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ORIGINAL RESEARCH

Blocking circ_0000520 Suppressed Breast Cancer Cell Growth, Migration and Invasion Partially via miR-1296/SP1 Axis Both in vitro and in vivo

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Background: Breast cancer (BCa) is an overwhelming malignant tumor mainly in women globally. Circular RNAs (circRNAs) are a special type of noncoding RNAs involved in competing endogenous RNA (ceRNA) network, a classic molecular mechanism of the tumorigenesis of human cancers, including BCa. Here, we intended to explore the role and mechanism of *hsa_circ_0000520* (*circ_0000520*) in BCa cells.

Methods: Expression of *circ_0000520*, *miRNA-1296-5p* (*miR-1296*) and *specificity protein 1* (*SP1*) was measured by real time-quantitative PCR and Western blotting. Cell growth was measured by cell counting kit-8, colony formation assay and flow cytometry method. Cell migration and invasion were assessed by transwell assays and Western blotting. Tumor growth was determined by xenograft models. The direct interaction among *circ_0000520*, *miR-1296* and *SP1* was confirmed by dual-luciferase reporter assay and RNA pull-down assay.

Results: *circ_0000520* was upregulated in BCa tumors and cell lines (T47D, MCF7, MDA-MB-231, BT549, and SKBR3), and *circ_0000520* high expression was associated with poor overall survival. Blocking *circ_0000520* suppressed cell viability, colony formation, migration and invasion, but promoted cell cycle arrest and apoptosis rate in MDA-MB-231 and MCF7 cells. *circ_0000520* could directly regulate *miR-1296* expression, and *SP1* was a novel target for *miR-1296*. Moreover, the anti-tumor role of *circ_0000520* silencing was abrogated by *miR-1296* downregulation or *SP1* restoration. Notably, tumor growth of MDA-MB-231 cells in mice was restrained by *circ_0000520* deletion.

Conclusion: *circ_0000520* knockdown could suppress cell growth, migration and invasion both in vitro and in vivo through regulating *miR-1296/SP1* pathway.

Keywords: *circ_0000520*, breast cancer, BCa, *miR-1296*, *SP1*

Introduction

Breast cancer (BCa) is a common cancer overwhelmingly affecting women globally, and is the leading cause of cancer death.[1] It is reported that the incidence of BCa has been rising in the last decade; while, the mortality remains the highest in female patients as well,[2] even though advances have been obtained on surgery, endocrine therapy and targeted therapy. Moreover, relapse and metastasis are always occurred in BCa patients, and metastatic breast cancer is refractory to conventional therapies.[3] As it's documented, it has not pinpointed the Achilles heel of BCa as the high heterogeneity at both morphological and molecular phenotypes.[4] Genetic changes are involved in tumorigenesis of BCa.[5,6] Therefore, novel genetic biomarkers are warranted to be discovered for the early detection and

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the understanding of the molecular pathways involved in BCa cell growth and metastasis.

The competing endogenous RNA (ceRNA) regulatory mechanism provides a promising look at the hallmarks of human malignant tumors, including BCa.[4] Essentially, ceRNA pathway consists of microRNAs (miRNAs), message RNAs (mRNAs), as well as other noncoding RNAs that possess competing shared sequences of miRNAs. Circular RNAs (circRNAs) are a peculiar class of endogenous long noncoding RNAs that are covalently closed, single-stranded transcripts with hundreds to thousands of nucleotides.[7] Unlike linear RNAs, circRNAs are resistant to the digestion of exonucleases or RNase R, due to the absence of 5′ cap and 3′ tail. Thus, circRNAs are more stable in cells and circulating environments, endowing themselves as novel, promising biomarkers for the diagnosis, treatment and prognosis of cancers.[8,9] As miRNA sponges, multiple circRNAs have been found to be functionally involved in BCa cells.[2] Herein, we wondered the role and mechanism of *hsa_circ_0000520* (*circ_0000520*) in BCa, whose expression is deregulated in BCa tumor tissues according to Gene Expression Omnibus database (GEO; GSE101123 dataset).

MiRNA-1296-5p (*miR-1296*) is a newly identified metastasis-associated miRNA in several cancers including BCa.[10] Moreover, very recently, its expression has also been claimed to be responsible for the prognosis of non-small cell lung cancer.[11] In BCa, *miR-1296* serves as a tumor suppressor.[12–14] However, *miR-1296*-centered ceRNA network remains largely uncovered in BCa.

Here, we intended to explore the biologic role of *circ_0000520* in cell growth and metastasis of BCa, and to figure out the interaction of *circ_0000520*, *miR-1296* and *specificity protein 1* (SP1), a basal transcriptional factor that widely regulates genes to contribute to the hallmarks of cancers including BCa.[15,16]

Materials and Methods

Tissue Samples

A total of 60 patients with primary BCa were recruited from China-Japan Union Hospital of Jilin University after receiving the approval of the Clinical Research Ethics Committee of this hospital. Then, 60 paired BCa tumor tissues and adjacent normal tissues were collected during radical surgery. The tissue collection was after the written informed consent was obtained from every participant, and this study was conducted in accordance with the Declaration of Helsinki. The tissue samples were put into liquid nitrogen immediately. After surgery, all the patients were received standard anti-BCa treatment, and were tracked for 60 months via telephone. The clinical characteristics of these 60 BCa patients are presented in Table 1.

Cell Culture and Cell Transfection

A series of human BCa cell lines including T47D (HTB-133), MCF7 (HTB-22), MDA-MB-231 (HTB-26), BT549 (HTB-122), and SKBR3 (HTB-30), and one normal breast epithelial cell line MCF10A (CRL-10,317) were purchased from American Type Culture Collection (Manassas, VA, USA). All the cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) in a humidified environment of sterile air (5% CO_2, 21% O_2 and 74% N_2) at 37°C.

For cell transfection, shRNAs targeting *circ_0000520* (sh-circ#1: sense 5′CGCTATGTGTTCTGGGAAA3′ and antisense 5′TTTCCCAGAACACATAGCG3′; sh-circ#2: sense 5′GAGGTGAGTTCCCAGAGAA3′ and antisense 5′TTCTCTGGGAACTCACCTC3′), *miR-1296* mimic (5′UUAGGGCCCUGGCUCCAUCUCC3′), and anti-

Table 1 Correlation Between circ_0000520 Expression and Clinicopathological Parameters of Breast Cancer Patients

Characteristics	Number	circ_0000520 Expression		*P*
		High	Low	
		30	30	
Age (years)				0.796
<50	31	16	15	
≥50	29	14	15	
Menopause				0.426
No	37	20	17	
Yes	23	10	13	
Tumor size (cm)				0.301
≤2	28	12	16	
>2	32	18	14	
Lymph node metastasis				0.020*
Yes	31	20	11	
No	29	10	19	
TNM stage				0.004*
I, II	29	9	20	
III, IV	31	21	10	

Note: *Statistically significant.
Abbreviation: TNM, tumor nodes metastasis.

miRNA against *miR-1296* (anti-miR-1296; 5′GGAGAUGGAGCCAGGGCCCUAA3′) were synthesized by GenePharma (Shanghai, China), as well as the corresponding negative controls including sh-NC (sense 5′CATTAAGCATGATGTCAACCAGACA3′ and antisense 5′TGTCTGGTTGACATCATGCTTAATG3′), miR-NC mimic (5′ UUCUCCGAACGUGUCACGUTT3′), and anti-NC (5′CAGUACUUUUGUGUAGUACAA3′). The pIRES2-EGFP vector (Clontech, Mountain View, CA, USA) was selected to construct *SP1* overexpression vector (pIRES2-EGFP-SP1). MDA-MB-231 and MCF7 cells were detached by 0.25% trypsin, and passaged at 40% confluency in 6-well plate for 24 h. The transfection was conducted following the manufacturer's protocol. Briefly, the cells were changed with fresh DMEM without FBS, and incubated with a mixture of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and nucleotides (50 nM oligonucleotides or 2 μg vectors) for 6 h. The DMEM medium was refreshed with complete medium for another 24 h for further analysis.

