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ORIGINAL ARTICLE

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Circ_ZFR affects FABP7 expression to regulate breast cancer progression by acting as a sponge for miR-223-3p

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Abstract

Background: Breast cancer (BC) is a common malignancy in women. Circular RNAs (circRNAs) have been reported to play a key role in the development of BC; however, the effect of circular RNA zinc finger RNA binding protein (circ_ZFR) in BC is unknown.

Methods: Abundances of circ_ZFR, fatty acid binding protein 7 (FABP7), and microRNA-223-3p (miR-223-3p) were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The circular structure of circ_ZFR was validated by RNase R treatment. Cell proliferation, migration, invasion, and apoptosis were assessed by colony formation, cell counting kit-8, Transwell, flow cytometry assays, respectively. All protein levels were determined by Western blot. Dual-luciferase reporter assay was used to confirm the relationship between miR-223-3p and circ_ZFR or FABP7. A xenograft model was established to understand the effect of circ_ZFR on BC cell growth in vivo.

Results: The expression levels of circ_ZFR and FABP7 were higher in BC tissues and cell lines, whereas miR-223-3p expression was lower. Knockdown of circ_ZFR or FABP7 in BC cells reduced proliferation, migration, invasion, and epithelial mesenchymal transition (EMT), and induced apoptosis in vitro, whereas the opposite effects were observed in circ_ZFR-overexpressed cells. Furthermore, circ_ZFR might act as a sponge for miR-223-3p to regulate FABP7 expression, thereby promoting the progression of BC cells in vitro and in vivo.

Conclusion: Circ_ZFR might act as a miRNA sponge for miR-223-3p to regulate FABP7, thereby promoting proliferation, migration, invasion, and EMT of BC cells, and inhibiting cell apoptosis.

KEYWORDS
breast cancer, Circ_ZFR, FABP7, miR-223-3p

INTRODUCTION

Breast cancer (BC) is the phenomenon that mammary gland epithelial cells proliferate out of control under the action of a variety of carcinogenic factors. BC is one of the most common malignant tumors among women in the world with high morbidity and mortality.[1] Despite significant improvements in surgical treatment, the recurrence rate and survival rate in BC patients remain unsatisfactory.[2,3] Recently, the function of circular RNAs (circRNAs) in cancer has attracted a lot of attention,[4] and finding circRNA molecules that can be used as an early diagnosis and targeted therapy for BC is a huge challenge.

CircRNAs are novel discovered non-coding RNAs (ncRNAs), and their 3′ heads are joined to the 5′ tails to form the closed loop structures.[5] Through the circRNA sequencing data, a large number of circRNAs have been identified in BC and they can involve in BC progression. For instance, circRNF20 could serve as a promoter of BC tumorigenesis and Warburg effect by sponging miR-487a.[6]

Circ_0001283 restrained cell proliferation and invasion in BC through functioning as miR-187 sponge.[7] Furthermore, hsa_circ_0061825 could trigger BC cell growth and metastasis by targeting miR-326.[8] Moreover, high-throughput circRNA microarray analysis has identified several differently expressed circRNAs in BC tissues, and circRNA zinc finger RNA binding protein (circ_ZFR) was one of the most upregulated circRNA in BC tissues.[9] Several research studies also confirmed that circ_ZFR exerted a carcinogenic function in hepatocellular carcinoma and papillary thyroid cancer (PTC).[10,11] However, the function and molecule mechanism of circ_ZFR in BC are largely unknown.

Fatty acid binding protein 7 (FABP7) is a member of the FABP intracellular lipid chaperone family, which is normally expressed in the mammary gland, and FABP7 was substantially increased in triple-negative BC than other BC subtypes.[12] Many studies have confirmed the pivotal role of FABP7 in BC progression and metastasis. For instance, high level of FABP7 correlated with the lower survival and higher incidence of brain metastases in BC patients and regulated the metabolic reprogramming process in HER^{2+} breast cancer cells.[13] Moreover, FABP7 targeted RXRβ and promoted cell proliferation in triple-negative breast cancer (TNBC), which could be a novel therapeutic target for the treatment of TNBC.[14] However, the upstream regulatory mechanism of FABP7 in BC is largely unknown.

