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CircRNA_069718 promotes cell proliferation and invasion in triple-negative breast cancer by activating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs) play critical roles in tumorigenesis. In the present study, we aimed to explore the potential regulatory mechanism of circRNA_069718 in triple-negative breast cancer (TNBC).

PATIENTS AND METHODS: CircRNA_069718 expression levels in TNBC tissues and cell lines were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). *In vitro* function assays were used to determine the functional roles of circRNA_069718 in TNBC and were explored by Cell Counting Kit-8 (CCK-8) assay, colony formation assay, transwell assay, and flow cytometric analysis. QRT-PCR and Western blot were used to explore the effects of circRNA_0023642 on the expression of Wnt/β-catenin pathway-related genes.

RESULTS: We found that circRNA_069718 expression was significantly increased in TNBC tissues and cell lines. High circRNA_069718 expression was significantly correlated with advanced TNM stage, lymph node metastasis, and poor overall survival of TNBC patients. Functionally, we showed that circRNA_069718 inhibition significantly reduced TNBC cells proliferation and invasion ability *in vitro*. Mechanically, we found that circRNA_069718 inhibition reduced the expression levels of Wnt/β-catenin pathway-related genes (β-catenin, c-myc, and cyclin D1).

CONCLUSIONS: Our findings suggested that circRNA_069718 promoted TNBC progression via Wnt/β-catenin pathway and could serve as a novel therapeutic target for TNBC treatment.

Key Words:
Triple-negative breast cancer, CircRNA_069718, Wnt/β-catenin.

Introduction

Breast cancer (BC) is one of the most common malignant tumors among women worldwide, which seriously threatens women's health[1]. Triple-negative breast cancer (TNBC) is one of the main subtypes of BC due to the lack of expression of estrogen receptor α (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[2,3]. Although therapeutic strategies have been developed, the survival rate remains poor due to the metastasis and resistance[4,5]. Therefore, effective identification and development of novel molecular approaches to the diagnosis and treatment of BC remain important.

Circular RNAs (circRNAs) are a novel type of non-coding RNA, which widely exist in mammalian cells[6]. CircRNAs are covalently closed, single-stranded transcripts without 5′ caps and 3′ tails that range in length from a few hundred to thousands of nucleotides[7,8]. Emerging evidence revealed that circRNAs are involved in tumorigenesis and cancer progression. For example, Zong et al[9] showed that circRNA_102231 expression was significantly upregulated and associated with advanced TNM stage, lymph node metastasis, and poor overall survival of lung cancer patients. Ge et al[10] revealed that circMTO1 inhibited colorectal cancer cells proliferation and invasion by regulating Wnt/β-catenin signaling pathway. Zou et al[11] suggested that circPCNXL2 sponged miR-153 to promote the proliferation and invasion of renal cancer cells by upregulating ZEB2 expression.

CircRNA_069718 (hsa_circ_0069718), located on human chromosome chr4: 52729602-52765544, is reported for overexpression in TNBC tissues[12]. However, the roles and molecular mechanisms of circRNA_069718 in BC remain unclear. Thus, the aim of this work was to evaluate the correlation of circRNA_069718 expression with clinicopathological features and prognosis in TNBC patients and to explore its effects on cells proliferation and invasion in TNBC cells.

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Patients and Methods

Patients and Specimens

A total of 35 paired TNBC tissues and adjacent non-tumor tissues were collected at the Department of General Surgery, Shidong Hospital, between 2012 and 2015. None of the patients had received any preoperative therapy. All samples from patients were immediately frozen in liquid nitrogen and stored at –80°C. This investigation has been approved by the Ethics Committee of Shidong Hospital and a written informed consent was obtained from all the patients.

Cell Culture and Treatment

Human BC cell lines (MCF-7, T47D, BT20, MDA-MB-468, and MDA-MB-231) and normal mammary epithelial cell lines (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), with 100 U/ml penicillin and 100 µg/mL streptomycin in a humidified incubator containing 5% CO_2 at 37°C.