Real Time-Quantitative PCR (RT-qPCR)

Total RNA in tissues and cells was isolated by RNAsimple total RNA kit (TIANGEN, Beijing, China). A total of 500 ng RNA was then used to synthesize cDNA using PrimeScript RT reagent Kit (Takara, Dalian, China), and the cDNA was further amplified using special primers and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (2×) (Takara). The miRNA expression was detected using Mir-X miRNA First-Strand Synthesis Kit (Takara) and Mir-X miRNA qRT-PCR TB Green® Kit (Takara). The primers including *circ_0000520* (forward 5′GTCTGAG ACTAGGGCCAGAGGC3′ and reverse 5′GACATGGG AGTGGAGTGACAGG3′), *miR-1296* (forward 5′TTGT TAGGGCCCTGGCTC3′ and reverse 5′GTGCAGGGT CCGAGGT3′), *SP1* (forward 5′AATTTGCCTGCCCT GAGTGC3′ and reverse 5′TTGGACCCATGCTAC CTTGC3′), *glyceraldehyde-phosphate dehydrogenase* (*GAPDH*; forward 5′TCACCAGGGCTGCTTTTAAC3′ and reverse 5′TGACGGTGCCATGGAATTTG3′), and *small nuclear U6* (*U6*; 5′ ATACAGAGAAAGTT AGCACGG3′ and reverse 5′GGAATGCTTCAAAGAG TTGTG3′). The amplification curve and melting curve were analyzed on ABI 7700 (Foster City, CA, USA), and comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) was used to calculate gene expression. The reaction system was conducted in four replicate wells for each sample.

Cell Counting Kit-8 (CCK-8) and Colony Formation Assay

After transfection, the MDA-MB-231 and MCF7 cells were immediately transferred onto 96-well plate at density of 10^4 cells per well for another 48 h. Afterwards, the cells were stained with 100 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) for 2 h at 37°C. Next, the absorbance at 450 nm was measured using a microplate reader. The assay was conducted in six replicate wells for each sample, and cells transfected with negative control were considered as 100% cell viability.

After transfection, the MDA-MB-231 and MCF7 cells were passaged in a new 6-well plate at 1000 cells per well. Then, the cells were replaced with fresh complete medium every three days, and cultivated for another 15 days. After that, the cells were washed with cold phosphate buffered saline (PBS), dried in the air, fixed with methanol for 15 min, and stained with 0.25% crystal violet for 20 min. The number of colony (more than 50 cells) was counted under a microscope.

Flow Cytometry (FCM)

After transfection for 24 h, the MDA-MB-231 and MCF7 cells were collected together with the floating cells. For cell apoptosis rate analysis, the cells were washed with cold PBS, re-suspended in the binding buffer, and then stained with 5 μL of Annexin V-fluorescein isothiocyanate (FITC; BD Biosciences, Franklin Lakes, NJ, USA) and 5 μL of propidium iodide (PI; BD Biosciences) for 30 min in the dark. For cell cycle analysis, the cell suspension was fixed with cold 75% ethanol at 4°C for 16 h prior to staining with 5 μL of PI (BD Biosciences) supplemented with 200 μg/mL of RNase A. Finally, the labelled cells were determined on FACScan flow cytometer (BD Biosciences) equipped with Cell Quest software (BD Biosciences). Apoptosis rate (%) was calculated with the percentage of cells in Annexin V+/PI+ and Annexin V+/PI- quadrants. Cell rate (%) in different cell cycle phases was calculated with the percentage of cells in G0/G1, S and G2/M phases, respectively.

Transwell Assays

Transfected cells were harvested to measure abilities of cell invasion and migration by transwell chambers (Corning, Corning, NY, USA) coated with matrigel (BD Biosciences) or not. For invasion assay, matrigel membrane was formed after being diluted in 1:10 serum-free DMEM and then placed at 37°C for 4–6 h. For migration and invasion assays, 5×10^5 transfected cells were re-suspended in 200 μL of DMEM

without FBS, then loaded in the upper layer of the chamber. The 400 μL of DMEM containing 10% FBS was transferred into the lower of the chamber. This transwell migration or invasion system was maintained for 48 h in normal cell culture condition. The cells on the bottom of chamber were fixed with methanol for 15 min and dyed with 0.25% crystal violet for 20 min. A total of 5 randomly fields (100×) were captured under microscope, and the numbers of migrated cells and invaded cells were counted.

Western Blotting

Western blotting was performed according to the standard methods as previously described. The primary antibodies including *E-cadherin* (*E-cad*; 20,874-1-AP, 1:50,000), *N-cadherin* (*N-cad*; 22,018-1-AP, 1:10,000), *SP1* (21,962-1-AP, 1:2500), and *GAPDH* (10,494-1-AP, 1:40,000) were from Proteintech (Deansgate, Manchester, UK). The secondary antibody goat-anti-rabbit (ab205718, 1:50,000) was from Abcam (Cambridge, UK). *GAPDH* was the internal control. The densitometry of Western blotting blots was measured using Image J software.

Dual-Luciferase Reporter Assay and RNA Pull-Down Assay

The synthetic *circ_0000520* and 3′ untranslated region of *SP1* (SP1 3′UTR) were inserted into pGL4 (Promega, Madison, WI, USA), respectively. The mutant of potential binding sites of *miR-1296* on *circ_0000520* and *SP1* 3′UTR were mutated into the complementary bases, and then inserted into pGL4 (Promega) as well. The recombinant vector pGL4 carrying *circ_0000520* wild type fragment (circ_0000520-WT), *circ_0000520* mutant fragment (circ_0000520-MUT), *SP1* 3′UTR wild type fragment (SP1 3′UTR-WT), and *SP1* 3′UTR mutant fragment (SP1 3′UTR-MUT) were co-transfected into MDA-MB-231 and MCF7 cells (in 24-well plate) together with miR-1296 mimic or miR-NC mimic. After transfection for 48 h, the cells were lysed with commercial lysis buffer (Ambion, Austin, TX, USA), and the luciferase activity was measured on Dual-luciferase Reporter Assay System (Promega). Every transfection group was repeated in three wells.

The biotin labelled *miR-1296* (Bio-miR-1296) and miR-NC (Bio-miR-NC) were from Sangon (Shanghai, China). MDA-MB-231 and MCF7 cells in 6-well plate were transfected with 50 nM of Bio-miR-1296/NC for 24 h. After that, the transfected cells were collected, washed with cold PBS, and lysed with lysis buffer (Ambion). Then, the cell lysate was incubated with M-280 streptavidin-coated magnetic beads (Sigma-Aldrich, St Louis, MO, USA) for 4 h at 6°C. By the way, the beads were pre-coated with RNase-free BSA and yeast tRNA. After washing, the RNAs pulled down were detected by RT-qPCR method.

Animal Experiment

MDA-MB-231 cells transfected with sh-circ#1 or sh-NC were harvested at a concentration of 2×10^7 cells/mL. A total of 8 female BALB/c mice (6-week-old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China), and randomly divided into sh-NC group (n=4) and sh-circ#1 group (n=4). Then, the mice were subcutaneously injected with 0.2 mL of MDA-MB-231 cells on both sides of posterior flanks prior to another 35 days feeding. During the feeding, the tumor size was measured every 7 days on the length and width, and the tumor weight was examined on the last day after the mice were euthanized. The tumor volume was calculated by the product of $0.5\times\text{length}\times\text{width}^2$. The animal experiment was approved by the Use and Care of Animals Committee of China-Japan Union Hospital of Jilin University, and was in accordance with the Laboratory Animals-Guideline of welfare and ethics published by "National Institutes of Health".

Statistical Analysis

Data were presented as the mean ± standard deviation from three independent experiments, and analyzed on GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The difference in groups was performed using paired sample Student's *t*-test and one-way analysis of variance. $P<0.05$ was considered as significant difference, and signed as *<0.01 was very significant difference, and signed as **and <0.001 was extremely significant difference, and signed as ***.