CircRNAs generally perform its biological functions by regulating microRNAs (miRNAs) activity to regulate gene expression.[15] MiRNAs are also ncRNA molecules, which play key regulatory roles in BC development, such as miR-199-5p,[16] miR-27a,[17] and miR-99a.[18] MiR-5119 could potentiate the immunotherapy for BC patients.[19] MiR-223 is located in the q12 segment of the human X chromosome, and it acts as a tumor promoter in multiple tumors, including colon cancer,[20] neuroblastoma,[21] and gastric cancer.[22] However, miR-223-3p could inhibit cell growth in osteosarcoma.[23] Moreover, miR-223-3p has been shown to repress the development of BC cells.[24] Bioinformatics analysis uncovered that miR-223-3p might be the target of circ_ZFR and target FABP7 in BC cells. Therefore, we set out to elucidate the correlation and potential mechanism among circ_ZFR, miR-223-3p, and FABP7 in BC.

In our study, circ_ZFR expression in BC tissues and cells were analyzed, and we also investigated its biological roles in BC cell progression. Furthermore, we identified the interaction among circ_ZFR, miR-223-3p, and FABP7. Overall, we aimed to explore new biomarkers for the diagnosis and prognosis of BC.

MATERIALS AND METHODS

Gene Expression Omnibus data

In this study, Gene Expression Omnibus (GEO) breast cancer microarray data GSE101124 and GSE62931, which were downloaded from the National Center for Biotechnology Information (NCBI) GEO dataset, were selected as the research object. The GSE101124 dataset contains high-throughput array data of circRNAs in eight BC patients' specimens (luminal A, $n = 4$; TNBC, $n = 4$) and three normal mammary gland tissues. The GSE62931 dataset consisted of 53 non-TNBC breast cancer tumor tissue chips and 53 TNBC tumor tissue chips. The threshold set for significant differences was log2|fold change|≥2 and $p < 0.05$.

Patient tissues and cell culture

BC tissues and adjacent normal tissues of 50 BC patients (ages 32–78; LA, $n = 10$; LB, $n = 8$; Her-2, $n = 6$; TNBC, $n = 26$) were obtained from Hunan Provincial People's Hospital. These samples were immediately frozen in liquid nitrogen and then stored in −80°C for further analysis. The study was carried out under the permission of the Ethics Committee of Hunan Provincial People's Hospital. The informed consent forms were obtained from all participators.

BC cell lines (MCF-7, MDA-MB-231, BT-549, and T-47D) and normal mammary epithelial cell line MCF-10A acquired from American Type Culture Collection (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) at 37°C with 5% CO_2. Additionally, DMEM was contained 10% fetal bovine serum (FBS) and 0.1% penicillin/streptomycin (Invitrogen).

Transfection

To knockdown circ_ZFR or FABP7, small interfering RNA (siRNA) against circ_ZFR (si-circ_ZFR) or FABP7 (si-FABP7) and the negative control (si-NC), short-hairpin targeting circ_ZFR (sh-circ_ZFR) and its control (sh-NC) were synthesized by GenePharma. MiR-223-3p mimic (miR-223-3p) or its inhibitor (anti-miR-223-3p) and their respective controls (miR-NC, anti-miR-NC) were also synthesized by GenePharma. Overexpression vectors of circ_ZFR and FABP7 and their corresponding controls (Vector, pcDNA) were provided by Sangon Biotech. Cells were harvested for subsequent experiments at 24 or 48 hours after transfection by Lipofectamine 3000 (Invitrogen).

Quantitative real-time polymerase chain reaction and RNase Rtreatment

The RNA in BC tissues and cells was isolated through TRIzol (Invitrogen) and was then hatched in RNase R (3 U/mg) for 20 min at 37°C. Reverse transcription was performed by using the PrimeScript RT Reagent (TaKaRa). Next, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted by using the TB Green Premix Ex Taq (TaKaRa). The internal reference for circRNA and mRNA quantification was GAPDH, whereas the internal control of miRNA was U6. The primer sequences were shown as below:

circ_ZFR, F: 5′-ATGGTCTGCAGTCCTGTGTG-3′ and R: 5′-TGGTGGCATGTTTTGTCATT-3′. ZFR, F: 5′-TCCCA ATGCTAAGGAGATGC-3′ and R: 5′-TTCTTCTCGTCTT CGCCAGT-3′. FABP7, F: 5′-CTCAGCACATTCAAGAAC ACG-3′ and R: 5′-CCATCCAGGCTAACAACAGAC-3′. miR-223-3p, F: 5′-GCGCGTGTCAGTTTGTCAAAT-3′ and R: 5′-AGTGCAGGGTCCGAGGTATT-3′. GAPDH, F: 5′-AATGGGCAGCCGTTAGGAAA-3′ and R: 5′-GCGCCCA ATACGACCAAATC-3′. U6, F: 5′-GCTTCGGCAGCACA TATACTAAAAT-3′ and R: 5′-CGCTTCACGAATTTGCG TGTCAT-3′.

