

Circular RNA hsa_circ_0008039 promotes proliferation, migration and invasion of breast cancer cells through upregulating CBX4 *via* sponging miR-515-5p

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Abstract. – **OBJECTIVE:** Breast cancer (BC) is the second most frequent malignancy worldwide. Hsa_circ_0008039 exerts the carcinogenic factors in BC. However, the pathogenesis of hsa_circ_0008039 involved in BC is still unclear.

PATIENTS AND METHODS: The expression levels of hsa_circ_0008039, microRNA-515-5p (miR-515-5p) and chromobox homolog 4 (CBX4) in BC tissues and cells were detected by real-time quantitative polymerase chain reaction (RT-qPCR). Cell proliferation, migration and invasion were assessed by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) and transwell assays, severally. The binding relationship among hsa_circ_0008039, miR-515-5p and CBX4 was predicted by starBase, then verified by the dual-luciferase reporter assay and immunoprecipitation (RIP) assay. The interaction between hsa_circ_0008039 and miR-515-5p was confirmed by RNA pull-down assay. The protein level of CBX4 was detected by Western blot assay. The biological role of hsa_circ_0008039 was detected by xenograft tumor model *in vivo*.

RESULTS: Hsa_circ_0008039 was upregulated in BC tissues and cells, and expedited proliferation, migration and invasion of BC cells. MiR-515-5p was downregulated in BC tissues and cells and worked as a target of hsa_circ_0008039. CBX4 was highly expressed in BC tissues and cells, and contributed to proliferation, migration and invasion of BC cells. Hsa_circ_0008039 enhanced CBX4 expression by competitively binding to miR-515-5p, thereby promoting BC development. Hsa_circ_0008039 knockdown repressed BC tumor growth *in vivo*.

CONCLUSIONS: These findings implicated that hsa_circ_0008039 contributed to proliferation, migration and invasion *in vitro* and promoted tumor growth *in vivo* by miR-515-5p/CBX4

axis in BC, suggesting a potential therapeutic strategy for BC treatment.

Key Words:

Hsa_circ_0008039, MiR-515-5p, CBX4, Proliferation, Metastasis, Breast cancer.

Introduction

Breast cancer (BC), a widespread malignant tumor, is the second most frequent malignancy and has the highest incidence in the world, with approximately 21,000,000 newly diagnosed worldwide in 2018¹. Although some therapeutic methods containing chemotherapy, radiation therapy and surgical operation have made substantial progress in recent decades, the overall prognosis of BC patients remains unsatisfactory². Hence, there is an imperative need to figure out the underlying mechanism of BC progression to develop more effective therapeutic targets.

Circular RNAs (circRNAs), a novel type of endogenous noncoding RNAs, are characterized by a circular covalently closed structure, participating in the regulation of gene expression in mammalian cells³. An extensive body of recent research has indicated that circRNAs could work as pivotal regulators in various biological processes⁴. Of note, Song et al⁵ displayed that hsa_circ_001564 boosted proliferation and inhibited cell cycle arrest, apoptosis by

binding to miR-29c-3p in osteosarcoma. Yao et al⁶ suggested that hsa_circ_0001946 could contribute to cell growth by activating Wnt/ β -catenin signaling pathway in lung adenocarcinoma. Noticeably, some researches manifested circRNAs acted as the potential diagnosis and therapeutic biomarkers in multiple cancers, including BC^{7,8}. Hsa_circ_0008039, located on chromosome 7, has been confirmed to exert the carcinogenic effect by promoting cell proliferation and migration via E2F3 in BC⁹. However, to date, the involvement of hsa_circ_0008039 in BC pathogenesis is still unclear.

MicroRNAs (miRNAs), about 22 nucleotides in length, have been reported to negatively regulate gene expression at the post-transcriptional level. Through competitively binding to the 3'-untranslated region (3'UTR) of target mRNAs, miRNAs reduce the stability or hinder translation of mRNA¹⁰. Rapidly accumulating evidence indicated that miRNA expression was tightly associated with the occurrence and development of various cancers, such as BC^{11,12}. MicroRNA-515-5p (miR-515-5p) was firstly reported as a fetal growth restriction (FGR) placenta-specific miRNA. Intriguingly, recent studies presented that miR-515-5p was downregulated, and miR-515-5p overexpression suppressed the formation and development in prostate cancer and non-small cell lung cancer^{13,14}. Moreover, several previous reports also confirmed that miR-515-5p could repress cell proliferation and migration in BC^{15,16}, suggesting the suppressive function of miR-515-5p in BC.

Chromobox homolog 4 (CBX4), located on chromosome 17q25.3, can function as a small ubiquitin-related modifier (SUMO) E3 ligase¹⁷. Interestingly, CBX4 has been reported as an oncogene or a tumor suppressor, in line with different cell content^{18,19}. CBX4 has been identified to exert the oncogenic activity in BC^{20,21}. However, the precise role of CBX4 in BC remains fuzzy.

In this research, we found that hsa_circ_0008039 was upregulated and the knock-down of hsa_circ_0008039 suppressed proliferation, migration and invasion of BC cells. Moreover, mechanism analysis suggested that hsa_circ_0008039 could contribute to BC progression *in vitro* and *in vivo* through miR-515-5p/CBX4 axis. Our study provided the evidence for the regulatory role of hsa_circ_0008039/miR-515-5p/CBX4, hinting a potential therapeutic strategy for BC treatment.

Patients and Methods

Clinical Samples and Cell Culture

The study was approved by the Ethical Committee of The People's Hospital of Jiaozuo. Before BC patients were enrolled in this research, written informed consent of every patient was obtained. BC tissues (n=35), as well as the matched normal tissues, were collected from The People's Hospital of Jiaozuo.

The human BC cell lines (MCF-7 and SKBR3) and the normal human breast epithelial cell line MCF10A were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained at 37°C with 5% CO₂ under moist atmosphere, and were cultured in Roswell Park Memorial Institute medium-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin mixture (100 units/mL penicillin, 100 μ g/mL streptomycin).