Results

circ_0000520 Was Upregulated in BCa Tumor Tissues and Cells

In order to explore the dysregulation of circRNAs in BCa, we grabbled in silicon data in GEO database. According to GSE101123 dataset, microarray-seq data showed that *circ_0000520* was comparatively abundant, and was highly expressed in BCa tumor tissues (Figure 1A). Thus, we identified *circ_0000520* expression status in recruited 60 BCa patients using RT-qPCR, and found that *circ_0000520* level in BCa tumors was 2.68-fold of

that in paired normal controls (Figure 1B). Additionally, the relationship between *circ_0000520* expression and clinicopathological parameters of this cohort of BCa patients was further analyzed. As a result, high expression of *circ_0000520* was associated with lymph node metastasis and TNM stage (Table 1). Moreover, 5-year overall survival rate of patients in *circ_0000520* low group (n=30) was 86.7%, paralleled with 63.3% in circ_0000520 high group (n=30) (Figure 1C). Similarly, expression of *circ_0000520* was overall upregulated in human BCa cell lines (T47D, MCF7, MDA-MB-231, BT549, and SKBR3) than normal MCF10A cells (Figure 1D). *circ_0000520* was located in gene *RPPH1* on chromosome 14:20,811,436–20,811,559, with a spliced sequence length of 123 nucleotides. Moreover, *circ_0000520* was resistant, while RPPH1 was sensitive to RNase R digestion in MDA-MB-231 and MCF7 cells (Figure 1E and F). These data suggested that *circ_0000520* was upregulated in BCa tumors and cells.

Blocking circ_0000520 Suppressed Cell Growth, Migration and Invasion of BCa Cells in vitro

Loss-of-functional experiments in vitro were carried out to investigate the biologic role of *circ_0000520* in BCa cells. First of all, *circ_0000520* was knocked down using shRNA transfection, and RT-qPCR data depicted a significant reduction of *circ_0000520* level in MDA-MB-231 and MCF7 cells due to sh-circ#1 and #2 transfection (Figure 2A); moreover, sh-circ#1 resulted in more than 55% silencing efficiency in both cells, thus sh-circ#1 was selected for the further functional analysis. We observed that cell viability and colony formation ability were attenuated in *circ_0000520*-silenced MDA-MB-231 and MCF7 cells, as examined by CCK-8 and colony formation assays (Figure 2B and C). FCM method was used to measure cell cycle and apoptosis, and indicated an increase of G0/G1-phase cells and apoptosis rate in MDA-MB-231 and MCF7 cells administrated with sh-circ#1 than sh-NC, as well as reduced

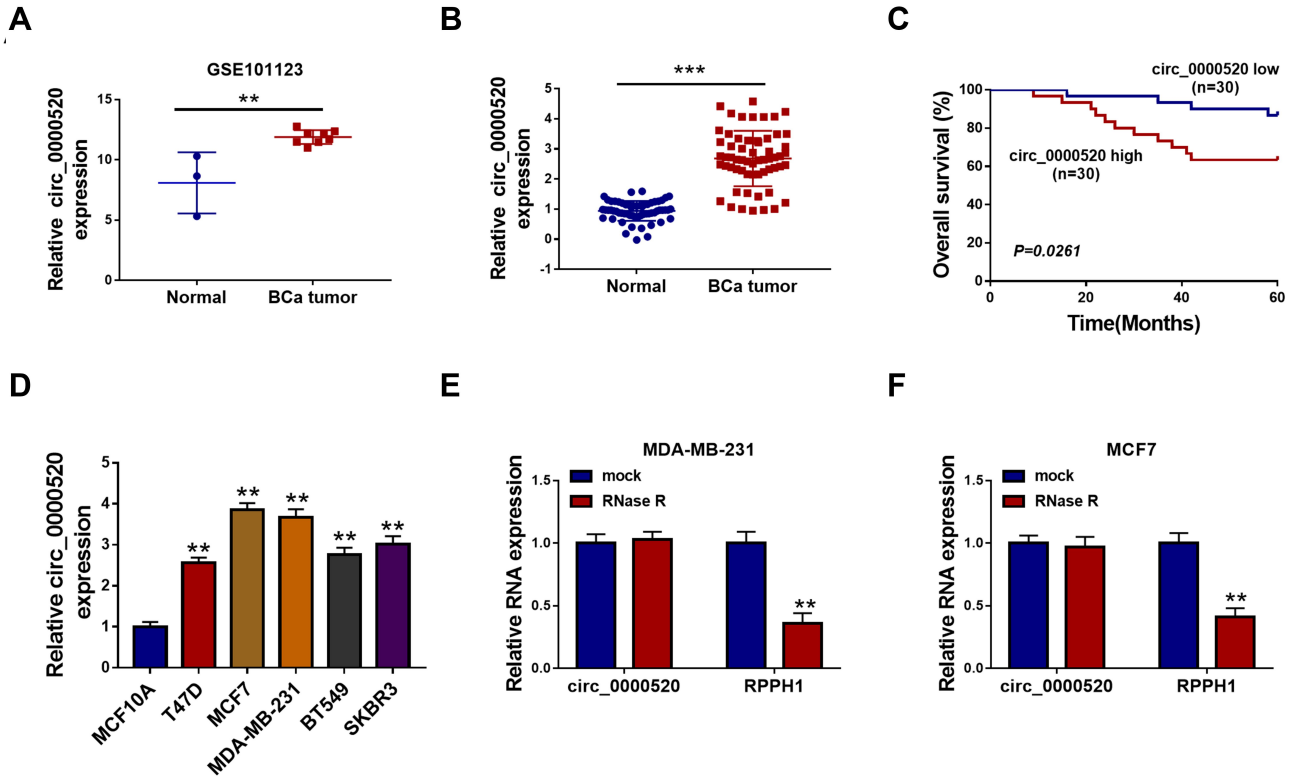

Figure 1 Expression of circular RNA *hsa_circ_0000520* (*circ_0000520*) in breast cancer (BCa) tissues and cells. (**A**) circRNAs chip determined the expression of *circ_0000520* in 8 BCa tumor tissues (BCa tumor) and 3 non-tumor breast tissues (Normal) according to GSE101123 dataset. (**B**) RT-qPCR measured relative *circ_0000520* expression in 60 paired BCa tumor and Normal groups. (**C**) Kaplan-Meier survival plots analyzed the overall survival (%) of these BCa cases with *circ_0000520* high (≥Median, n=30) or low (<Median, n=30). (**D**) RT-qPCR tested *circ_0000520* expression in human BCa cell lines (T47D, MCF7, MDA-MB-231, BT549, and SKBR3) and normal breast epithelial cell line (MCF10A). (**E** and **F**) RT-qPCR validated relative RNA expression of *circ_0000520* and its parent gene *RPPH1* with or without RNase R treatment in MDA-MB-231 and MCF7 cells. **Represented *P*<0.01 and ***Represented *P*<0.001.