Cell colony formation and proliferation assay

For cell colony formation, MCF-7 and MDA-MB-231 cells were seeded in the 6-well plates in the fresh medium. The colonies were stained with 0.1% crystal violet for 20 minutes (Solarbio). Fourteen days later, the colony number in each well was photographed and calculated.

For cell proliferation assay, cells seeded in the 96-well plates were transfected. At the specified point of time (0, 1, 2, and 3 day) after transfection, 10 μL cell counting kit-8 solution (CCK-8, Solarbio) was added to the cells. After incubating for 3 hours, a microplate reader was used to examine the optical density (OD) value of cells at 450 nm.

Transwell assay

Cell migration and invasion ability were analyzed by Transwell assay. Matrigel (Solarbio) was diluted and coated on the upper compartment of the chambers in invasion assay. Briefly, 200 μL cells resuspended in serum-free medium were added to the upper compartment of the chambers and 600 μL medium supplemented with 10% FBS was replenished to the lower compartment. Twenty-four hours later, the cells passing through the chambers were fixed and stained with 0.1% crystal violet for 20 minutes and then washed by phosphate-buffered saline (PBS). The stained cells were observed and photographed by a microscope.

Western blot assay

BC tissues and cells were lysed in radioimmunoprecipitation assay

buffer (RIPA) solution (Beyotime) contained protease inhibitors, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF, Beyotime) membranes. After being sealed in 5% skim milk for 2 hours, the membranes were hatched together with the primary antibodies targeting Snail (1:1000, Abcam), Twist1 (1:1000, Thermo Fisher Scientific), E-cadherin (1:500, Abcam), FABP7 (1:500, Thermo Fisher Scientific), or GAPDH (1:2000, Abcam) at 4°C overnight. The samples were then incubated with secondary antibodies goat anti-rabbit IgG H&L (HRP) or rabbit anti-mouse IgG H&L (HRP) (1:4000, Abcam) for 1 hour. The protein bands were examined by using an ECL reagent (Thermo Fisher Scientific).

Cell apoptosis assay

Cell apoptosis was detected using an annexin V-fluorescein isothiocynate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences). BC cells were harvested at 48 hours post-transfection, and cells were stained with 5 μL annexin V-FITC and 5 μL PI for 15 minutes in the dark. Flow cytometry was used to analyze cell apoptosis (BD Biosciences).

Dual-luciferase reporter assay

The sequences of circ_ZFR contained wild-type (wt) or mutant type (mut) miR-223-3p binding sites were inserted into the pmirGLO vector (Promega), which named circ_ZFR-wt and circ_ZFR-mut, respectively. FABP7-wt with wild-type miR-223-3p binding sites and FABP7-mut mutant type miR-223-3p binding sites were constructed. Next, the reporter vectors were co-transfected into MCF-7 and MDA-MB-231 cells with miR-223-3p or miR-NC, respectively. The luciferase activity following a 24 hours transfection was examined by a dual-luciferase reporter assay kit (Promega).

Animal experiments

The animal tests were approved by the Animal Care and Use Committee of Hunan Provincial People's Hospital. MDA-MB-231 cells were used in Xenograft models, and cells transfected sh-circ_ZFR or sh-NC were subcutaneously injected into the female nude mice (4-week-old, $n = 5$ each group). Tumor volume was examined by a caliper once a week. Four weeks later, mice were sacrificed and the tumors weight was detected. Furthermore, the tumors were cut off for further analysis.

Statistical analysis

Data were shown as the mean ± standard deviation (SD) from at least three separate tests. χ^2 test was used to analyze the differences between two groups, and for the comparison among multiple groups, one-way analysis of variance (ANOVA) was carried out. Pearson correlation coefficient was used to evaluate the correlation among circ_ZFR, miR-223-3p, and FABP7 expression in BC tissues. $p < 0.05$ was considered as the expression of statistical significance.