The siRNAs of circRNA_069718 (si-circRNA_069718) and si-NC were obtained from GenePharma (Shanghai, China). These oligonucleotides were transiently transfected into cells using Lipofectamine 3000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Isolation and Quantitative Real Time-PCR

Total RNA was extracted from tissues or cells using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNAs were synthesized using the Reverse Transcription System Bestar qPCR RT Kit according to the manufacturer's instruction with ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The fold change was determined using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. All experiments were triplicated. The primer sequences were as follows: CircRNA_069718 (forward: 5'-CAGATATGCGTTTGACTTTGC-3', reverse: 5'-CGTCTTGGCCAATGTCTTCT-3'); GAPDH (forward: 5'-AGCCACATCGCTCAGACAC-3', reverse: 5'-GCCCAATACGACCAAATCC-3').

Western Blot

Proteins were extracted using a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Samples containing 50 µg of proteins were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring on a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA) at 300 mA for 100 min, the membrane was blocked in 5% skim milk for 2 h, incubated with rabbit anti-N-cadherin, rabbit anti-E-cadherin, rabbit anti-β-catenin, rabbit anti-c-myc, rabbit anti-cyclin D1, and rabbit anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Then, the membranes were washed three times and incubated with goat anti-rabbit IgG HRP (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. Proteins were visualized with enhanced chemiluminescence reagents (ECL; Pierce, Rockford, IL, USA).

CCK-8 Assay

Cell viability was measured using the cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, transfected cells (5×10^3 cells/well) were seeded in 96-well plates. Following incubation for 24, 48, and 72 h, 10 µL of CCK-8 solutions were added to each well and incubated for 2 h. The absorbance was measured at 450 nm with a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Transfected cells (1×10^3 cells/well) were plated into 6-well plates. After 2 weeks of incubation, the colonies were fixed using 4% paraformaldehyde (Beyotime, Shanghai, China) for 15 min and stained using 0.1% crystal violet (Yeasen, Shanghai, China) for 10 min. Next, formed colonies were counted and photographed (Nikon, Tokyo, Japan).

Transwell Invasion Assay

1×10^5 transfected cells in 100 µL serum-free medium were added to the upper chamber pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and the lower chamber was supplemented with 500 µL medium containing 10% FBS. After incubation for 24 h, cells in the lower chamber were stained using 0.1% crystal violet and then counted under a light microscope (Nikon, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed by the SPSS 21.0 software (IBM, Armonk, NY, USA).

Data were compared between two groups or multiple groups by Student's *t*-test or one-way analysis of variance (ANOVA) followed by LSD test, respectively. The Kaplan-Meier method and log-rank test were used to evaluate the prognosis of TNBC patients. $p < 0.05$ was considered significant.

Results

CircRNA_069718 Upregulates in TNBC

The previous study showed that circRNA_069718 was one of the most upregulated circRNAs in TNBC by high-throughput circular RNA microarray assays[12]. However, the roles and underlying mechanisms remain unclear. In the present research, we firstly explored circRNA_069718 expression in 35 paired TNBC tissues and adjacent non-tumor tissues. Results showed that circRNA_069718 expression was significantly increased in TNBC tissues compared with adjacent non-tumor tissues (Figure 1A). Of note, high circRNA_069718 expression was associated with TNBC patients with lymph node metastasis (Figure 1B) and advanced TNM stage (Figure 1C). Furthermore, the Kaplan-Meier analysis revealed that TNBC patients with high circRNA_069718 expression had a poor overall survival compared to low expression (Figure 1D).

CircRNA_069718 Promotes BC Cells Proliferation

QRT-PCR was used to determine circRNA_069718 expression in BC cells. Results showed that circRNA_069718 expression was significantly increased in BC cells (MCF-