Cell Transfection

For the overexpression of hsa_circ_0008039 or CBX4, the pcDNA 3.1 vectors (pcDNA, Invitrogen, Carlsbad, CA, USA) containing the fragment of hsa_circ_0008039 or CBX4 were constructed by RiboBio (Guangzhou, China), named as hsa_circ_0008039/pcDNA 3.1 (hsa_circ_0008039) or CBX4/pcDNA 3.1. Short hairpin RNA (shRNA) against hsa_circ_0008039 (sh-hsa_circ_0008039), shRNA against CBX4 (sh-CBX4) and their corresponding negative control (sh-control), miR-515-5p mimic and its negative control (NC mimic), miR-515-5p inhibitor and its negative control (NC inhibitor) were purchased from GenePharma (Shanghai, China). All plasmids and oligonucleotides were transfected into MCF-7 and SKBR3 cells referring to the instructions of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from BC tissues and cells as per the operation manual of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand complementary DNA (cDNA) of hsa_circ_0008039, miR-515-5p and CBX4 was generated with a PrimeScript™ RT Master Mix Kit (TaKaRa, Otsu, Shiga, Japan). Then, RT-qPCR was enforced on an ABI7300 system (Applied Biosystems, Foster City, CA, USA) using an

SYBR Premix DimerEraser Kit (TaKaRa, Otsu, Shiga, Japan). The relative expression of hsa_circ_0008039, miR-515-5p and CBX4 was calculated by the $2^{-\Delta\Delta C_t}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the expression of hsa_circ_0008039 and CBX4, and U6 small nuclear RNA (snRNA) was applied to normalize miR-515-5p expression level. The specific primers were as follows:

Hsa_circ_0008039: 5'-AACGTGCTCTTC-GCTCACCT-3' (sense), 5'-CGTACAGCTCAGCCCTTCA-3' (antisense);

MiR-515-5p: 5'-TTCTCCAAAAGAAAG-CACTTTCTG-3' (sense), 5'-TGGTGTCGTG-GAGTCG-3' (antisense);

CBX4: 5'-AGTGGAGTATCTGGTGAAAT-GGA-3' (sense), 5'-TCCTGCCTTTCCCT-GTTCTG-3' (antisense);

GAPDH: 5'-GTCAACGGATTTGGTCT-GTATT-3' (sense), 5'-AGTCTTCTGGGTGG-CAGTGAT-3' (antisense);

U6: 5'-ACGAATACCGGCGTGAGAAA-3' (sense), 5'-TCGTGAAAGACCGCAGCAAA-3' (antisense).

Cell Viability Assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) was implemented to assess proliferation ability of BC cells. Generally, MCF-7 and SKBR3 cells were seeded in 96-well plates at a density of 5×10^4 cells/well. Then, 20 μ L MTT (5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added in each well at the indicated time points (0 h, 24 h, 48 h, or 72 h), followed by incubation for another 4 h. After discarding the supernatant, 150 μ L dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added to stop the reaction. At last, the absorbance at a wavelength of 490 nm was determined using a microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA).

Cell Migration and Invasion Assay

Transwell chambers (Corning Incorporated, Corning, NY, USA) were applied to detect the migration and invasion of BC cells as per the supplier's direction. Notably, the transwell chamber coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for invasion assay. In brief, cell suspensions in serum-free medium (1×10^5 cells/well for migration, and 5×10^4 for invasion) were introduced into the upper chambers. The complete medium was added to the lower chambers.

At 24 h post-incubation, a cotton swab was used to wipe off the cells inside the upper chamber, while the migrated/invaded BC cells were fixed and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). At last, the staining cells were photographed and counted by using a microscope (Nikon, Tokyo, Japan).

Dual-Luciferase Reporter Assay

Partial fragments of hsa_circ_0008039 and CBX4 3'-UTR possessing wild-type or mutant-type miR-515-5p binding sites were obtained. Then, these sequences were inserted into psiCHECK luciferase reporter vectors (Promega, Madison, WI, USA), termed as hsa_circ_0008039-WT, hsa_circ_0008039-MUT, CBX4 3'-UTR-WT and CBX4 3'-UTR-MUT reporter plasmids. Subsequently, MCF-7 and SKBR3 cells were co-transfected with these constructed reporter plasmids and miR-515-5p mimic or negative control by Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA), respectively. Following cultivation for 48 h, the luciferase activities were assessed by the dual-luciferase reporter assay kit (Promega, Madison, WI, USA) based on the supplier's direction.

RNA Immunoprecipitation (RIP) Assay

To confirm the potential binding between miR-515-5p and hsa_circ_0008039 or CBX4, RIP assay was performed by using the Magna RIP kit (Millipore, Billerica, MA, USA). Generally, MCF-7 and SKBR3 cells were collected and lysed in complete RIP. Then, the cell extracts were maintained in complete RIP buffer containing magnetic beads with antibodies of Argonaute2 (Ago2, ab32381, 1:2000, Abcam, Cambridge, MA, USA) or immunoglobulin G (IgG, 1:100, ab172730, Abcam, Cambridge, MA, USA) as a negative control. After that, the complexes were captured and digested with proteinase K to extract RNA fraction. At last, RT-qPCR was performed to detect the enrichments of hsa_circ_0008039, miR-515-5p and CBX4.

RNA Pull-Down Assay

Biotinylated miR-NC (NC), miR-515-5p-WT and miR-515-5p-MUT were transfected into MCF-7 and SKBR3 cells with Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA, USA). After sonicating the samples, cell lysates were incubated with dynabeads M-280 Streptavidin (Invitrogen, Carlsbad, CA, USA). Finally, RNA enrichment was detected by RT-qPCR.

Western Blot Assay

Western blot was implemented with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system referring to the previous description²². Generally, separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Following blockage with 5% skim milk, the membranes were probed with primary antibodies CBX4 (HPA008228, 1:1000, Sigma-Aldrich, St. Louis, MO, USA) or GAPDH (G9545, 1:1000, Sigma-Aldrich, St. Louis, MO, USA). Then, the horseradish peroxidase (HRP)-conjugated secondary antibody (ab0208, 1:10000, Abcam, Cambridge, MA, USA) was incubated with the membranes. At last, bands were exposed by detection reagents (Amersham Biosciences, Little Chalfont, UK).

Tumor Xenograft Assay

For stable expression of sh-hsa_circ_0008039, the shRNA sequence of hsa_circ_0008039 was inserted into Lentiviral expression vector pLVX-IRES-neo (Invitrogen, Carlsbad, CA, USA) generating sh-hsa_circ_0008039 lentivirus plasmid. Then, MCF-7 cells were transfected with sh-hsa_circ_0008039 lentivirus plasmid, sh-control plasmid (pLVX-IRES-neo empty vector). The animal experiment was approved by the Institutional Committee for Animal Research of The People's Hospital of Jiaozuo. Six-week-old male BALB/C nude mice were obtained from the National Laboratory Animal Center (Beijing, China). Then, these mice were randomly divided into 2 groups (n=6 per group). Transfected MCF-7 cells (7×10^6) were subcutaneously injected into the left flank of the nude mice. Tumor volume was measured every week. 4 weeks later, tumors were excised and weighed. Besides, the expression levels of hsa_circ_0008039, miR-515-5p and CBX4 were assessed by RT-qPCR in xenograft tumors, and the protein level of CBX4 in xenograft tumors was measured by Western blot.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism7 (La Jolla, CA, USA). Student's *t*-test was used for comparisons between groups, and multiple groups were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. The values were presented as mean $\pm$ standard deviation (SD) from three independent experiments. $p < 0.05$ indicated a significant difference.