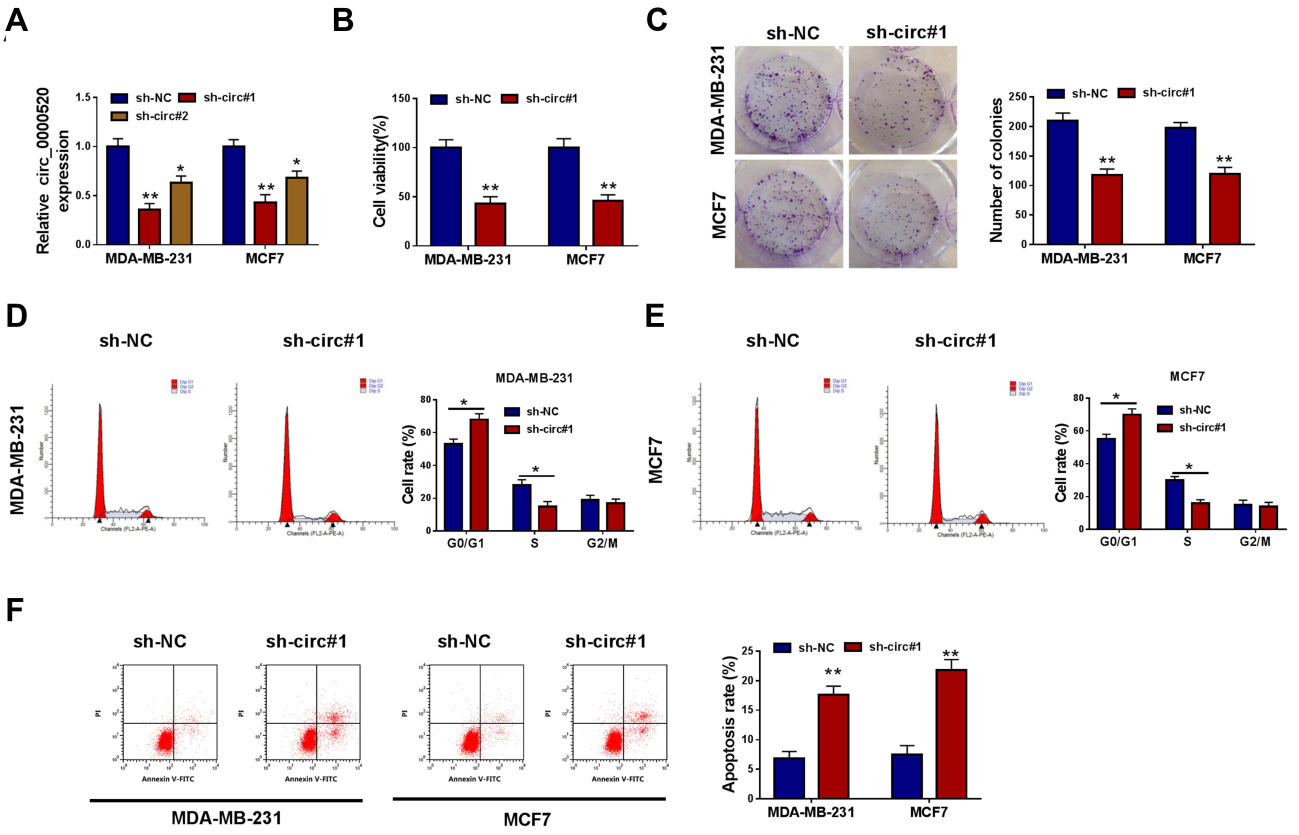

Figure 2 Blocking *circ_0000520* suppressed cell growth of BCa cells in vitro. MDA-MB-231 and MCF7 cells were administrated with shRNAs against *circ_0000520* (sh-circ#1 and #2) or scrambled shRNA (sh-NC). (**A**) RT-qPCR measured relative *circ_0000520* expression on 24 h. (**B**) CCK-8 assay detected cell viability (%) on 48 h, and (**C**) colony formation assay measured colony numbers on 15 d. (**D** and **E**) Flow cytometry (FCM) method examined cell rate (%) in different cell cycle phases on 24 h. (**F**) FCM method evaluated apoptosis rate (%) on 24 h. *Represented $P<0.05$ and **Represented $P<0.01$.

Abbreviations: Annexin V-FITC, fluorescein isothiocyanate-coated annexin V; PI, propidium iodide.

S-phase cells (Figure 2D–F). Transwell assays and Western blotting evaluated cell migration and invasion abilities. Introducing sh-circ#1 decreased migrated cell number and invaded cell number in MDA-MB-231 and MCF7 cells, compared to sh-NC introduction (Figure 3A and B). Besides, *E-cad* expression was promoted, and *N-cad* expression was inhibited by *circ_0000520* silencing (Figure 3C). Taken above results together, blocking *circ_0000520* via transfection suppressed cell viability, colony formation, migration and invasion, but promoted cell cycle arrest and apoptosis in MDA-MB-231 and MCF7 cells, suggesting a suppressive role of *circ_0000520* knockdown in BCa cell growth, migration and invasion in vitro.

miR-1296 Was Targeted by circ_0000520 in BCa Cells

We confirmed a tumor-suppressive role of *circ_0000520* knockdown in BCa, and its molecular mechanism was further identified. With separation of nuclear and cytoplasmic fractions, we found that *circ_0000520* was enriched in cytoplasmic fractions, similar to *GAPDH* (Figure 4A), suggesting *circ_0000520* was probably mainly distributed in the cytoplasm. Thus, we assumed that *circ_0000520* could associate with miRNAs sponging. Furthermore, circinteractome, circBank and starbase were employed to predict the potential target miRNAs of *circ_0000520*. As shown in Figure 4B, *miR-1296* was the common one among these three websites. Then, *miR-1296* was the only candidate miRNA for further identification. Dual-luciferase reporter assay showed a decrease of circ_0000520-WT vector in *miR-1296* mimic-transfected MDA-MB-231 and MCF7 cells (Figure 4C); RNA pull-down assay illuminated an enrichment of *circ_0000520* in Bio-miR-1296 pull-down contents in MDA-MB-231 and MCF7 cells (Figure 4D); in addition, *miR-1296* expression could be upregulated in the presence of sh-circ#1 (Figure 4E). Expression status of *miR-1296* was lower in BCa tumor tissues

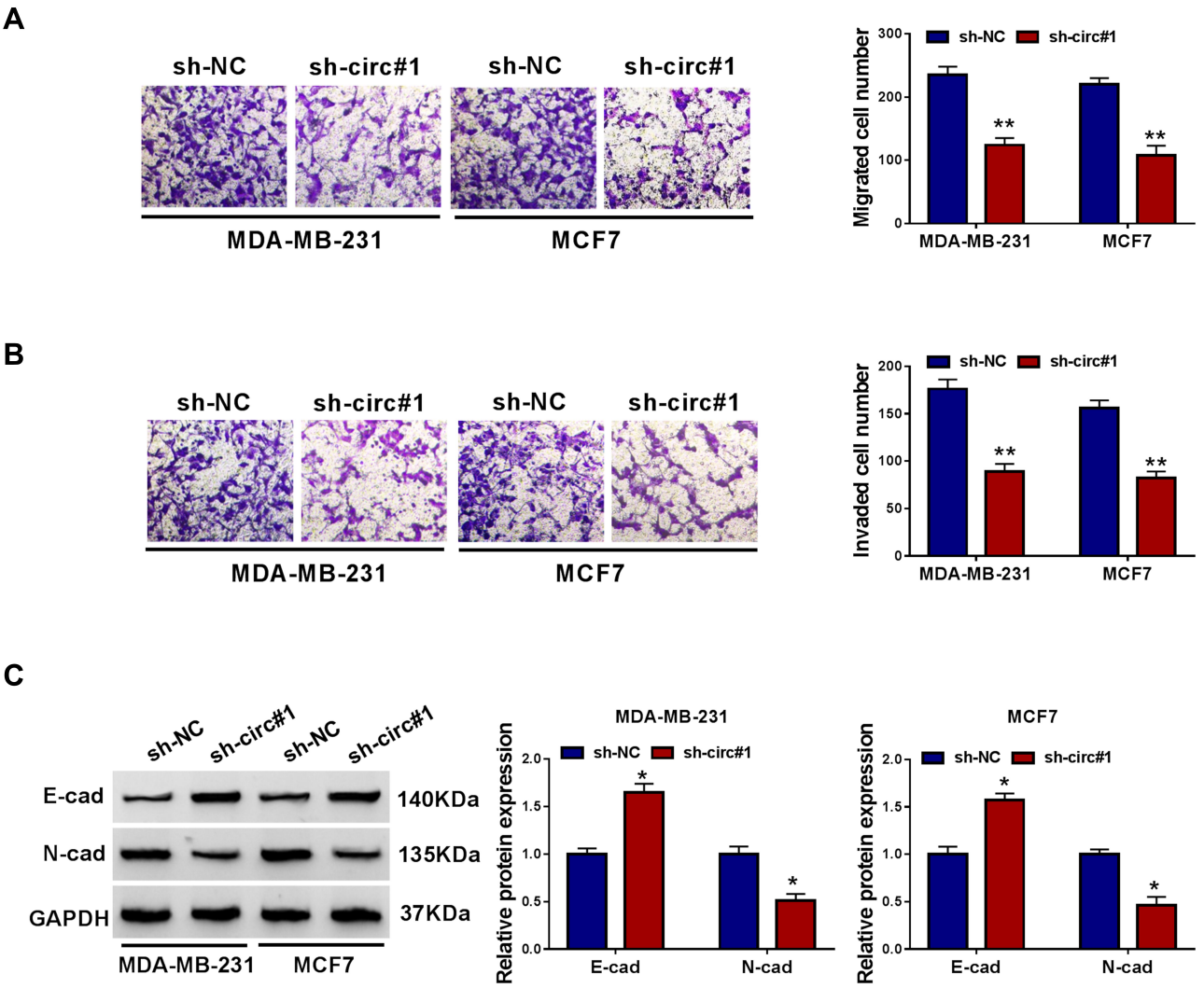

Figure 3 Blocking *circ_0000520* inhibited cell migration and invasion of BCa cells in vitro. MDA-MB-231 and MCF7 cells were administrated with sh-circ#1 or sh-NC. (**A** and **B**) Transwell assay measured migrated cell number and invaded cell number on 48 h. The most representative images were presented. (**C**) Western blotting evaluated *E-cadherin* (*E-cad*) and *N-cadherin* (*N-cad*) protein expression on 24 h. The most representative images were presented. *Represented $P<0.05$ and **Represented $P<0.01$.