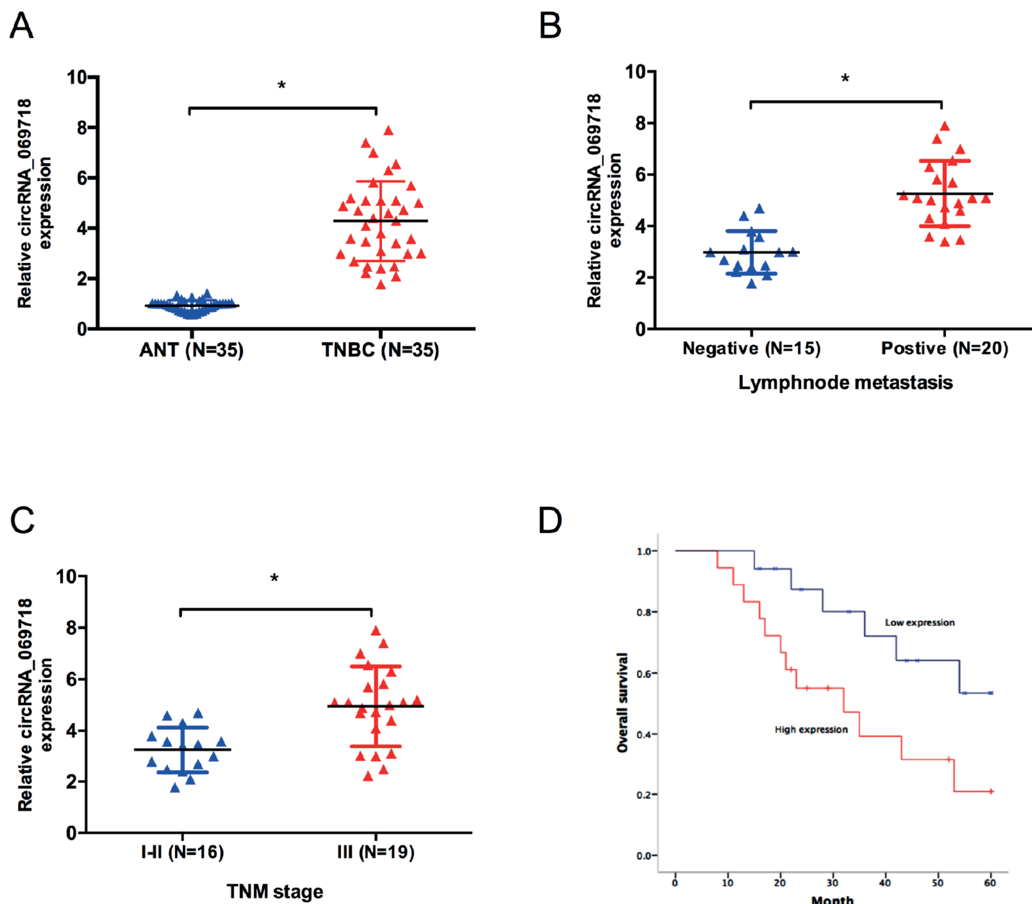


Figure 1. High expression of circRNA_069718 in TNBC tissues. ***A,*** Relative circRNA_069718 expression in TNBC tissues and adjacent non-tumor (ANT) tissues. ***B,*** High circRNA_069718 expression was associated with lymph node metastasis. ***C,*** High circRNA_069718 expression was associated with advanced TNM stage. ***D,*** Kaplan-Meier analysis showed TNBC patients with high circRNA_069718 expression had a poor overall survival. *$p < 0.05$.

7, T47D, BT20, MDA-MB-468, and MDA-MB-231) (Figure 2A). To explore the effect of circRNA_069718 on BC cell growth, MCF-7 and MDA-MB-468 cells were transfected with si-circRNA_069718 or si-NC (Figure 2B). CCK-8 assay demonstrated that circRNA_069718 inhibition significantly decreased BC cells proliferation ability compared to si-NC group (Figure 2C). Similarly, colony formation assay showed that the visible colonies numbers were reduced in si-circRNA_069718 group compared to the si-NC group (Figure 2D). Moreover, flow cytometry assay revealed that circRNA_069718 suppression could arrest BC cells in G0/G1 phase (Figure 2E).

CircRNA_069718 Promotes BC Cells Invasion

We further explored the effect of circRNA_069718 on BC cells invasion. Transwell invasion assay showed that circRNA_069718 suppression reduced BC cells invasive abilities *in vitro* (Figure 3A). Epithelial-mesenchymal transition (EMT) plays critical roles in metastasis of tumor cells. Here, we performed qRT-PCR and Western blot to explore the effect of circRNA_069718 on EMT-related genes (epithelial marker E-cadherin, mesenchymal markers N-cadherin). Results showed that circRNA_069718 suppression reduced N-cadherin expression, but increased E-cadherin expression at both mRNA (Figure 3B) and proteins levels (Figure 3C) in BC cells.

