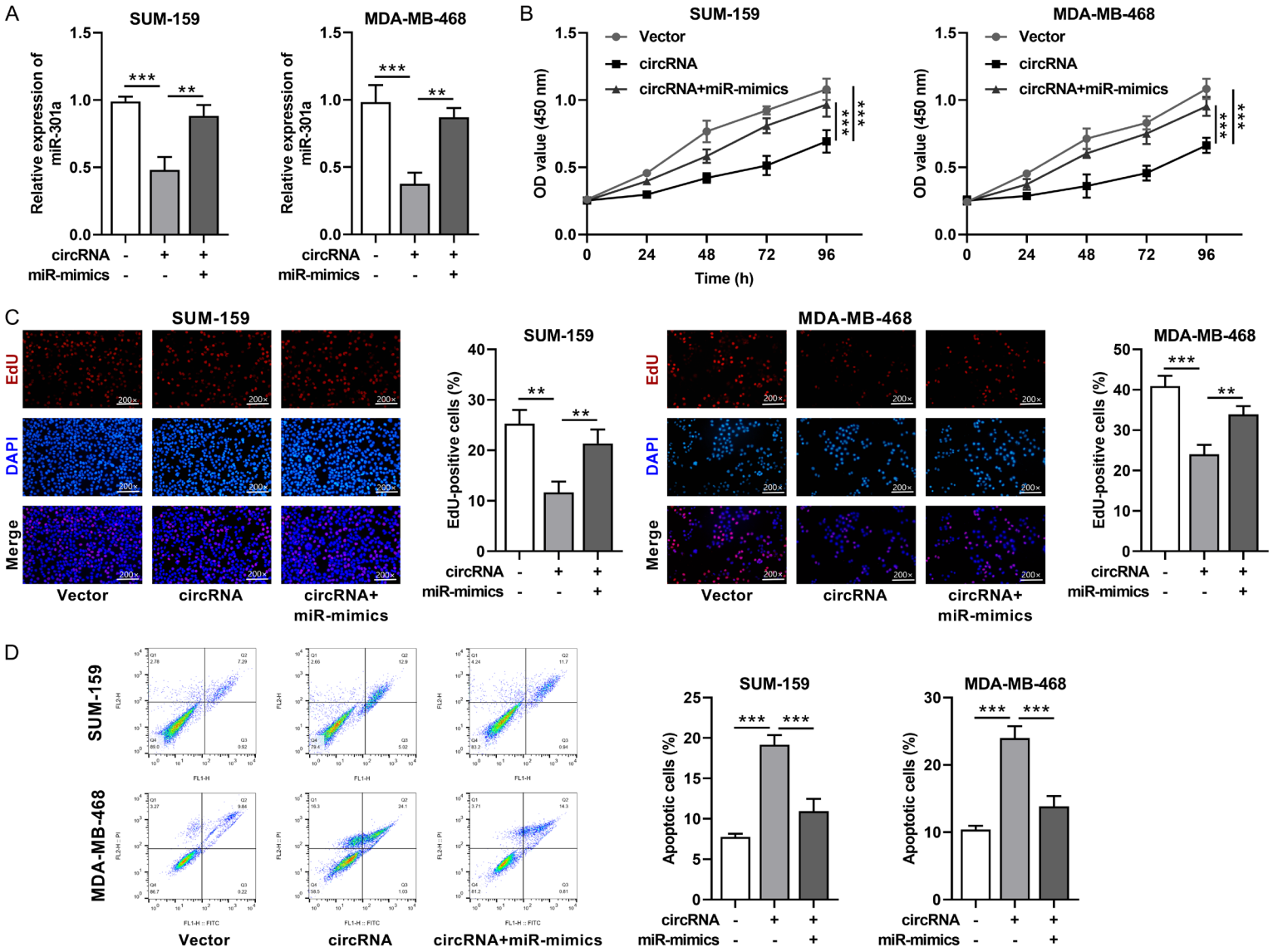

Figure 4. Circ_0000190 inhibits TNBC cell proliferation by downregulating miR-301a expression. A: Effects of circ_0000190 overexpression and miR-301a mimics on miR-301a expression. B, C: circ_0000190 inhibits TNBC cell proliferation by down-regulating miR-301a. D: miR-301a overexpression reverses the promoting effect of circ_0000190 overexpression on TNBC cell apoptosis. *P<0.05, **P<0.01, ***P<0.001.