Results

Hsa_circ_0008039 Promoted Proliferation, Migration, Invasion of BC Cells

To investigate the function of hsa_circ_0008039 in BC, its expression level was detected by RT-qPCR assay. As compared with the normal human breast tissues (n=35), hsa_circ_0008039 was highly expressed in BC tissues (n=35) (Figure 1A). Also, we observed that the level of hsa_circ_0008039 was notably increased in BC cell lines (MCF-7 and SKBR3) relative to the normal human breast epithelial cell line MCF10A (Figure 1B). To further explore the role of hsa_circ_0008039 in BC cells, we knocked down hsa_circ_0008039 in MCF-7 and SKBR3 cells. As presented in Figure 1C, hsa_circ_0008039 level was strikingly downregulated in sh-hsa_circ_0008039-transfected MCF-7 and SKBR3 cells in comparison with cells transfected with sh-control. Then, proliferation, migration and invasion were assessed by the knockdown system. The results showed that depletion of hsa_circ_0008039 suppressed the proliferative ability of MCF-7 and SKBR3 cells (Figure 1D and 1E). Furthermore, decreases of migration (Figure 1F and 1G) and invasion (Figure 1H and 1I) were also viewed due to knockdown of hsa_circ_0008039 in MCF-7 and SKBR3 cells. All these results proved the involvement of hsa_circ_0008039 in BC progression.

MiR-515-5p was a Direct Target of hsa_circ_0008039

Growing evidence suggested that circRNA can exert its function by interacting with miRNA²³. Thus, the potential target miRNAs of hsa_circ_0008039 were predicted by the web-based bioinformatics software starBase2. As shown in Figure 3A, miR-515-5p was found to harbor some complementary binding sites with hsa_circ_0008039. To confirm this prediction, we executed luciferase reporter assay. The data suggested that the overexpression of miR-515-5p evidently reduced the luciferase activity of hsa_circ_0008039-WT reporter but not that of hsa_circ_0008039-MUT reporter (Figure 2B and 2C). Moreover, in order to further confirm the binding relationship between hsa_circ_0008039 and miR-515-5p in MCF-7 and SKBR3 cells, RIP assay was enforced. In this assay, we observed that cell extracts between hsa_circ_0008039 and miR-515-5p were notably enriched in anti-Ago2 group when compared with the IgG control group