CircRNA_069718 Can Modulate Wnt/β-Catenin Pathway in BC

Wnt/β-catenin pathway plays important roles in tumorigenesis and cell invasion. Thus, we explored whether circRNA_069718 could regulate the Wnt/β-catenin pathway in BC cells. We found that circRNA_069718 suppression decreased β-catenin, c-Myc, and cyclin D1 expression at both mRNA (Figure 4A) and proteins (Figure 4B) levels in BC cells. These data suggested that circRNA_069718 could modulate the activity of Wnt/β-catenin pathway in BC progression.

Discussion

Triple-negative breast cancer (TNBC) is one of the main breast cancer subtypes, which is a serious threat to the health of females worldwide[13]. Although therapeutic strategies have been developed and widely used, the survival rates of TNBC remain unsatisfied[14]. This may be partly due to the inherently aggressive clinical behaviors and lack of proper therapeutic targets[15]. Thus, there is an urgent need to explore novel therapeutic targets of TNBC patients.

Recently, several circRNAs have been reported[16] to be involved in BC progression. For example, Zeng et al[17] showed that circANKS1B acted as an oncogenic circRNA in BC progression. Zhou et al[18] found that circRNA_0023642 promoted migration and invasion of gastric cancer cells by regulating EMT. Zhao et al[19] suggested that circRNA-BARD1 overexpression in BC cells with TCDD treatment could promote cell apoptosis via miR-3942/BARD1 axis.

In the present study, we showed that circRNA_069718 expression was significantly increased in TNBC tissues. High circRNA_069718 expression was associated with lymph node metastasis, advanced TNM stage, and poor overall survival of TNBC patients. CCK-8 and colony formation assays showed that circRNA_069718 inhibition significantly decreased BC cells viability *in vitro*. Flow cytometry revealed that circRNA_069718 suppression arrested BC cells in G0/G1 phase. Transwell invasion assay suggested that circRNA_069718 suppression reduced BC cells invasive abilities *in vitro*. Moreover, we found that circRNA_069718 suppression reduced N-cadherin expression, but promoted E-cadherin expression in BC cells. These data indicated that circRNA_069718 might serve as a tumor oncogenic circRNA in BC progression.

Wnt/β-catenin pathway is a key cascade tightly associated with cancer progression[20,21]. Activation of the Wnt/β-catenin pathway could promote tumor invasion by the upregulation of factors regulating the EMT processes[22]. For example, Xu et al[23] showed that TRIM29 prevented hepatocellular carcinoma progression by inhibiting Wnt/β-catenin signaling pathway. Chen et al[24] suggested that lncRNA SNHG7 suppression inhibited cell proliferation and migration in bladder cancer by activating the Wnt/β-catenin pathway. Yang et al[25] found that silencing of cZNF292 suppressed human glioma tube formation via the Wnt/β-catenin signaling pathway. In our work, we showed that circRNA_069718 inhibition decreased β-catenin, c-Myc, and cyclin D1 expression both in mRNA and protein levels in BC cells. These results indicated that circRNA_069718 might modulate the Wnt/β-catenin pathway in BC progression.