in *circ_0000520*-dependent manner (Figure 4F and G). Thereby, we validated *circ_0000520* as a sponge for *miR-1296*.

Silencing miR-1296 Countermanded the Suppressive Role of circ_0000520 Knockdown in BCa Cells in vitro

Circ_0000520 knockdown was accompanied with upregulation of *miR-1296*, thus we examined the effect of *miR-1296* inhibition on the anti-tumor role of *circ_0000520* knockdown in BCa cells. The exogenous administration of anti-miR-1296 could lead to *miR-1296* inhibition in MDA-MB-231 and MCF7 cells (Figure 5A). Rescue experiments demonstrated that the low cell viability and colony formation ability in *circ_0000520*-silenced MDA-MB-231 and MCF7 cells were elevated by anti-miR-1296 transfection (Figure 5B and C); the sh-circ#1-mediated high rates of G0/G1-phase cells and apoptotic cells were descended by *miR-1296* deletion (Figure 5D–F); cell migration and invasion capacities were facilitated by *circ_0000520* downregulation, and this promoting activity was partially reversed in the presence of anti-miR-1296, as evidenced by increased migrated cells and invaded cells (Figure 5G and H), as well as higher *E-cad* and lower *N-cad* expression (Figure 5I and J). These before-mentioned outcomes indicated that silencing *miR-1296* could countermand the suppressive role of *circ_0000520* knockdown in BCa cell growth, migration and invasion in vitro.

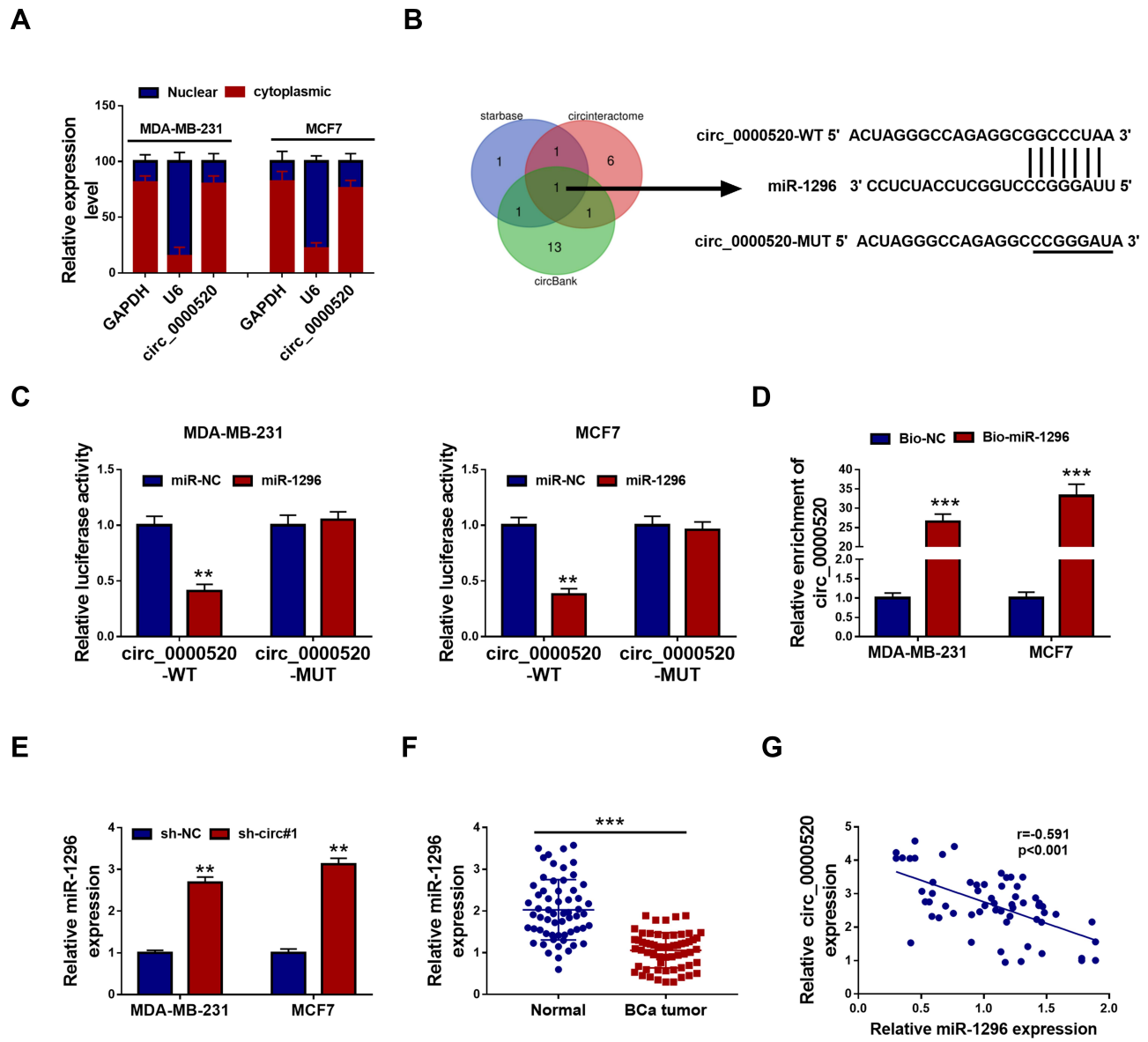

Figure 4 *MiRNA-1296-5p* (*miR-1296*) was targeted by *circ_0000520* in BCa cells. (**A**) RT-qPCR analyzed nuclear and cytoplasmic RNA expression levels of *glyceraldehyde-phosphate dehydrogenase* (*GAPDH*), *small nuclear U6* (*U6*) and *circ_0000520*. (**B**) Bioinformatics algorithms predicted potential target miRNAs of *circ_0000520*. (**C**) Dual-luciferase reporter assay identified the direct interaction between *circ_0000520* and *miR-1296* in MDA-MB-231 and MCF7 cells. (**D**) RNA pull-down assay validated the enrichment of *circ_0000520* by biotin labelled *miR-1296* (Bio-miR-1296) or miR-NC (Bio-NC) in MDA-MB-231 and MCF7 cells. (**E** and **F**) RT-qPCR tested *miR-1296* expression in MDA-MB-231 and MCF7 cells transfected with sh-circ#1 or sh-NC, and in paired BCa tumor and Normal groups (n=60). (**G**) Pearson correlation coefficient (r) analysis determined the relationship between *circ_0000520* and *miR-1296* expression in these BCa patients (n=60). **Represented *P*<0.01 and ***Represented *P*<0.001.
Abbreviations: miR-1296, miR-1296 mimic; miR-NC, miR-NC mimic.

Ectopic Expression of SP1 Counteracted the Suppressive Role of circ_0000520 Knockdown in BCa Cells in vitro Through Directly Regulated by miR-1296

The arithmetic of starbase also predicted a potential binding sequence of *miR-1296* on *SP1* 3′UTR (Figure 6A). Luciferase activity of SP1 3′UTR-WT was also decreased in MDA-MB-231 and MCF7 cells introduced with miR-1296 mimic (Figure 6B). Besides, *SP1* protein expression was inhibited by *miR-1296* mimic transfection, and promoted by anti-miR-1296 transfection (Figure 6C). These data supported a direct interaction between *miR-1296* and *SP1* through target binding.