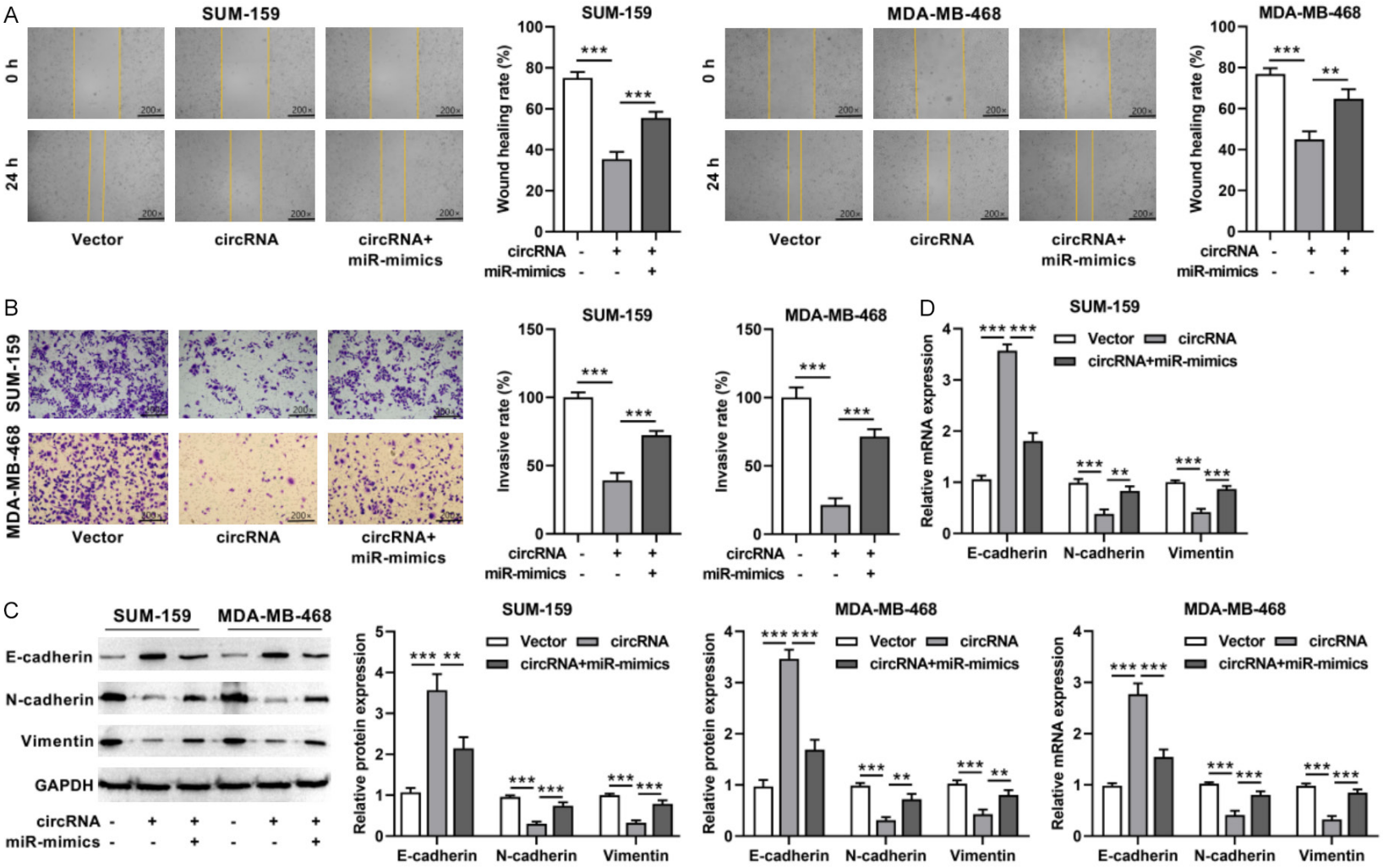

Figure 5. Circ_0000190 inhibits TNBC migration, invasion, and EMT by downregulating miR-301a. A: Overexpression of miR-301a reverses the inhibitory effect of circ_0000190 overexpression on cell migration. B: Overexpression of miR-301a reverses the inhibitory effect of circ_0000190 overexpression on cell invasion. C, D: Verexpression of miR-301a reverses the inhibitory effect of circ_0000190 overexpression on EMT process in cells. *P<0.05, **P<0.01, ***P<0.001. EMT: epithelial-mesenchymal transition. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