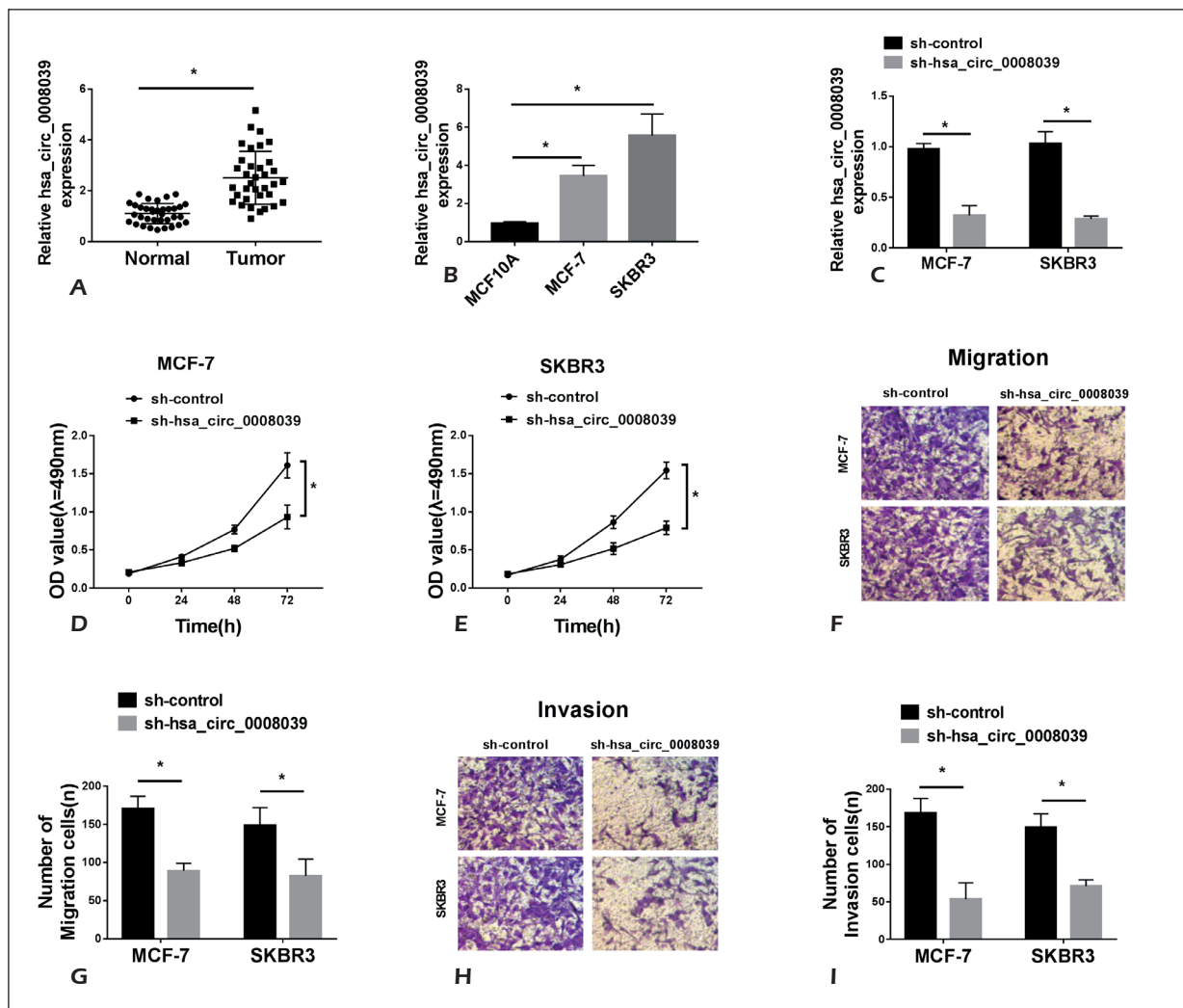


Figure 1. Identification of IgH gene rearrangement using FR3A and FR2A primers. 1: control; 2-5: FR3A primers; M: marker; 6-9: FR2A primers. Figure 1. Hsa_circ_0008039 facilitated proliferation, migration, invasion of BC cells. **A**, RT-qPCR assay was performed to detect the expression of hsa_circ_0008039 in 35 pairs of BC tumor tissues and adjacent normal tissues. **B**, Hsa_circ_0008039 expression level in BC cell lines (MCF-7 and SKBR3) and normal human breast epithelial cell line (MCF10A) was detected by RT-qPCR assay. **C**, Transfection efficiency of sh-hsa_circ_0008039 in MCF-7 and SKBR3 cells was validated via RT-qPCR assay. **D-E**, Cell viability was detected by MTT assay in sh-hsa_circ_0008039-transfected MCF-7 and SKBR3 cells. **F-G**, Cell migration capacity was assessed by transwell assay in sh-hsa_circ_0008039-transfected MCF-7 and SKBR3 cells. **H-I**, Cell invasion ability was detected by transwell assay in sh-hsa_circ_0008039-transfected MCF-7 and SKBR3 cells. * $p < 0.05$.

(Figure 2D and 2E), in agreement with bioinformatics analysis and luciferase assay. Besides, we implemented RNA pull-down assay to further verify the interaction between hsa_circ_0008039 and miR-515-5p. The results displayed that hsa_circ_0008039 enrichment was significantly increased in Bio-miR-515-5p-WT group in contrast to the Bio-miR-515-5p-MUT group and Bio-NC group (Figure 2F). In a word, hsa_circ_0008039 directly interacted with miR-515-5p. Additionally, we found that miR-515-5p expressed at lower level in both BC tissues and cells compared to respec-

tive control groups (Figure 2G and 2H). Meanwhile, RT-qPCR results disclosed that silencing of hsa_circ_0008039 induced the expression level of miR-515-5p in MCF-7 and SKBR3 cells (Figure 2I). Collectively, hsa_circ_0008039 interacted with miR-515-5p to hinder its expression.

CBX4 Accelerated Proliferation, Migration, Invasion of BC Cells

CBX4, an oncogenic factor, has been proved to be closely related with proliferation of BC²¹. Therefore, in this study, we observed that the lev-

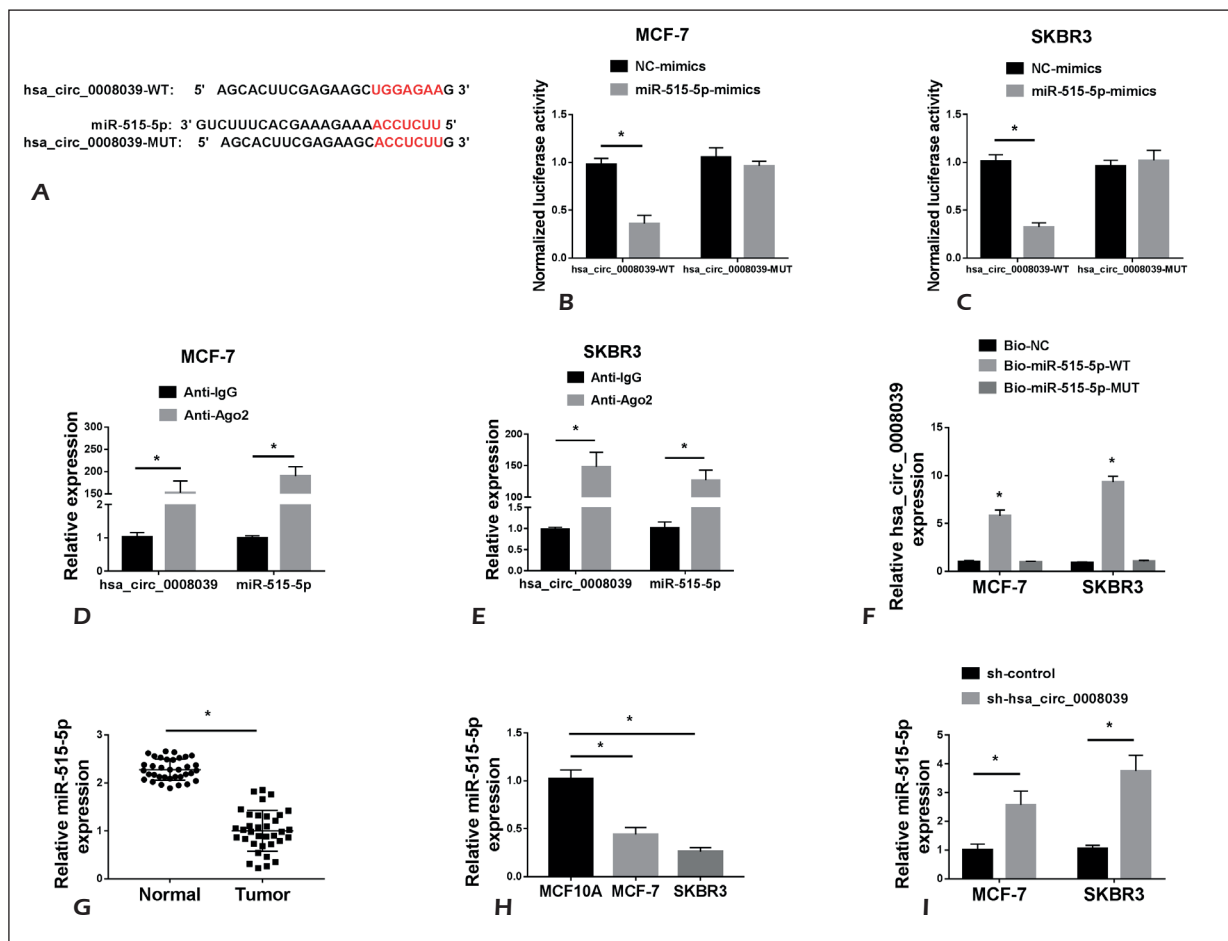


Figure 2. MiR-515-5p was a direct target of hsa_circ_0008039. **A**, Putative binding regions between hsa_circ_0008039 and miR-515-5p were exhibited. **B-C**, The effects of miR-515-5p overexpression on luciferase activity of hsa_circ_0008039-WT and hsa_circ_0008039-MUT reporters were measured by dual luciferase reporter assay in MCF-7 and SKBR3 cells. **D-E**, TRIP assay was performed in MCF-7 and SKBR3 cells extracts to examine miR-515-5p endogenously associated with hsa_circ_0008039. **F**, RNA pull-down assay was executed to detect the physical interactions between hsa_circ_0008039 and miR-515-5p in MCF-7 and SKBR3 cells. **G**, RT-qPCR assay was employed to measure the expression of miR-515-5p in 35 pairs of BC tumor tissues and adjacent normal tissues. **H**, Expression of miR-515-5p was detected by RT-qPCR in BC cell lines (MCF-7 and SKBR3) and normal human breast epithelial cell line (MCF10A). **I**, MiR-515-5p level was examined by RT-qPCR assay in MCF-7 and SKBR3 cells transfected with sh-control and sh-hsa_circ_0008039. * $p < 0.05$.