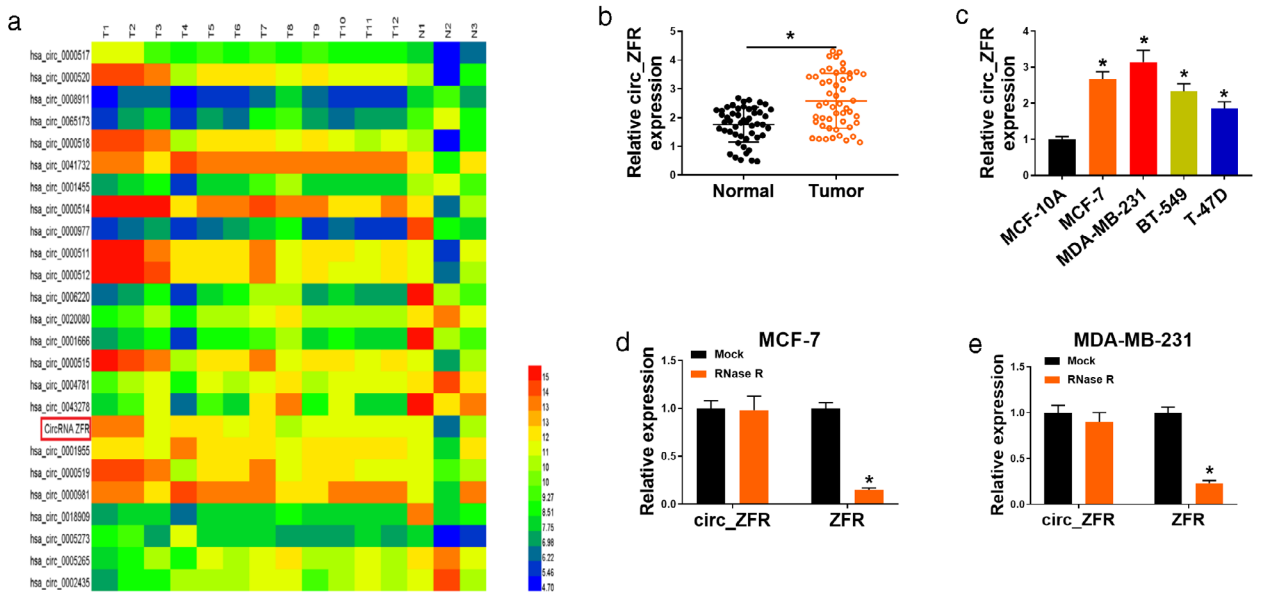

FIGURE 1 Circ_ZFR was upregulated in BC tissues and cell lines. (a) Twenty-five upregulated circRNAs in BC tissues compared with normal tissues were presented according to the GSE101124 dataset. (b),(c) relative expression of circ_ZFR in 50 cases of BC tissues and BC cell lines (MCF-7, MDA-MB-231, BT-549, and T-47D) compared with normal tissues and cell line (MCF-10A) was measured by qRT-PCR. (d),(e) MCF-7 and MDA-MB-231 cells were treated with or without RNase R for 20 minutes, and relative expression of circ_ZFR and its linear transcript ZFR were detected by qRT-PCR. $^*p < 0.05$

RESULTS

Circ_ZFR was up-regulated in BC tissues and cell lines

To investigate the roles of circRNAs in the tumorigenesis of BC, we first analyzed the expression profile of circRNAs in BC tissues based on the open GEO database (GSE101124). As displayed in Figure 1(a), 25 circRNAs were significantly upregulated in BC tissues compared to normal tissues. Among them, circ_ZFR was one of the most upregulated circRNAs in BC tissues, and has been reported to exert crucial functions in several tumors,[25–27] therefore, it was chosen as the subsequent study subject to investigate its function in BC. Subsequently, the expression of circ_ZFR was detected by qRT-PCR in 50 BC patients and adjacent normal tissues. The results showed that circ_ZFR expression was highly expressed in BC tissues compared with normal tissues (Figure 1(b)). Moreover, circ_ZFR expression in BC cell lines (MCF-7, MDA-MB-231, BT-549, and T-47D) was obviously increased in contrast with the normal mammary epithelial cells MCF-10A, especially in MCF-7 and MDA-MB-231 cell lines (Figure 1(c)). To confirm the circular form and loop structure of circ_ZFR, the expression of circ_ZFR and its linear transcript ZFR in MCF-7 and MDA-MB-231 cells treated with or without RNase R were examined by qRT-PCR. The results indicated that the linear form of ZFR was digested by RNase R, whereas circ_ZFR was resistance to digestion by RNase R exonuclease (Figure 1(d),(e)). These data implied that circular RNA circ_ZFR was upregulated in BC.