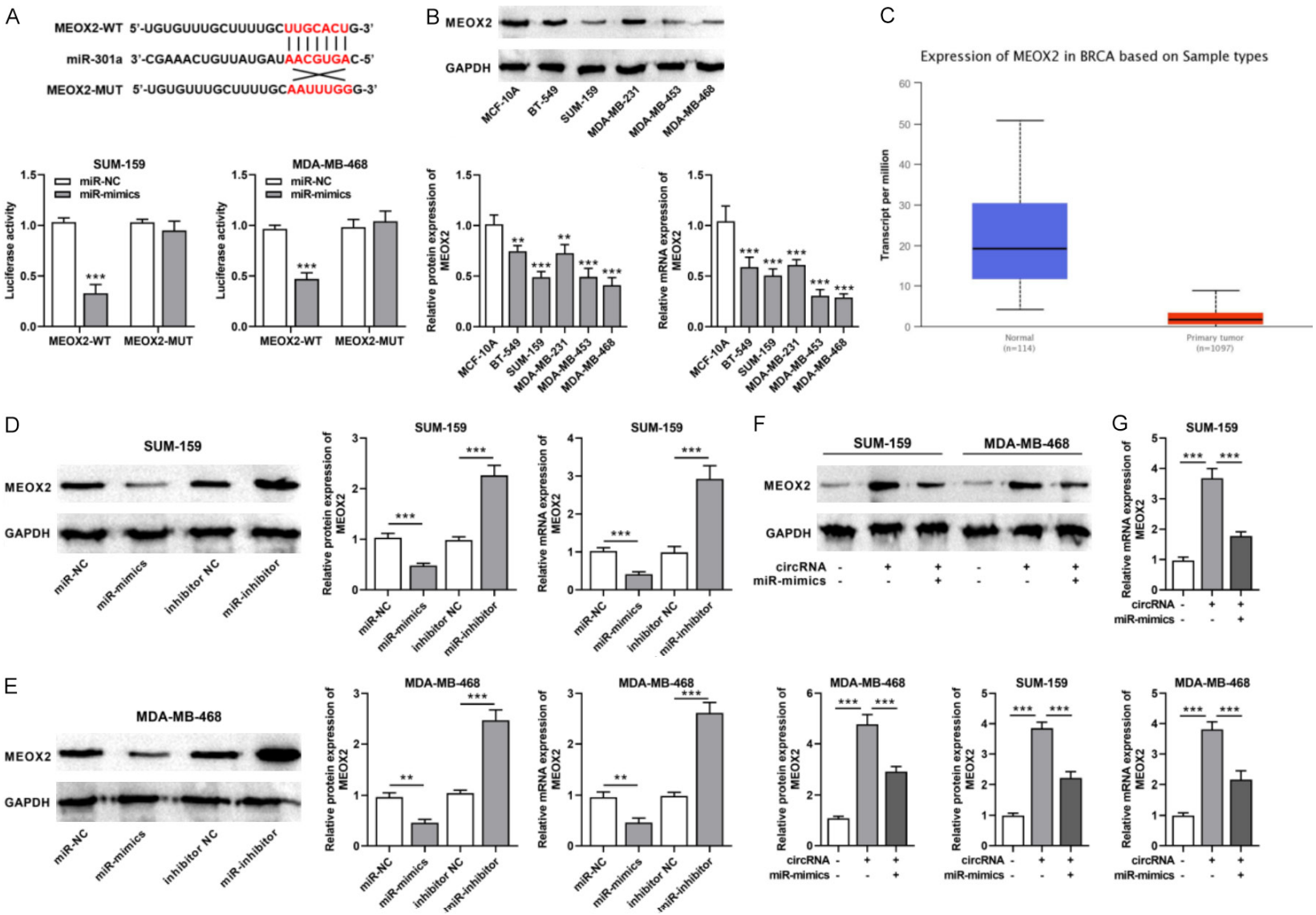

Figure 6. MEOX2 acts as an endogenous competitive RNA for miR-301a. A: Dual luciferase report assay to verify the binding site of miR-301a and MEOX2. B, C: Differences in MEOX2 expression between breast cancer cell lines and normal breast epithelial cells. D, E: Effects of miR-301a overexpression on MEOX2 expression in TNBC cell lines. F, G: Overexpression of miR-301a partially inhibited the inhibitory effect of circ_0000190 overexpression on MEOX2 expression. *P<0.05, **P<0.01, ***P<0.001. MEOX2: mesenchyme homeobox 2. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