el of CBX4 was prominently upregulated in BC tumor tissues vs. adjacent normal tissues (Figure 3A and 3B). To further verify the role of CBX4 in BC cells, its expression was tested in MCF-7 and SKBR3 cells. As illustrated in Figure 3C and 3D, the apparent increase of CBX4 was confirmed in BC cells. Based on the above data, we knocked down CBX4 in MCF-7 and SKBR3 cells. Western blot results presented the transfection efficiency of sh-CBX4 in BC cells (Figure 3E). Afterwards, we used the knockdown system to further detect the biological function of CBX4 in the development of BC. Firstly, the results of MTT assay suggested that the knockdown of CBX4 blocked the

proliferative ability of MCF-7 and SKBR3 cells (Figure 3F). Similarly, the capacity of migration and invasion was markedly reduced by downregulating of CBX4 in MCF-7 and SKBR3 cells (Figure 3G). All these findings indicated that CBX4 might exert the carcinogenic effect in BC.

Hsa_circ_0008039 Elevated CBX4 Expression by Competitively Binding to miR-515-5p in BC Cells

Given that the circRNAs could act as a miRNA sponge to regulate mRNA, the underlying targets of miR-515-5p were predicted. As displayed in Figure 4A, there existed some potential binding

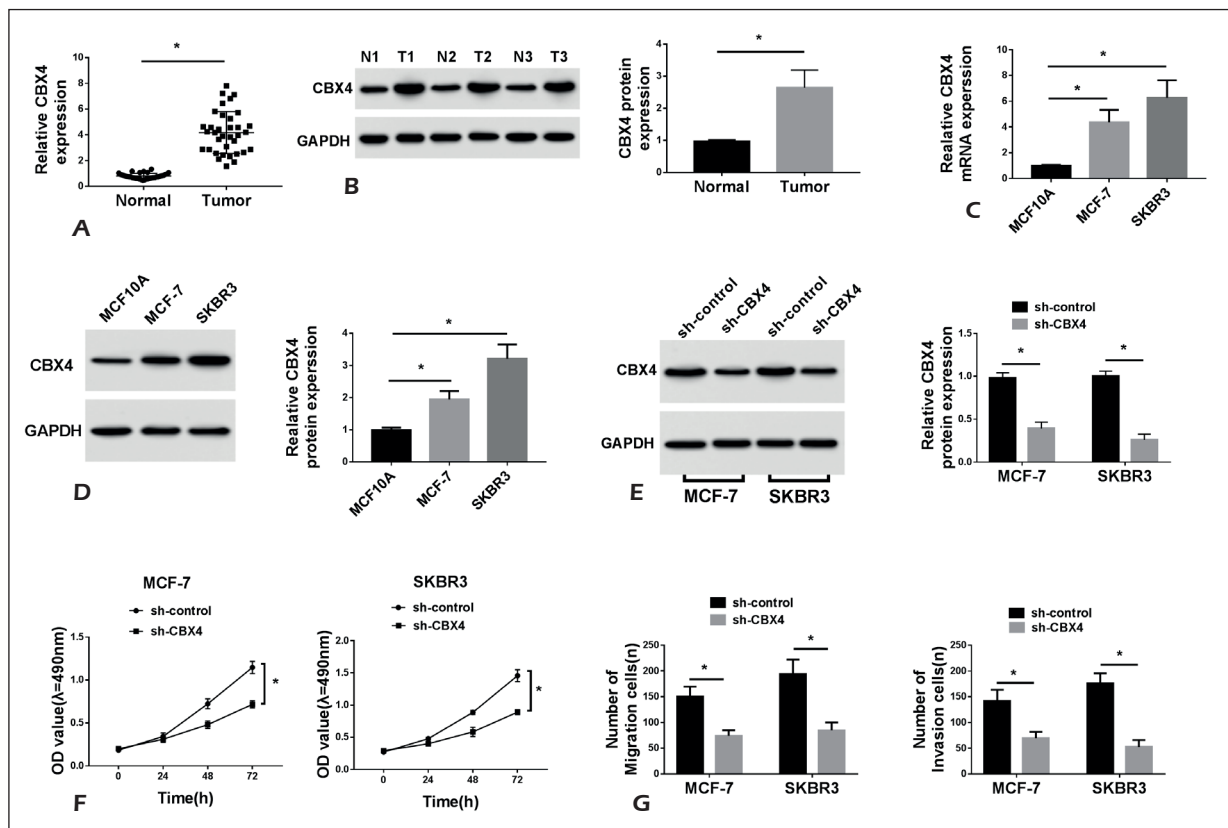


Figure 3. CBX4 promoted proliferation, migration, invasion of BC cells. **A**, The relative expression of CBX4 was detected by RT-qPCR assay in 35 pairs of BC tumor tissues and adjacent normal tissues. **B**, The protein level of CBX4 was detected by Western blot assay in BC tissues and adjacent normal tissues. **C**, The mRNA level of CBX4 was assessed by RT-qPCR assay in normal human breast epithelial cell line (MCF10A) and BC cell lines (MCF-7 and SKBR3). **D**, Western blot assay was employed to test the expression of CBX4 in normal human breast epithelial cell line (MCF10A) and BC cell lines (MCF-7 and SKBR3). **E**, The protein level of CBX4 was measured by Western blot assay in MCF-7 and SKBR3 cells transfected with sh-control and sh-CBX4. **F**, MTT analysis of proliferation in sh-CBX4-transfected MCF-7 and SKBR3 cells was conducted. **G**, Transwell analysis of migration and invasion capacity in sh-CBX4-transfected H460 and A549 cells was performed. * $p < 0.05$.

sites between miR-515-5p and CBX4. Consistent with bioinformatics analysis, luciferase reporter assay suggested that the overexpression of miR-515-5p suppressed the luciferase activity of CBX4 3'UTR-WT reporter, while had no effect on the luciferase activity of CBX4 3'UTR-MUT in MCF-7 and SKBR3 cells (Figure 4B and 4C). Importantly, RIP assay further proved the binding between hsa_circ_0008039, miR-515-5p and CBX4. As shown in Figure 4D and 4E, compared with the IgG control group, cell extracts between hsa_circ_0008039, miR-515-5p and CBX4 were strikingly enhanced in anti-Ago2 group. Also, the effects of hsa_circ_0008039 and miR-515-5p on CBX4 expression were detected in BC cells. RT-qPCR results revealed that deficiency of hsa_circ_0008039 led to an overt decrease of CBX4 mRNA level, while the reintroduction of miR-

515-5p inhibitor effectively abrogated the changes (Figure 4F). Similar to the RT-qPCR results, hsa_circ_0008039 knockdown repressed the protein expression of CBX4, which was conspicuously relieved by suppression of miR-515-5p (Figure 4G and 4H). Our study is the first to demonstrate that hsa_circ_0008039 acted as a competitive endogenous RNA (ceRNA) of miR-515-5p to improve CBX4 expression in BC cells.