The presence of sh-circ#1 led to lower level of *SP1* in MDA-MB-231 and MCF7 cells, which could be improved by anti-miR-1296 (Figure 6D). Moreover, *SP1* overexpression vector transfection caused *SP1* upregulation (Figure

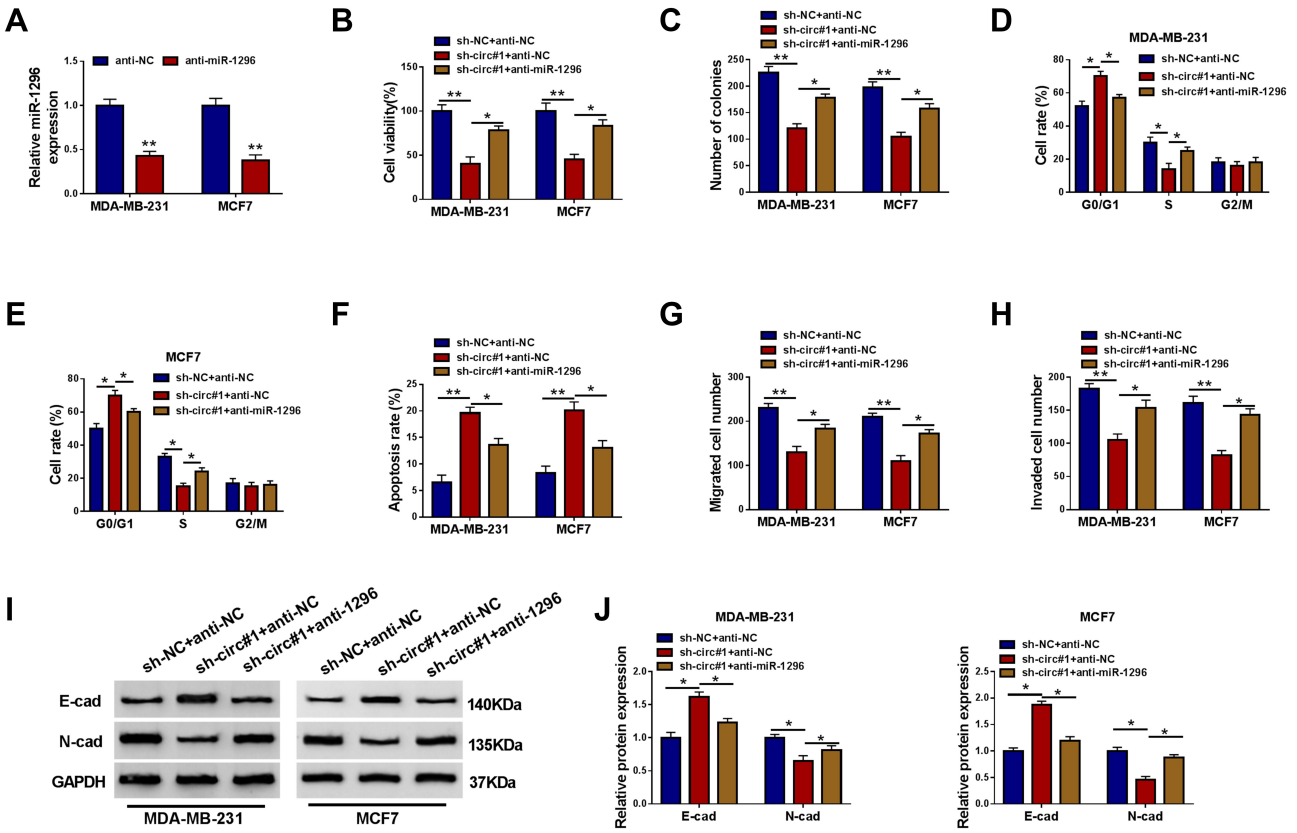

Figure 5 The effect of *miR-1296* downregulation on the role of *circ_0000520* knockdown in BCa cells in vitro. (**A**) RT-qPCR measured *miR-1296* expression in MDA-MB-231 and MCF7 cells introduced with anti-miRNA against *miR-1296* (anti-miR-1296) or its negative control anti-NC. (**B**–**J**) MDA-MB-231 and MCF7 cells were co-transfected with sh-NC and anti-miR-NC, sh-circ#1 and anti-NC, sh-circ#1 and anti-miR-1296. (**B**) CCK-8 assay detected cell viability (%) on 48 h, and (**C**) colony formation assay measured colony numbers on 15 d. (**D** and **E**) FCM method examined cell rate (%) in different cell cycle phases on 24 h. (**F**) FCM method evaluated apoptosis rate (%) on 24 h. (**G** and **H**) Transwell assay measured migrated cell number and invaded cell number on 48 h. (**I** and **J**) Western blotting evaluated E-cad and N-cad protein expression on 24 h. *Represented $P<0.05$ and **Represented $P<0.01$.

7A). Restoration of *SP1* could rescue cell growth of *circ_0000520*-silenced MDA-MB-231 and MCF7 cells in vitro, as evidenced by increased cell viability and colony formation ability (Figure 7B and C), as well as S-phase cell rate (Figure 7D and E). Cell apoptosis rate was enhanced by sh-circ#1, and this effect was abolished by *SP1* overexpression via transfection (Figure 7F). Cell migration and invasion of MDA-MB-231 and MCF7 cells were facilitated when *circ_0000520* was knocked down, which could be further counteracted due to *SP1* upregulation (Figure 7G–J). Collectively, we proposed the anti-tumor role of *circ_0000520* downregulation was dependent on *SP1* inhibition via *miR-1296*.

Silencing circ_0000520 Restrained Tumorigenesis of BCa Cells in vivo

We already defined *circ_0000520* as an oncogene in BCa cells in vitro, the role of *circ_0000520* in tumorigenesis of BCa cells was studied in depth in vivo. Subcutaneous xenograft tumor models displayed a restrained tumor growth in sh-circ#1 group, as supported by reduced tumor volume and tumor weight (Figure 8A and B). Notably, the gene expression in neoplasm tissues was further testified. RT-qPCR analysis demonstrated a low expression of *circ_0000520* and *SP1*, and Western blotting data declared a high expression of miR-1296 in mice neoplasms in circ#1 group (Figure 8C–E). These results preliminarily revealed a tumor-suppressive role of *circ_0000520* silencing in tumorigenesis of BCa cells in vivo through regulating *miR-1296* and *SP1*.

Discussion

Very recently, circRNAs had been as emerging new biomarkers for BCa, and potential role of several circRNAs were uncovered. For example, *circ-TFF1* was indicated to serve as an enhancer in cell proliferation, migration, invasion and epithelial-mesenchymal transition of BCa cells through absorbing *miR-326*,[17] and *circ_002178* was a tumor