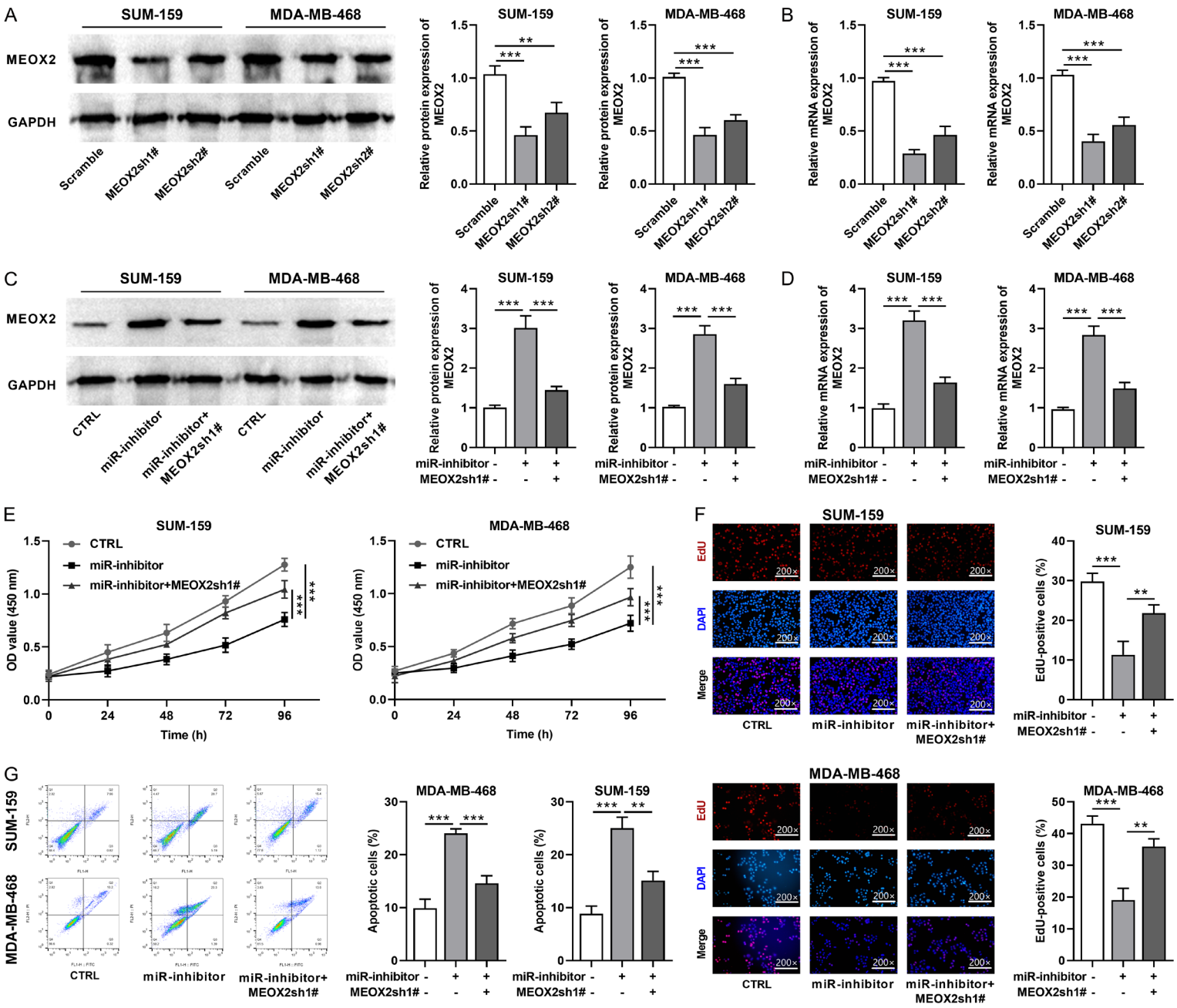
A
SUM-159
MDA-MB-468
MEOX2
GAPDH
Scramble
MEOX2sh1#
MEOX2sh2#
Relative protein expression of MEOX2
B
Relative mRNA expression of MEOX2
C
CTRL
miR-inhibitor
miR-inhibitor+ MEOX2sh1#
D
miR-inhibitor
MEOX2sh1#
E
CTRL
miR-inhibitor
miR-inhibitor+MEOX2sh1#
OD value (450 nm)
Time (h)
F
EdU
DAPI
Merge
200×
EdU-positive cells (%)
G
Apoptotic cells (%)