Hsa_circ_0008039 Facilitated to Proliferation and Metastasis of BC Cells by Sponging miR-515-5p to Upregulate CBX4

As mentioned above, we inferred that the regulatory effect of hsa_circ_0008039 on BC progression was mediated by miR-515-5p/CBX4 axis. To prove the inference, we first constructed the

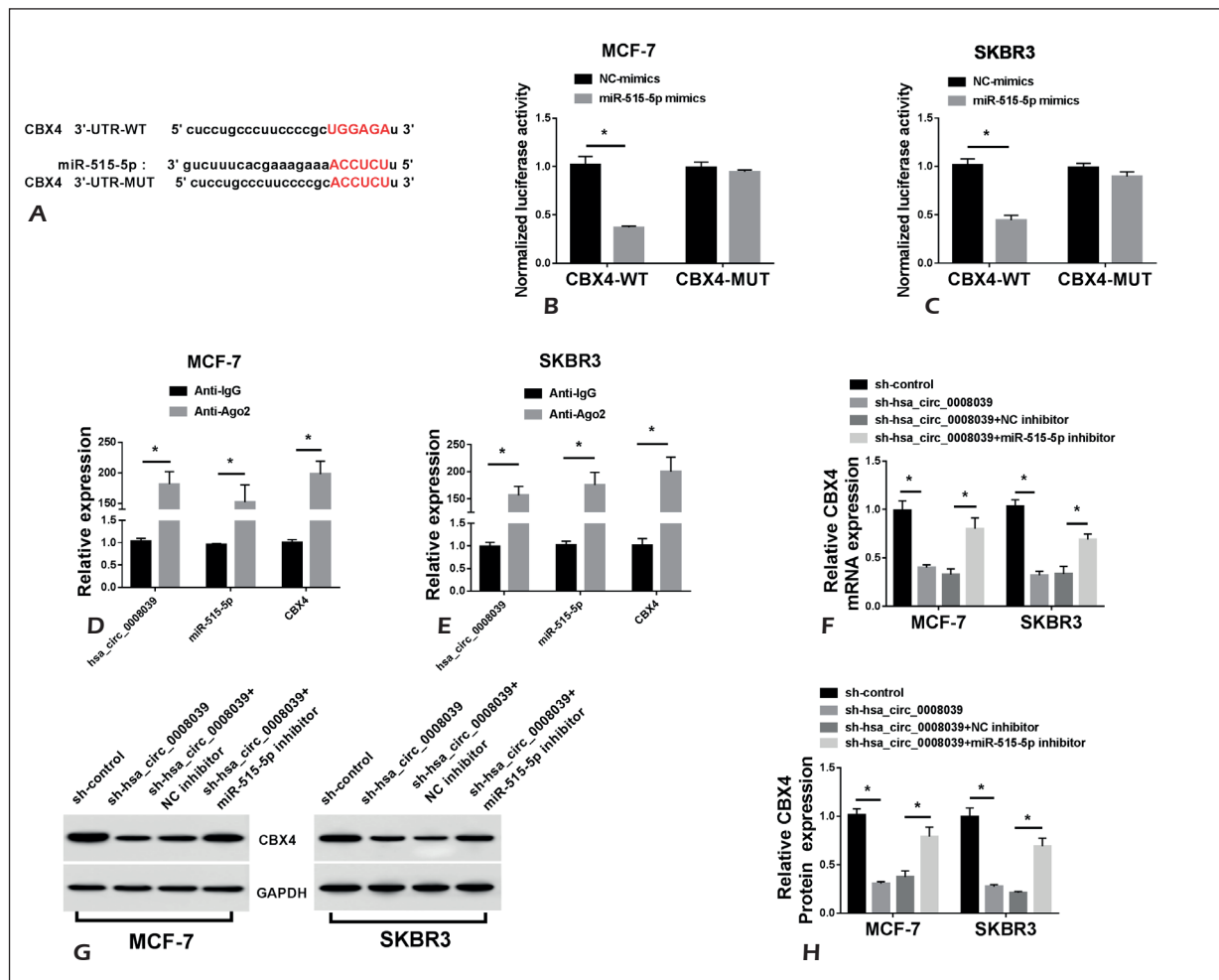


Figure 4. Hsa_circ_0008039 elevated CBX4 expression by sponging miR-515-5p in BC cells. **A**, The predicted binding sites between miR-515-5p and CBX4 3'UTR-WT and the sequences of CBX4 3'UTR-MUT were shown. **B-C**, The interactions between miR-515-5p and CBX4 3'UTR were testified by luciferase activity analysis. **D-E**, RIP assay was performed in MCF-7 and SKBR3 cells to determine the binding condition between hsa_circ_0008039, miR-515-5p and CBX4 3'UTR. **F**, The mRNA level of CBX4 was tested by RT-qPCR assay in MCF-7 and SKBR3 cells transfected with sh-control, sh-hsa_circ_0008039, sh-hsa_circ_0008039 + NC inhibitor and sh-hsa_circ_0008039 + miR-515-5p inhibitor. **G-H**, The protein level of CBX4 was detected by Western blot assay in transfected MCF-7 and SKBR3 cells. * $p < 0.05$.

overexpression system of CBX4 in BC cells. The transfection efficiency was detected and exhibited in Figure 5A and 5B. Functionally, miR-515-5p deficiency or CBX4 overexpression could abolish the suppressive effect of hsa_circ_0008039 knockdown on cell proliferation (Figure 5C and 5D). Similarly, the inhibition of cell migration and invasion caused by hsa_circ_0008039 downregulation was counteracted by reintroducing miR-515-5p inhibition or pcDNA3.1/CBX4 (Figure 5E and 5F). Collectively, we concluded that hsa_circ_0008039 accelerated BC cell growth and metastasis via sponging miR-515-5p to increase CBX4 *in vitro*.