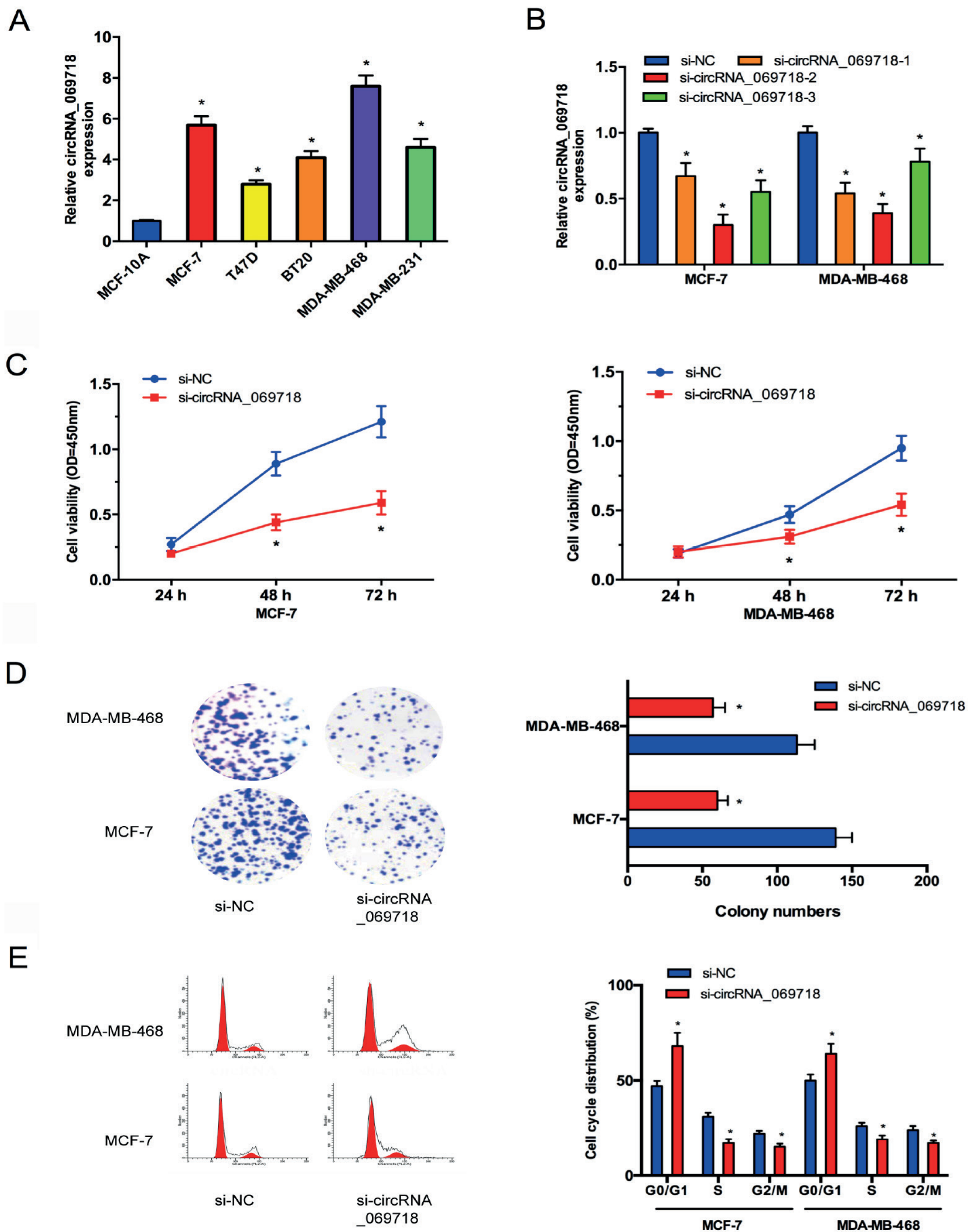


Figure 2. Downregulation of circRNA_069718 inhibits BC cells proliferation. ***A,*** Relative circRNA_069718 expression in BC cell lines. ***B,*** CircRNA_069718 expression in BC cells transfected with si-circRNA_069718 or si-NC. ***C,*** CCK-8 assay was used to evaluate the proliferation ability of BC cells transfected with si-circRNA_069718 or si-NC. ***D,*** Colony formation assay was used to analyze the colony formation ability of BC cells transfected with si-circRNA_069718 or si-NC. ***E,*** Flow cytometry assay was used to explore the cell cycle distribution of BC cells transfected with si-circRNA_069718 or si-NC. $*p < 0.05$.