Figure 7. Knocking down miR-301a inhibits TNBC cell proliferation by upregulating MEOX2 levels. A, B: MEOX2-shRNA sequence screening. C, D: Effect of miR-301a knockdown on MEOX2 expression. E, F: Effects of miR-301a and MEOX2 knockdown on cell proliferation. G: Effects of miR-301a knockdown and MEOX2 knockdown on apoptosis. *P<0.05, **P<0.01, ***P<0.001. MEOX2: mesenchyme homeobox 2. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

inhibitor + MEOX2 sh1# group was increased (P<0.01), indicating that MEOX2 knockdown partially reversed the inhibitory effect of miR-301a knockdown on cell migration (**Figure 8A**). Compared with miR-301a inhibitor group, cell invasion ability of miR-301a inhibitor + MEOX2 sh1# group was increased (P<0.01) (**Figure 8B**). Compared with miR-301a inhibitor group, E-cadherin mRNA and protein of miR-301a inhibitor + MEOX2 sh1# group were significantly decreased (P<0.001). The mRNA and protein expressions of N-cadherin and Vimentin were significantly increased (P<0.001) (**Figure 8C**, **8D**). The results showed that MEOX2 knockdown partially reversed the inhibitory effect of miR-301a knockdown on EMT.

Circ_0000190 inhibits the growth of xenograft tumors in vivo

The results of xenograft tumor growth test in mice showed that the tumor volume and weight in the circ_0000190 overexpression group were significantly reduced compared with the no-load group (P<0.001) (**Figure 9A**, **9B**). Compared with the no-load group, the expression of circ_0000190 in tumor tissues of the circ_0000190 overexpression group was significantly increased (P<0.001), and the expression of MEOX2 mRNA and protein was increased (P<0.001), but the expression of miR-301a was decreased (P<0.001) (**Figure 9C-E**).

Discussion

In addition to surgical resection and radiotherapy, chemotherapy remains a cornerstone of triple-negative breast cancer (TNBC) treatment, aiming to eradicate tumors and suppress metastatic spread [16, 17]. However, systemic chemotherapy often leads to off-target toxicity and cancer cell dissemination due to its non-specific mechanism of action [18, 19]. The intrinsic heterogeneity of TNBC and the absence of molecular targets contribute to therapeutic variability, limiting the efficacy of standardized treatment regimens [20, 21]. Notably, the U.S. Food and Drug Administration (FDA) has yet to approve a targeted therapy for TNBC, highlighting the urgent need for novel therapeutic strategies [20, 21].

Recent advances in high-throughput RNA sequencing and bioinformatics have revealed that circular RNAs (circRNAs) are ubiquitously expressed in eukaryotic cells and exhibit dynamic regulation across developmental stages and physiological conditions [22]. Due to their covalently closed loop structure, circRNAs are resistant to RNase degradation, conferring enhanced stability, abundance, and evolutionary conservation compared to linear RNAs [23, 24]. These properties make circRNAs promising biomarkers for clinical applications. Functionally, circRNAs act as miRNA sponges, RNA-binding protein (RBP) scaffolds, translational templates, and transcriptional regulators, playing pivotal roles in disease pathogenesis [25, 26]. Accumulating evidence links circRNAs to neurological disorders [27], cardiovascular diseases [28], Alzheimer's disease [29], osteoarthritis [30], diabetes [31], and cancer [32]. In particular, circRNAs have been implicated in tumor growth, metastasis, and therapy resistance in TNBC [33]. For instance, circSEPT9 is upregulated in TNBC tissues and correlates with advanced clinical stage and poor prognosis. Its knockdown suppresses proliferation, migration, and invasion, while inducing apoptosis and autophagy in TNBC cells, both in vitro and in vivo [26].