Circ_ZFR promoted cell proliferation, migration, invasion, and epithelial mesenchymal transition in BC

To investigate the role of circ_ZFR in BC, MCF-7 and MDA-MB-231 cells were transfected si-NC, si-circ_ZFR, vector or circ_ZFR overexpression vector, respectively. Circ_ZFR expression was decreased by half in cells with si-circ_ZFR transfection, whereas it was five times enhanced by transfection of circ_ZFR (Figure 2(a)). The colony formation assay showed that knockdown of circ_ZFR decreased the number of clones of MCF-7 and MDA-MB-231 cells (Figure 2(b)). CCK-8 assay indicated that depletion of circ_ZFR inhibited the proliferation of MCF-7 and MDA-MB-231 cells (Figure 2(c)). The Transwell assay data showed that the migration and invasion ability of MCF-7 and MDA-MB-231 cells were inhibited (Figure 2(d),(e)). Furthermore, the protein levels of epithelial mesenchymal transition (EMT) regulators Snail, Twist1, and E-cadherin in MCF-7 and MDA-MB-231 cells were detected by Western blot. As shown in Figure 2(f), the protein levels of Snail and Twist1 were decreased in cells transfected with si-circ_ZFR compared with si-NC group, whereas E-cadherin expression was increased by circ_ZFR depletion, indicating that circ_ZFR depletion inhibited EMT in BC cells. On the contrary, circ_ZFR overexpression obviously enhanced the ability of colony formation (Figure 2(g)), proliferation (Figure 2(h)), migration (Figure 2(i)), and invasion (Figure 2(j)) in BC cells. In addition, overexpression of circ_ZFR also increased the protein levels of Snail and Twist1, and decreased the expression of E-cadherin in MCF-7 and MDA-MB-231 cells (Figure 2(k)). These data indicated that circ_ZFR possesses