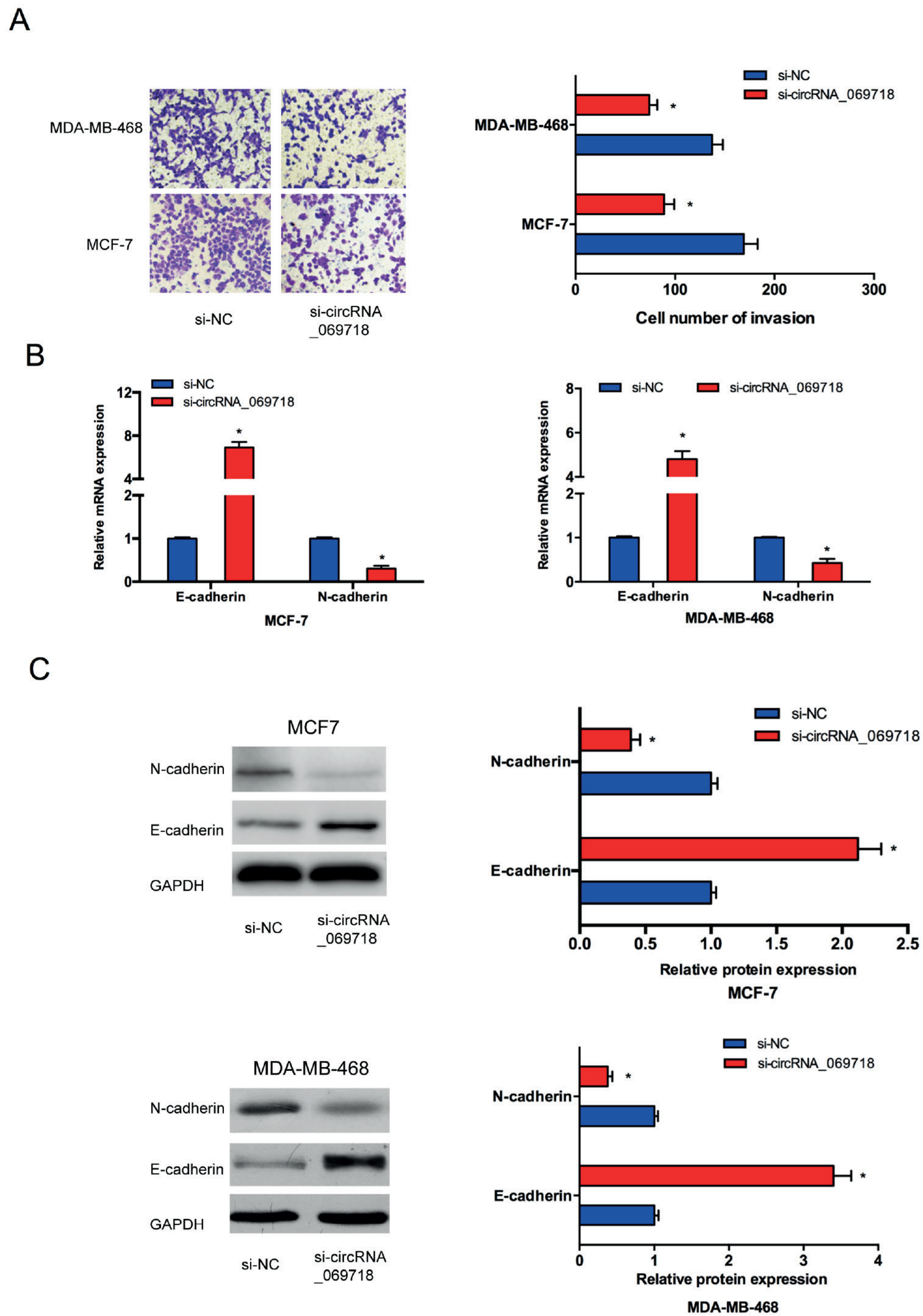
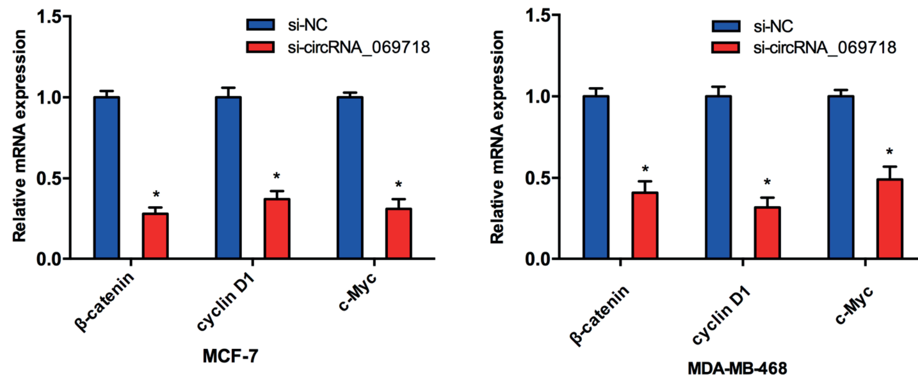