Hsa_circ_0008039 Knockdown Inhibited BC Cell Growth *In Vivo*

Additionally, to detect the biologic function of hsa_circ_0008039 on cell growth *in vivo*, mice xenograft models of BC were established. As presented in Figure 6A and 6B, tumor volume and weight declined in presence of hsa_circ_0008039 downregulation, supporting that stable knockdown of hsa_circ_0008039 significantly repressed cell growth of BC *in vivo*. Besides, RT-qPCR analysis manifested that hsa_circ_0008039 and CBX4 mRNA levels were reduced in tumor tissues derived from sh-hsa_circ_0008039-transfected MCF-7 cells (Figure 6C and 6E), whereas

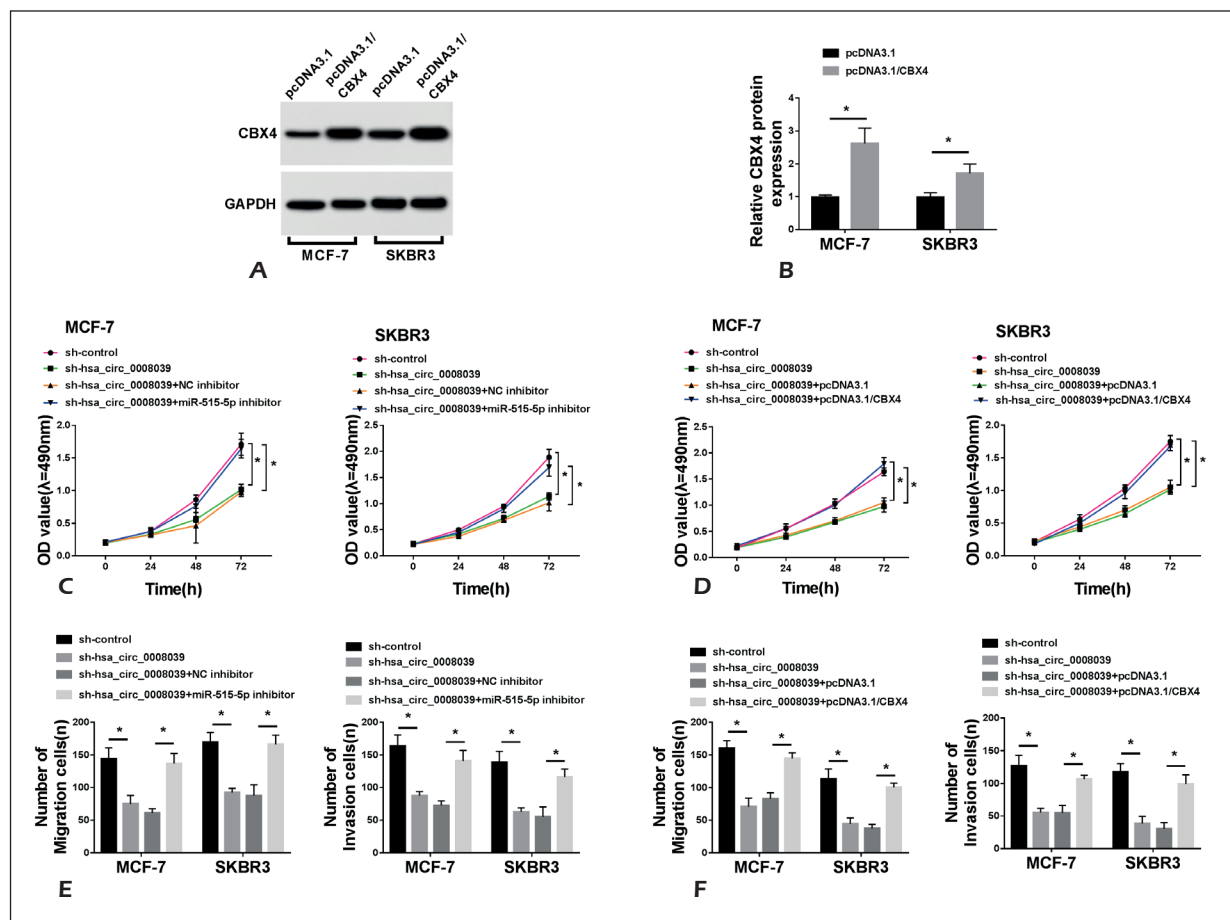


Figure 5. Hsa_circ_0008039 contributed to BC cells proliferation and metastasis by sponging miR-515-5p to upregulate CBX4. **A-B**, Transfection efficiency of pcDNA3.1/CBX4 in MCF-7 and SKBR3 cells was detected by RT-qPCR. **C-D**, Proliferation analysis in MCF-7 and SKBR3 cells transfected with sh-control, sh-hsa_circ_0008039, sh-hsa_circ_0008039 + pcDNA3.1 and sh-hsa_circ_0008039 + pcDNA3.1/CBX4 was implemented using MTT assay. **E-F**, The analysis of migration and invasion in transfected MCF-7 and SKBR3 cells was executed by transwell assay. * $p < 0.05$.

miR-515-5p mRNA level was elevated (Figure 6D). Simultaneously, Western blot results also confirmed that the protein level of CBX4 was downregulated in xenografts (Figure 6F and 6G). All of these might suggest that hsa_circ_0008039 deletion suppressed BC cell growth by regulating miR-515-5p/CBX4 axis *in vivo*.

Discussion

Mounting evidence suggests the pivotal roles of circRNAs in the modulation of numerous biological processes^{24,25}. It has been confirmed that circRNAs participate in the formation and development of many kinds of cancers, including BC^{26,27}. Noteworthy, previous studies have displayed that hsa_circ_0008039 is highly expressed and the

knockdown of hsa_circ_0008039 repressed proliferation and migration in BC⁹. However, the exact function and mechanism of hsa_circ_0008039 remain unclear in BC.