Circ_0000190, located on human chromosome 1 (chr1: 224553580-224559125), has been implicated in various cancers [34]. In this study, we observed significant downregulation of hsa_circ_0000190 in TNBC cell lines compared to normal breast epithelial cells. Overexpression of circ_0000190 suppressed cell proliferation, migration, invasion, and EMT, while promoting apoptosis, suggesting a tumor-suppressive role in TNBC. These findings align with previous reports in cervical cancer, where circ_0000190 inhibits tumor progression by modulating proliferation and apoptosis [14]. Similarly, Wang et al. [35] demonstrated that circ_0000190 is downregulated in gastric cancer, where it inhibits tumorigenesis by regulat-

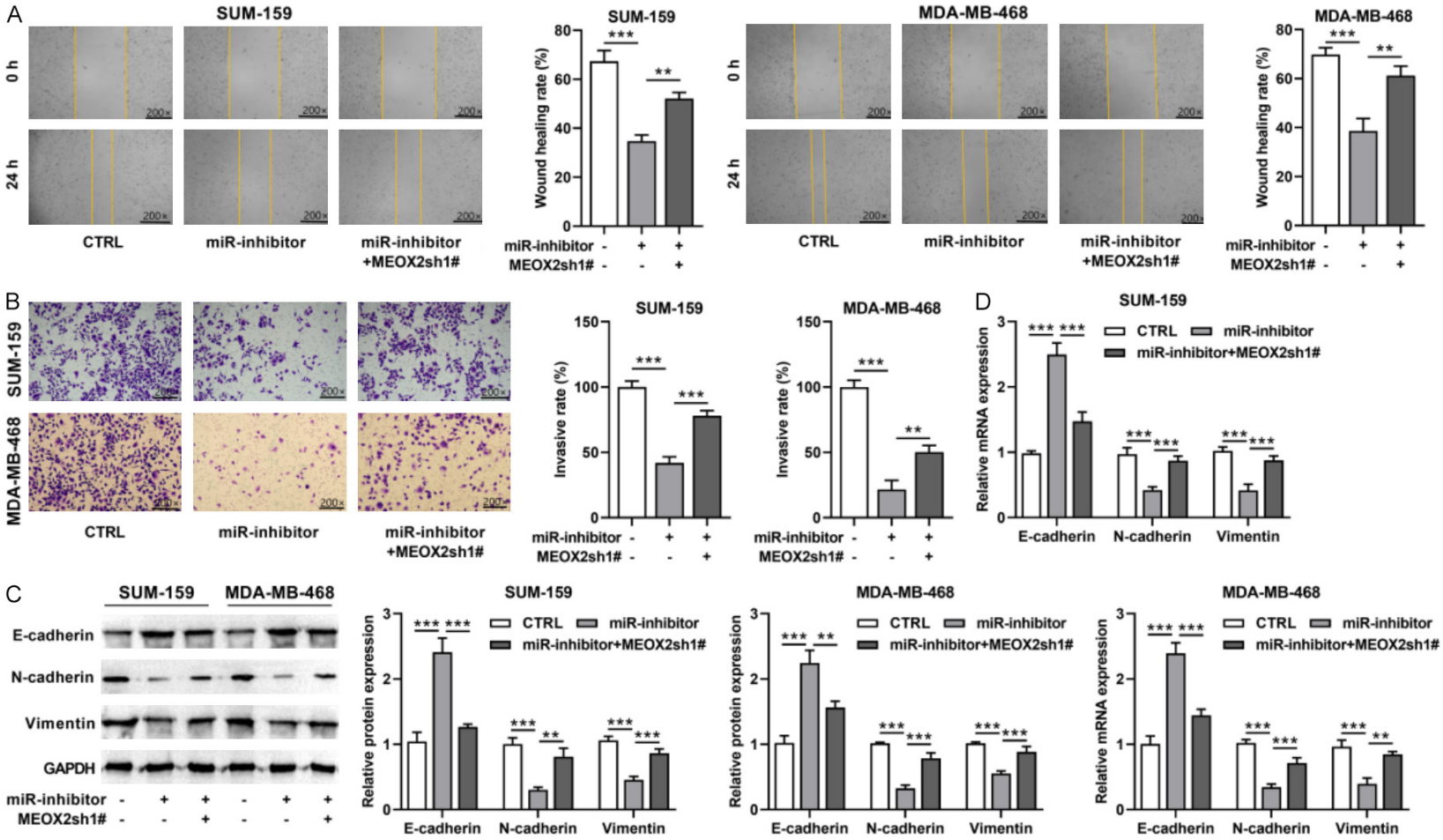

Figure 8. Inhibition of miR-301a suppresses TNBC cell migration, invasion, and EMT by upregulating MEOX2 levels. A: Effects of miR-301a and MEOX2 knockdown on TNBC cell migration. B: Effects of knocking down miR-301a and MEOX2 on TNBC cell invasion. C, D: Effects of miR-301a knockdown and MEOX2 knockdown on EMT process. *P<0.05, **P<0.01, ***P<0.001. MEOX2: mesenchyme homeobox 2. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