Figure 3. Downregulation of circRNA_069718 reduces BC cells invasion. ***A,*** Transwell invasion assay was performed to explore the invasion ability of BC cells transfected with si-circRNA_069718 or si-NC. ***B,*** QRT-PCR was used to determine EMT-related genes (E-cadherin, N-cadherin) expression in BC cells transfected with si-circRNA_069718 or si-NC. ***C,*** Western blot was used to determine EMT-related genes (E-cadherin, N-cadherin) expression in BC cells transfected with si-circRNA_069718 or si-NC. *$p < 0.05$.



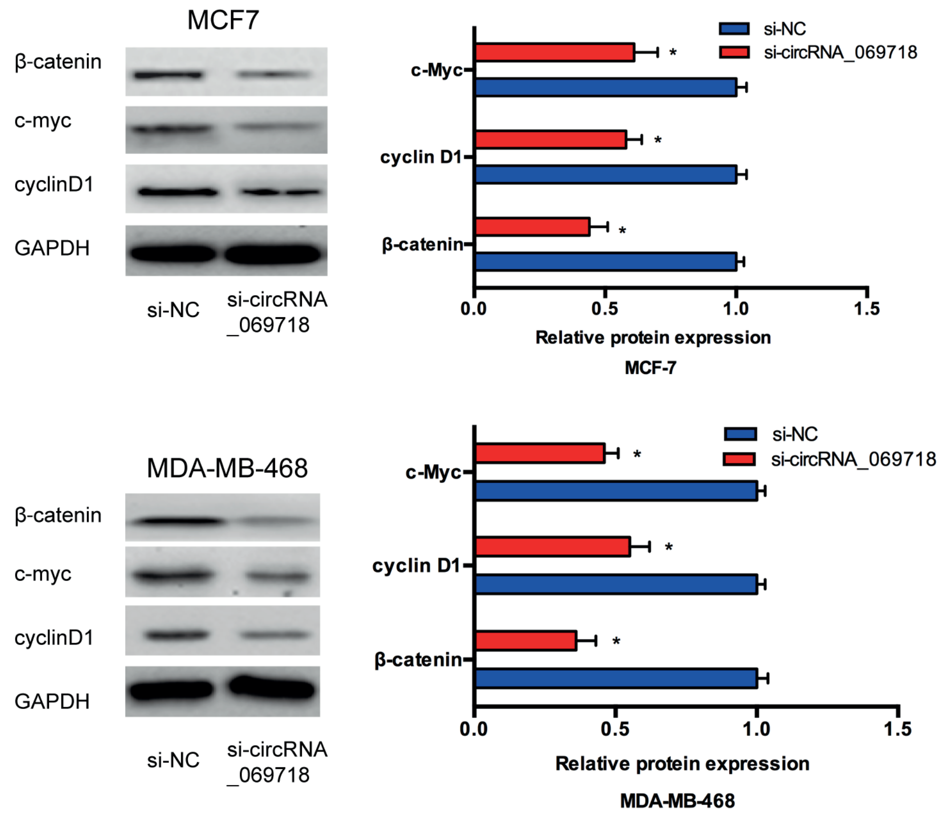


Figure 4. Downregulation of circRNA_069718 suppresses Wnt/β-catenin pathway. ***A,*** QRT-PCR was used to explore β-catenin, cyclin D1, and c-Myc expression in BC cells. ***B,*** Western blot was performed to assess β-catenin, cyclin D1, and c-Myc expression in BC cells. *$p < 0.05$.

Conclusions

We showed that circRNA_069718 could act as a tumor oncogenic circRNA in TNBC progression. The knockdown of circRNA_069718 could reduce cell proliferation and invasion in TNBC by inactivating the Wnt/β-catenin pathway. Thus, our study suggested that circRNA_069718 could function as a potential therapeutic target for BC treatment.

Conflict of interest

The authors declare no conflicts of interest.

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