In this study, we showed that hsa_circ_0008039 was significantly upregulated in BC tissues and cells. Functionally, hsa_circ_0008039 was identified to exert the carcinogenic effect by accelerating proliferation, migration and invasion in BC progression. A substantial body of evidence proved that circRNAs could exert the role by the interaction of miRNA^{28,29}. In this study, there are some common complementary sequences between hsa_circ_0008039 and miR-515-5p in BC cells. Then, their direct interaction was confirmed by dual-luciferase reporter assay, RIP assay and RNA pull-down assay. Furthermore, our results exhibited that miR-515-5p was downregulated in

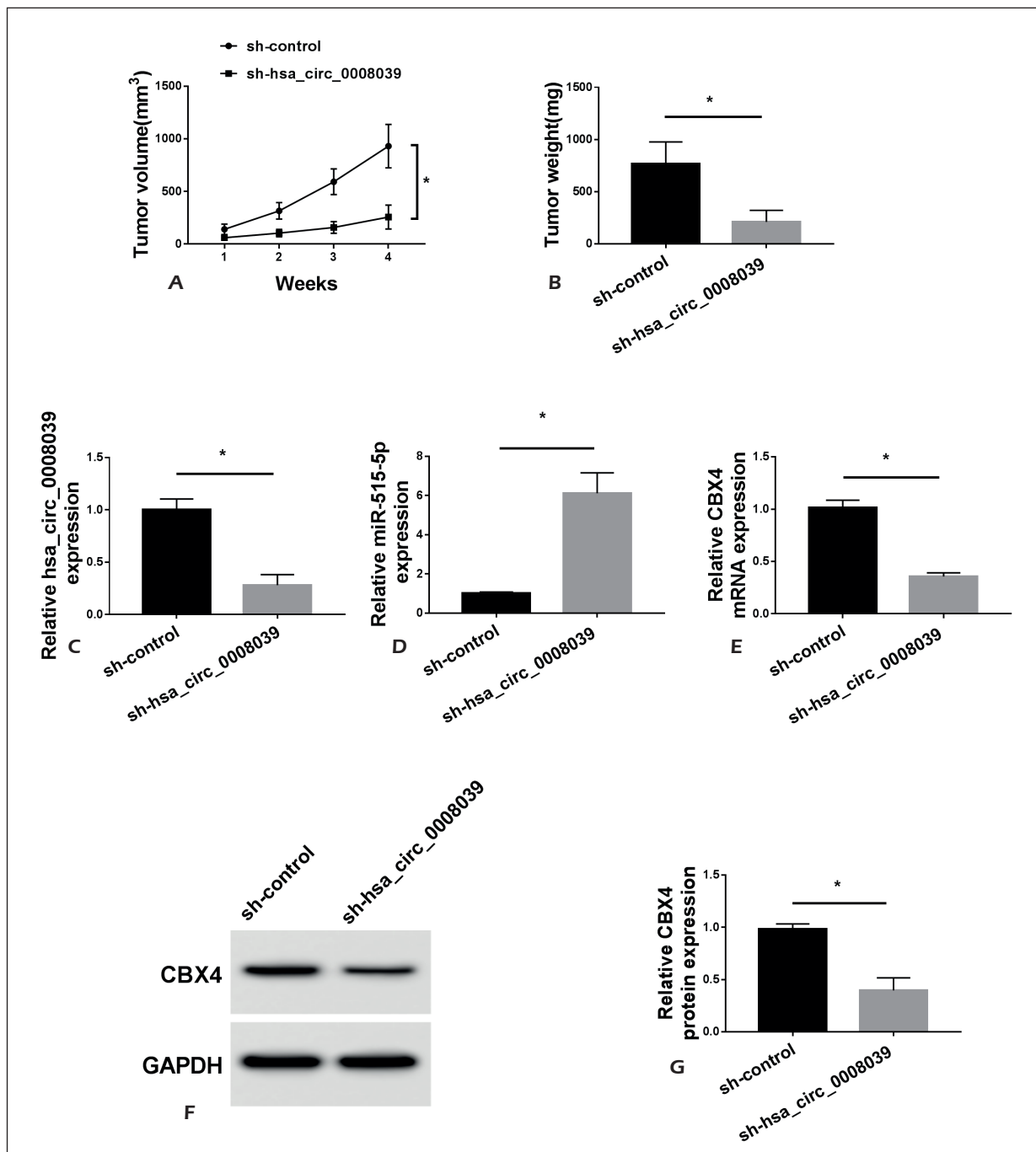


Figure 6. Hsa_circ_0008039 knockdown inhibited BC cell growth in vivo. **A-B**, Tumor volume and tumor weight were measured. **C**, The level of sh-hsa_circ_0008039 in xenografts was detected by RT-qPCR. **D-E**, Expression levels of miR-515-5p and CBX4 were measured by RT-qPCR in xenografts. **F-G**, The protein level of CBX4 in xenografts was detected by Western blot assay. * $p < 0.05$.

BC tissues and cells, in agreement with recent report³⁰. Also, the miR-515-5p level was negatively correlated with hsa_circ_0008039 in BC cells. Thus, hsa_circ_0008039 interacted with miR-515-5p to inhibit its expression.

Previous research showed that CBX4 could act as oncogenic factors in the development of BC²¹. In this investigation, we demonstrated that CBX4 level was upregulated in BC tissues and cells. Simultaneously, our results suggested that CBX4 deficiency inhibited

BC cell proliferation, migration and invasion, supporting the potential oncogenic role. It has been confirmed that circRNA could act as the ceRNA to isolate miRNA away from miRNA^{31,32}. Thus, in this study, we first proved the binding among hsa_circ_0008039, miR-515-5p and CBX4 by RIP assay. Then, to further investigate whether hsa_circ_0008039 could act as a ceRNA of miR-515-5p to regulate CBX4 expression, the effect of hsa_circ_0008039 on CBX4 level was explored in BC cells. In our study, RT-qPCR assay and Western blot assay proved that miR-515-5p inhibition partly reversed the suppression effect of hsa_circ_0008039 deficiency on CBX4 level in BC cells, supporting that circRNA worked as a ceRNA of miRNA to regulate mRNA expression. Based on the above findings, we speculated that hsa_circ_0008039/miR-515-5p/CBX4 regulatory axis might be involved in the development of BC. To validate the inference, rescue assays were conducted. As expected, miR-515-5p deletion or CBX4 overexpression could reverse hsa_circ_0008039 knockdown-mediated decrease in proliferation, migration and invasion of BC cells *in vitro*.

Next, the mice xenograft models of BC further verified that the downregulation of hsa_circ_0008039 repressed tumor growth *in vivo*. Synchronously, hsa_circ_0008039 silencing led to an enhancement of miR-515-5p level and caused a decrease of CBX4 expression, demonstrating that hsa_circ_0008039 deficiency hindered tumor growth by modulating the miR-515-5p/CBX4 axis *in vivo*.

Conclusions

The novelty of this study is that hsa_circ_0008039 served as the ceRNA of miR-515-5p to upregulate CBX4 expression, thereby facilitating BC development. Our findings elucidated an underlying molecular basis of hsa_circ_0008039 in progress and offered a possible therapeutic target for BC treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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