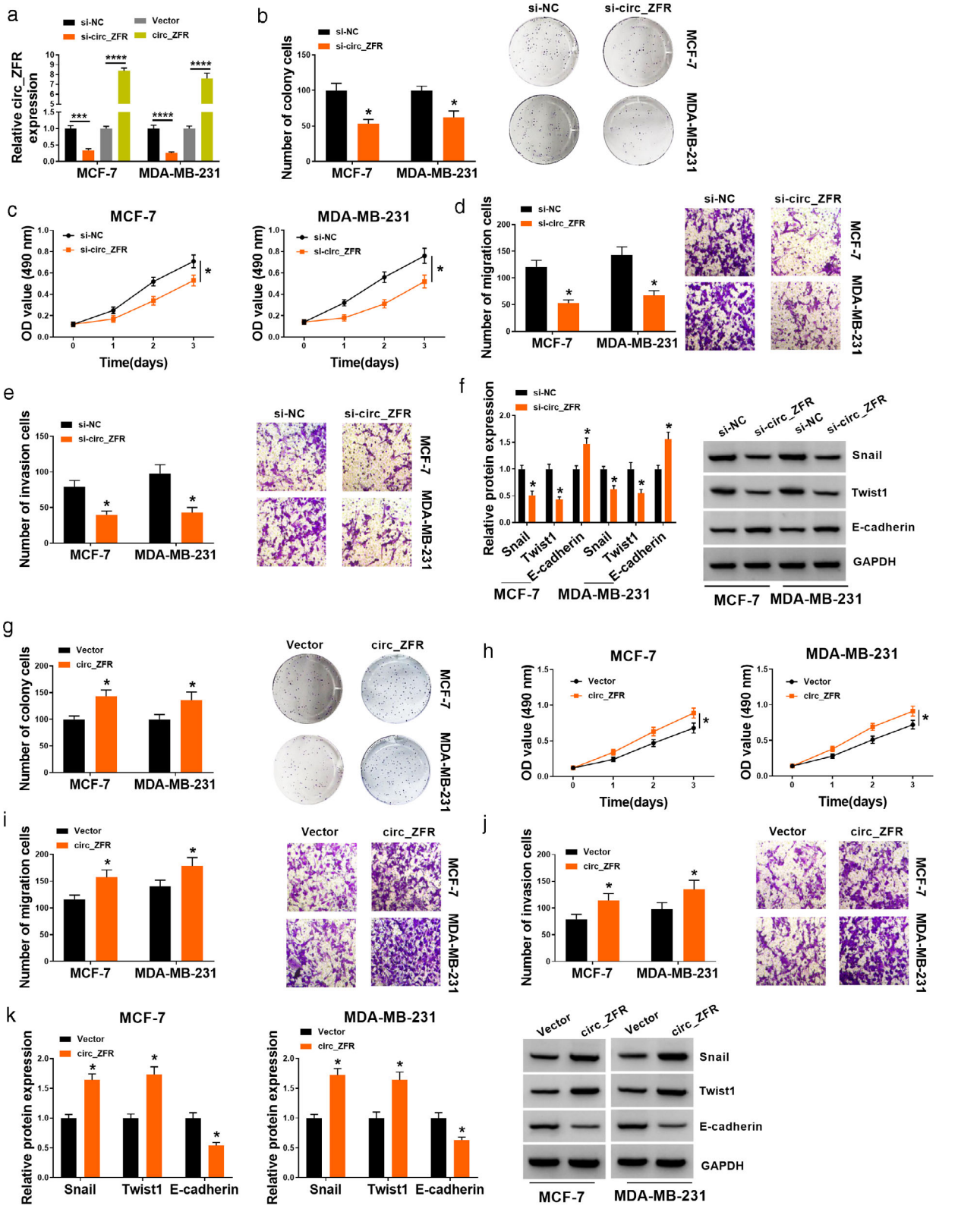

FIGURE 2 Circ_ZFR promoted cell proliferation, migration, invasion, and EMT in BC. (a) Relative expression of circ_ZFR in MCF-7 and MDA-MB-231 cells transfected with si-NC, si-circ_ZFR, vector or circ_ZFR was examined by qRT-PCR. (b)–(f) MCF-7 and MDA-MB-231 cells were transfected with si-NC or si-circ_ZFR. (b) MCF-7 and MDA-MB-231 cells were transfected with si-NC or si-circ_ZFR, and were allowed to incubate for 14 days, and the colony numbers were counted. (c) CCK-8 assay were performed to check cell proliferation ability, the OD value at 490 nm was recorded at 0, 1, 2, or 3 days on transfection. (d),(e) After transfection for 24 hours, cell migration and invasion abilities were assessed using Transwell assay. (f) Protein levels of Snail, Twist1, and E-cadherin were measured by Western blot assay. (g)–(k) MCF-7 and MDA-MB-231 cells were transfected with Vector or circ_ZFR, respectively. (g) Fourteen days on transfection, colony numbers was examined. (h) CCK-8 assay was used to detect cell viability, and the OD value of BC cells was detected by at 0, 1, 2, or 3 days on transfection. (i),(j) Twenty-four hours on transfection, the migration and invasion of transfected BC cells were assessed by Transwell assay. (k) Western blot assay was used to examine the protein levels of Snail, Twist1, and E-cadherin. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.001$