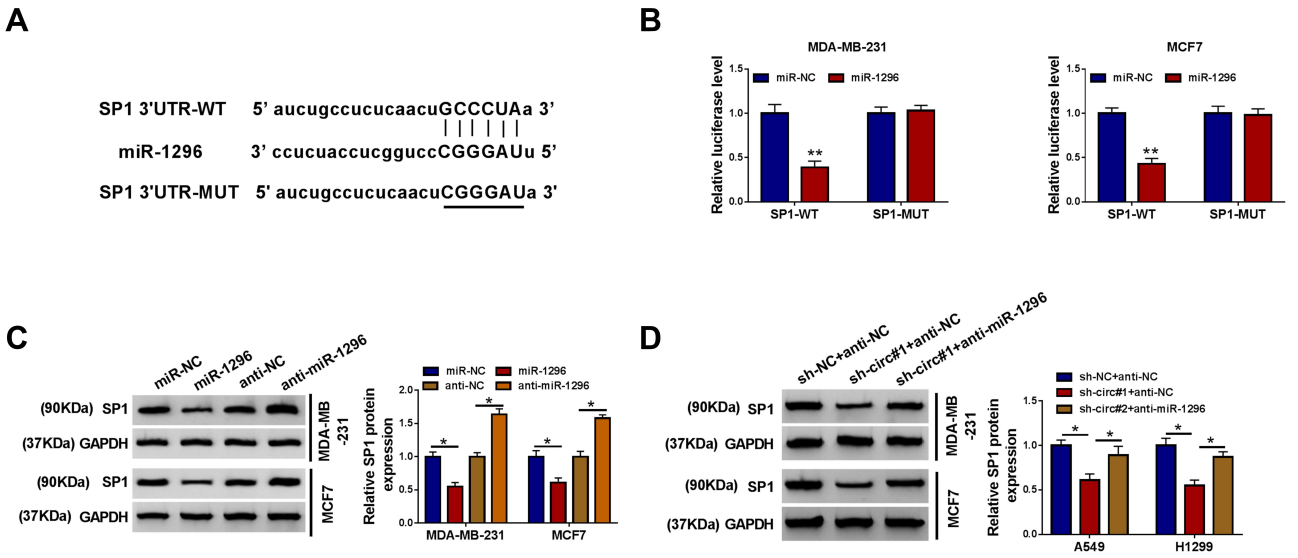

Figure 6 *Specificity protein 1* (*SP1*) was a direct target gene for *miR-1296*. (**A**) The starbase website predicted a potential binding site of *miR-1296* on 3′ untranslated region of *SP1* (SP1 3′UTR). (**B**) Dual-luciferase reporter assay identified the direct interaction between *SP1* 3′UTR and *miR-1296* in MDA-MB-231 and MCF7 cells. (**C** and **D**) Western blotting examined *SP1* protein expression in MDA-MB-231 and MCF7 cells transfected with miR-1296, miR-NC, anti-miR-1296, or anti-NC, and co-transfected with sh-NC and anti-NC, sh-circ#1 and anti-NC, or sh-circ#1 and anti-miR-1296. *Represented $P<0.05$ and **Represented $P<0.01$.

suppressor in BCa cell viability, energy metabolism and tube formation ability by sponging *miR-328-3p*.[18] Furthermore, circRNA-miRNA networks were proposed to be responsible for the development, prognosis and therapy of BCa.[19] However, the link between circRNAs and BCa progression had not been well disclosed. Herewith, we researched the differently expressed circRNAs in GEO database (GSE101123 dataset), and found one interested circRNA, that was *circ_0000520*. Subsequently, we aimed to identify the expression of *circ_0000520* in a group of BCa patients, and to figure out the biological role of *circ_0000520* in BCa cells, as well as the underlying *circ_0000520*-miRNA-mRNA pathway.

Together with RT-qPCR data in BCa tumor tissues, we considered that *circ_0000520* was upregulated in BCa. The high expression of *circ_0000520* seemed to predict a shorter 5-year overall survival in this cohort of BCa patients. Functional experiments displayed *circ_0000520* could be an oncogene in BCa cell growth, migration and invasion both in vitro and in vivo. Therefore, we suggested *circ_0000520* as a potential potent biomarker in the prediction and prognosis of BCa, due to its unique structure property. Thus, it could be better to further investigate the expression of *circ_0000520* in the plasma of patients, together with the relationship between plasma *circ_0000520* and either clinical features or BCa cell processes.[20,21] According to previous studies, *circ_0000520* was abnormally expressed in hepatocellular carcinoma (HCC) and gastric cancer (GCa).[21,22] In HCC, *circ_0000520* existed in hepatic tissue, and was highly expressed in HCC tissues (n=3) than non-tumor tissues depending on circRNA microarray scanning.[22] In GCa, *circ_0000520* was downregulated in tumor tissues, plasma and GCa cell lines from circRNA microarray (n=5) and RT-qPCR (n=56);[21] moreover, plasma *circ_0000520* showed a remarkable high diagnostic value in GCa patients. Taken our data and above two publications together, it might conclude that *circ_0000520* existed in tissues in a content-dependent manner and could be a biomarker in cancers.

The circinteractome database provided a *circ_0000520*-miRNA-mRNA interaction network, including *miR-556-5p*, *miR-224*, *miR-146b-3p*, and *miR-1296*.[21] Here, we found that *miR-1296* was the only one common potential target miRNA according to circinteractome, circBank and starbase. Thus, we took *miR-1296* as a candidate target for *circ_0000520*, and further testified this hypothesis using dual-luciferase reporter assay and RNA pull-down assay. *MiR-1296* had also been declared to exhibit tumor suppressive role in BCa. For example, Phan et al[14] probably gave birth to the first evidence of the anti-tumor activity of *miR-1296* through the decrease of cell proliferation and S-phase cells in triple-negative BCa cells (MDA-MB-231 and MDA-MB-436) by directly regulating *cyclinD1*. Chen et al[12] noticed that *miR-1296* overexpression inhibited clonogenic capacity and drug-resistance of *human epidermal growth factor receptor 2* (*HER2*)-positive BCa cells