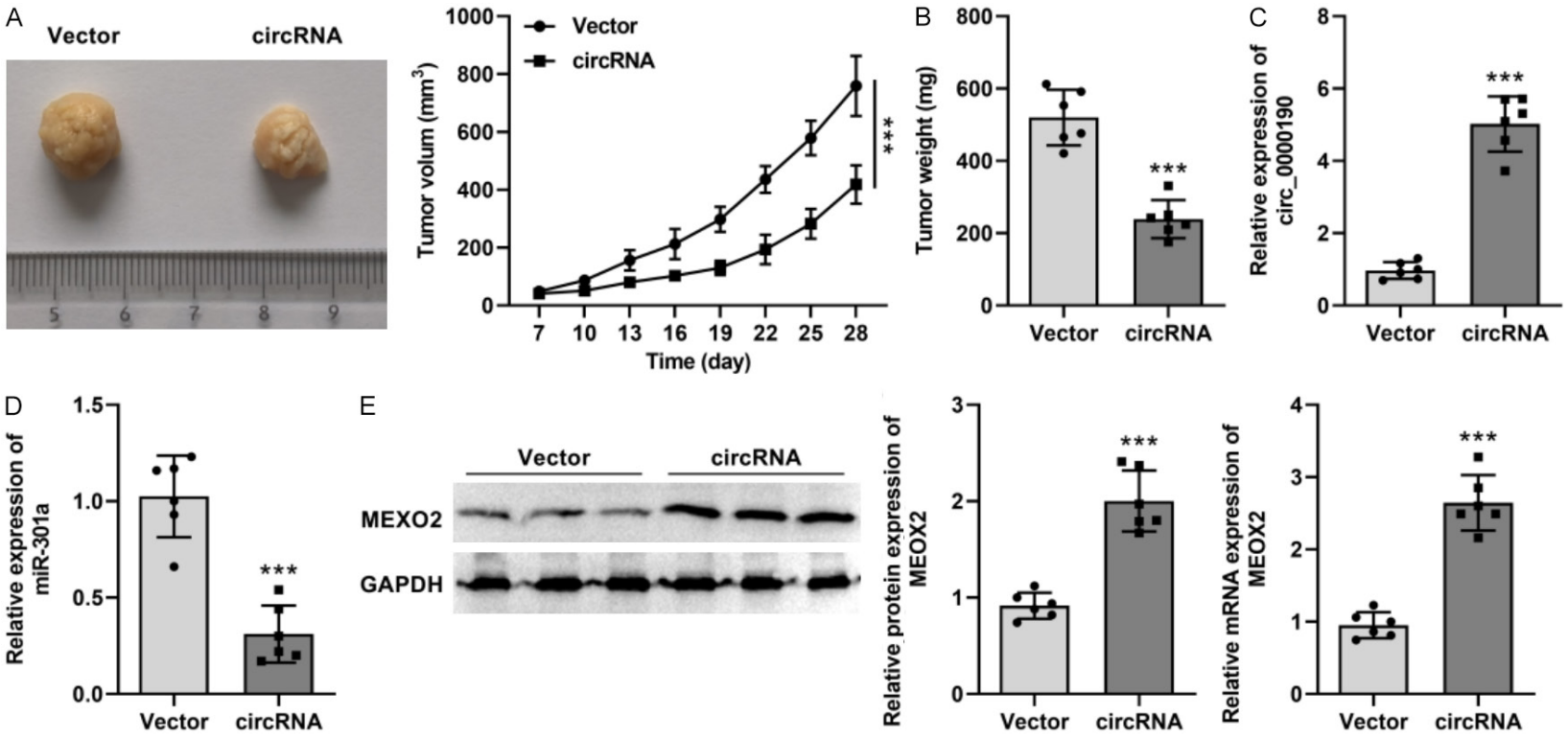

Figure 9. Circ_0000190 inhibits the growth of xenograft tumors in vivo. A, B: Effect of circ_0000190 overexpression on xenograft tumor growth in mice. C-E: Effect of circ_0000190 overexpression on miR-301a and MEOX2 expression in mouse tumor tissue. *P<0.05, **P<0.01, ***P<0.001. MEOX2: mesenchyme homeobox 2. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

ing caspase-3, p27, and cyclin D1. However, Luo et al. [36] reported elevated circ_0000190 levels in the blood of advanced cancer patients, correlating with poor response to anti-PD-L1 therapy. Mechanistically, circ_0000190 promotes ERK1/2 phosphorylation, cell proliferation, migration, and xenograft tumor growth [37]. These context-dependent discrepancies suggest that circ_0000190 may function as either an oncogene or tumor suppressor, depending on cancer type and signaling milieu.

Over 80% of circRNAs originate from exonic sequences, sharing homology with their linear counterparts [38]. As competitive endogenous RNAs (ceRNAs), circRNAs sequester miRNAs, thereby modulating gene expression in cancer [38]. Dysregulation of key circRNAs can disrupt ceRNA networks, contributing to tumorigenesis. For example, circTADA2As inhibits breast cancer progression via the miR-203a-3p/SOCS3 axis [39], while hsa_circ_001783 promotes tumorigenesis by suppressing miR-200c-3p [40]. In our study, circ_0000190 knockdown upregulated miR-301a, whereas its overexpression downregulated miR-301a, confirming its role as a molecular sponge. Notably, miR-301a mimics reversed the anti-tumor effects of circ_0000190, reinforcing the functional interplay between these molecules.