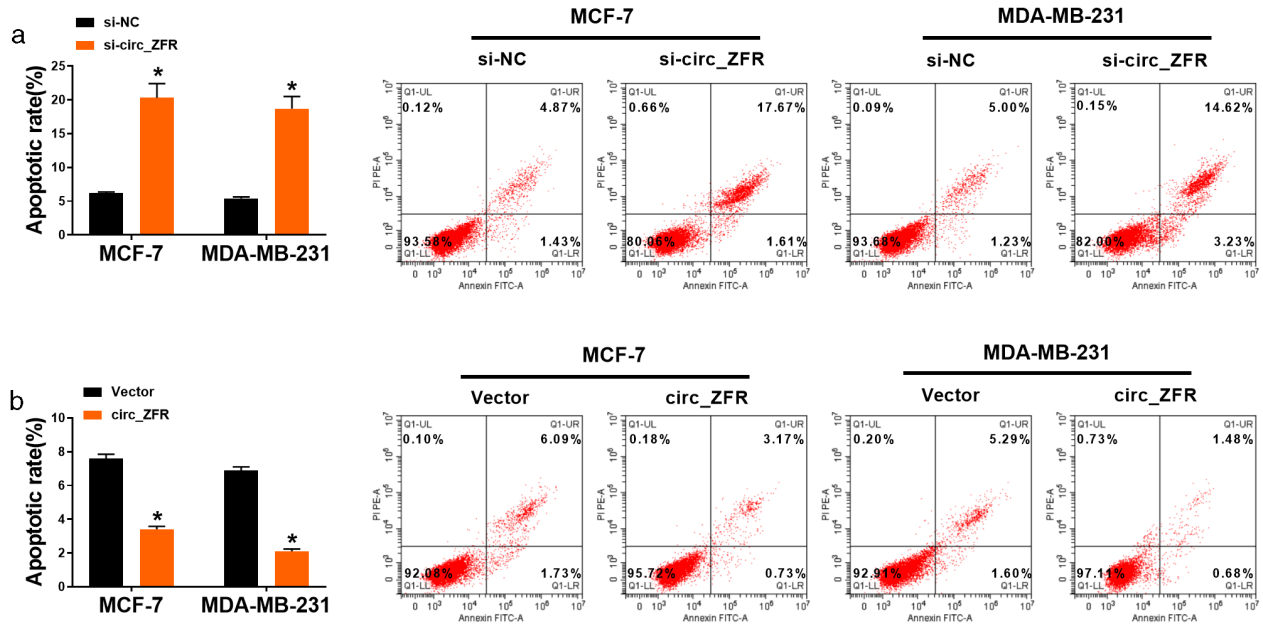

FIGURE 3 Circ_ZFR inhibited cell apoptosis in BC. (a),(b) The apoptosis rate of MCF-7 and MDA-MB-231 cells transfected with si-NC, si-circ_ZFR, vector or circ_ZFR for 48 hours were determined by flow cytometry assay. *$p < 0.05$

a tumor promotion role in BC cells partly by affecting cell proliferation, migration, invasion, and EMT.

Circ_ZFR inhibited cell apoptosis in BC

Flow cytometry analysis was then performed to detect whether circ_ZFR affected cell apoptosis in BC. As shown in Figure 3(a),(b), the apoptosis rate of MCF-7 and MDA-MB-231 cells was increased by transfection of si-circ_ZFR, but was decreased by overexpression of circ_ZFR. The results supported that circ_ZFR could hinder apoptosis of BC cells.

Downregulation of FABP7 repressed proliferation, migration, invasion, and EMT of BC cells, and promoted cell apoptosis

We also investigated the differentially expressed mRNAs in BC tissues according to the GSE62931 microarray dataset downloaded from GEO database (Figure 4(a)). FABP7 was upregulated in TNBC tumor tissues in contrast with the non-TNBC tumor tissues, and Cordero et al.[28] have indicated that FABP7 was a key metabolic regulator in BC; FABP7 was chosen as the research object. The mRNA and protein levels of FABP7 were also increased in 50 BC tissues compared to the adjacent normal tissues (Figure 4(b),(d)). Meanwhile, we found that FABP7 expression was positively correlated ($r = 0.5292$, $p < 0.0001$) with circ_ZFR expression in BC tissues (Figure 4(c)). The mRNA and protein levels of FABP7 were markedly facilitated in BC cell lines compared to MCF-10A cell line (Figure 4(e),(f)). To explore the functional role of FABP7 in BC, we knocked down the mRNA and protein expression of FABP7 by transfection with si-FABP7 in MCF-7 and MDA-MB-231 cells (Figure 4(g),(h)). Our data showed that colony formation (Figure 4(i)), proliferation (Figure 4(j),(k)), migration (Figure 4(l)), and invasion (Figure 4(m)) capabilities were significantly suppressed by downregulation of FABP7. In addition, down-regulation of FABP7 decreased the protein levels of Snail and Twist1, but elevated the protein level of E-cadherin in MCF-7 and MDA-MB-231 cells (Figure 4(n)). The apoptosis rate of MCF-7 and MDA-MB-231 cells were also inhibited by transfection of si-FABP7 (Figure 4(o)). These results suggested that FABP7 played an oncogenic role in BC cells, and FABP7 depletion could inhibit BC progression in vitro.