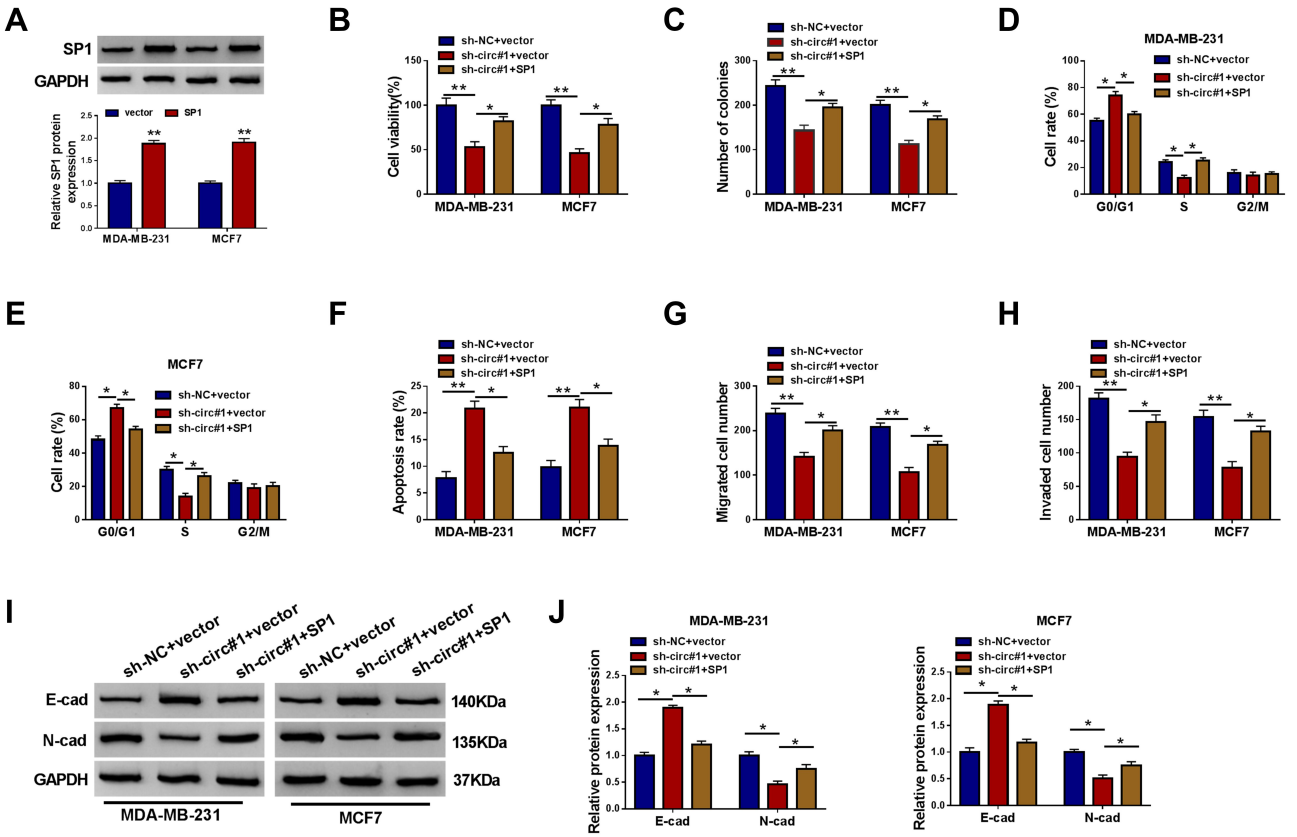

Figure 7 The effect of *SP1* restoration on the role of *circ_0000520* knockdown in BCa cells in vitro. (**A**) Western blotting measured *SP1* protein expression in MDA-MB-231 and MCF7 cells introduced with pIRES2-EGFP-SP1 vector (SP1) or pIRES2-EGFP vector (vector). (**B**–**J**) MDA-MB-231 and MCF7 cells were co-transfected with sh-NC and vector, or sh-circ#1 and vector, sh-circ#1 and SP1. (**B**) CCK-8 assay detected cell viability (%) on 48 h, and (**C**) colony formation assay measured colony numbers on 15 d. (**D** and **E**) FCM method examined cell rate (%) in different cell cycle phases on 24 h. (**F**) FCM method evaluated apoptosis rate (%) on 24 h. (**G** and **H**) Transwell assay measured migrated cell number and invaded cell number on 48 h. (**I** and **J**) Western blotting evaluated E-cad and N-cad protein expression on 24 h. *Represented $P<0.05$ and **Represented $P<0.01$.

(SK-BR-3 and BT-474) by targeting *HER2*. In addition, a number of miRNAs including *miR-1296* were associated with chemoresistance and self-renewal capability of MCF7 (*estrogen receptor* (*ER*)-positive) cells.[13] Here, we suggested a similar inhibitory role of *miR-1296* in BCa cells MDA-MB-231 and MCF7 cells. The higher level of *miR-1296* was underlay the suppressive effect of *circ_0000520* silencing on cell viability, colony formation, migration and invasion of BCa cells, as well as the promoting effect on cell apoptosis and cell cycle arrest. The target of *miR-1296* was also validated, and *SP1* was a novel downstream target of this miRNA.

SP1, belonging to the *SP/Kruppel like factor* family, was well-documented to be overexpressed in different cancers.[15] In BCa, *SP1* was 71.67% positive in tumor tissues (n=60), and was correlated with the cell proliferation, invasion and metastasis.[23] Mechanically, on one hand, a regulatory axis of miRNAs and *SP1* had been established. For example, *miR-200b*, *miR-539* and *miR-411* targeted *SP1* to suppress cell proliferation, cell cycle progression, cisplatin resistance, epithelial-mesenchymal transition, migration and invasion of BCa cells.[24–26] Furthermore, an auto-regulatory loop of *miR-22-SP1-c-Myc-miR-22* was also observed to participate in BCa invasion and metastasis.[27] On the other hand, an indirect interaction between miRNAs and *SP1* had also been discovered. For instance, *miR-106b* could depress the expression of *ZBTB4*, a suppressor of SPs including *SP1*, to regulate *E2H2* activity, thus affecting tumor growth and colonization of BCa cells in vivo.[28] Besides, Mertens-Talcott et al[29] early pointed out *miR-27a-ZBTB10* axis to modulate G2/M checkpoint in MDA-MB-231 cells, and *ZBTB10* was also a SPs repressor. Here, our data identified that *SP1* was one target of *miR-1296* in BCa, and could be regulated by *circ_0000520*. Functionally, restoration of *SP1* could improve cell viability, S-phase cells, colony formation ability, and migratory and invasive capacities, but decrease cell apoptosis rate in MDA-MB-231 and MCF7 cells. Lower expression of *SP1* was hidden in the suppression of *circ_0000520* knockdown on cell proliferation, invasion

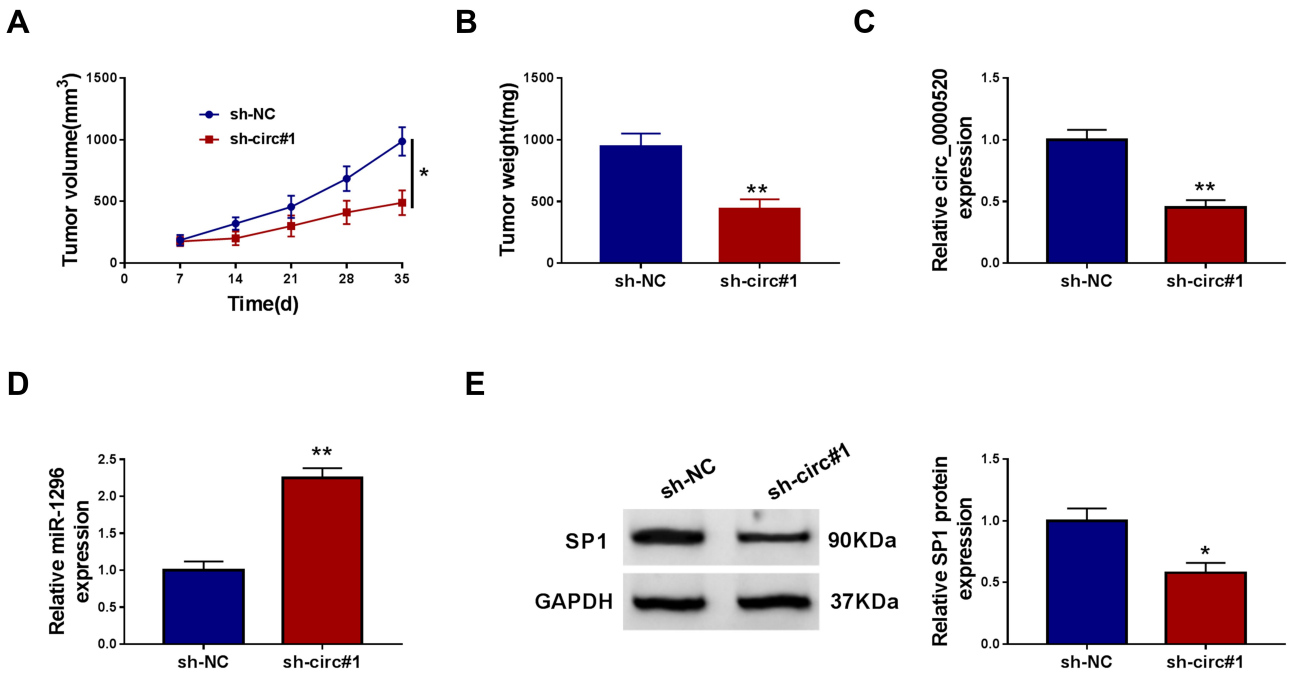

Figure 8 Silencing *circ_0000520* restrained tumorigenesis of BCa cells in vivo. MDA-MB-231 cells transfected with sh-circ#1 or sh-NC were subcutaneously transplanted into both sides of posterior flanks in nude mice (n=4). (**A**) Tumor volume was calculated every 7 days after implantation. (**B**) Tumor weight was measured after implantation for 35 days. (**C** and **D**) RT-qPCR measured *circ_0000520* and *miR-1296* expression, and (**E**) Western blotting detected *SP1* protein expression in xenograft neoplasm tissues. *Represented $P<0.05$ and **Represented $P<0.01$.

and tumor growth, which was in line with the previous data in SKBR3 and MDA-MBA-231 cells.[30] The pity of this study was that the relevant signaling pathway underlying had not further explored, such as *Smad3* signaling, *Erk*, *NF-κB* signaling pathways.[31,32]

In conclusion, this present study showed an upregulation of *circ_0000520* in BCa tumor tissues and cell lines, and an anti-tumor role of *circ_0000520* inhibition on cell viability, cell cycle progression, apoptosis, migration and invasion in MDA-MB-231 and MCF7 cells, as well as on tumor growth in vivo. The underlying molecular mechanism of *circ_0000520* in BCa cells was through regulating *miR-1296-SP1* axis. Thereby, we suggested *circ_0000520-miR-1296-SP1* pathway as a potential target for the treatment and prognosis of BCa.

Disclosure

The authors report no funding and no conflicts of interest for this work.

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