miR-301a is a well-documented oncogene in multiple cancers. Li et al. [41] reported its upregulation in renal cell carcinoma (RCC), where it promotes tumor growth and cell cycle progression. Similarly, Yin et al. [42] observed elevated miR-301a in TNBC, where it enhances proliferation and invasion via the Cip2a/ERK/CREB feedback loop. Our findings further demonstrate that MEOX2, a direct target of miR-301a, is suppressed by miR-301a overexpression in TNBC. MEOX2 knockdown attenuated the anti-tumor effects of miR-301a silencing, underscoring its critical role in TNBC progression. These results align with Cao et al. [43], who identified MEOX2 as a miR-301a target in lung cancer, and Huang et al. [44], who implicated MEOX2 in angiogenesis regulation via the miR-1200/MEOX2 axis.

MEOX2, a homeobox-containing transcription factor, regulates tissue development and cell cycle arrest by activating p16 and p21 [45, 46]. Its dual role as an oncogene or tumor suppressor varies across cancers. In lung cancer, MEOX2 overexpression correlates with chemoresistance and poor prognosis [47], whereas low MEOX2 expression predicts shorter survival in hepatocellular carcinoma (HCC) and laryngeal cancer [48]. Tian et al. [49] further demonstrated that MEOX2 upregulation is associated with higher-grade gliomas and worse clinical outcomes, suggesting context-dependent functions.

Conclusion

Our study elucidates a novel ceRNA network in which circ_0000190 suppresses TNBC progression by sponging miR-301a and upregulating MEOX2. These findings highlight circ_0000190 as a potential therapeutic target for TNBC, offering new avenues for precision oncology.

Acknowledgements

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Disclosure of conflict of interest

None.

Authors' contribution

Heng Liu and Xiunan Li contributed to the conception and design of the study; Gangyue Wang supervised the present study. Heng Liu , Yu Ren, Zhenlie Fan performed the experiments; Xin Tang contributed to the analysis of data; Heng Liu wrote the manuscript; All authors reviewed and approved the final version of the manuscript.

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