Circ_ZFR directly targeted miR-223-3p and FABP7 was a target gene for miR-223-3p

It has been confirmed that circRNA could function as a miRNAs sponge to regulate target mRNAs expression.[29] Given the apparent relationship between FABP7 and circ_ZFR, we next investigated the target miRNAs for circ_ZFR and FABP7 by Circinteractome or Starbase v3.0 online software. Among the predicted miRNAs, miR-223-3p was predicted by both databases (Figure 5(a)). In MCF-7 and MDA-MB-231 cells, miR-223-3p level was significantly elevated in BC cells with circ_ZFR knockdown, but was decreased in BC cells with circ_ZFR overexpression (Figure 5(b)). The transfection effects of miR-223-3p mimic or inhibitor were verified by qRT-PCR assay, and we found that miR-223-3p expression was nearly 150-fold increased by miR-223-3p mimic relative to scramble control, whereas

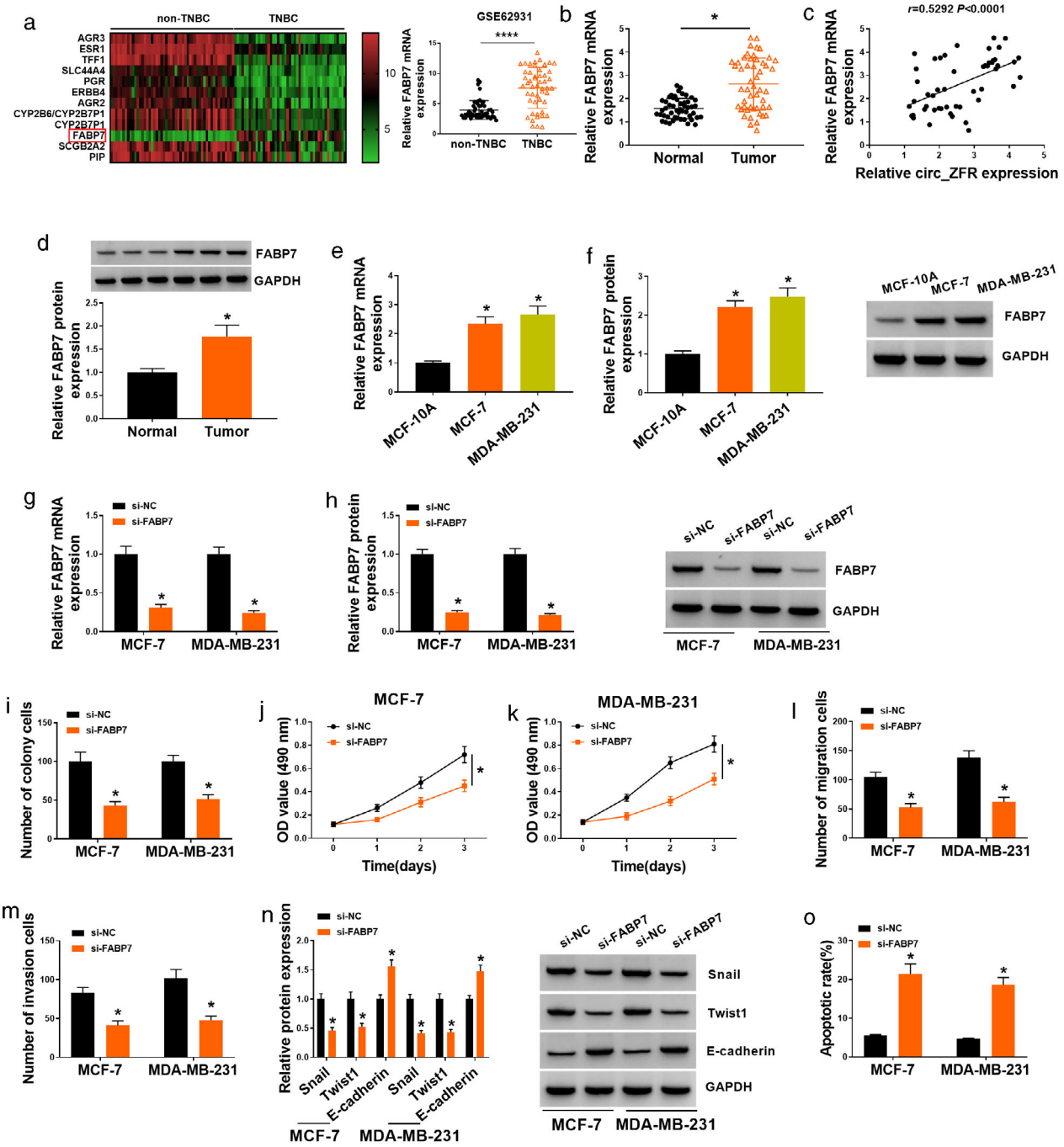

FIGURE 4 Downregulation of FABP7 repressed proliferation, migration, invasion, and EMT, but promoted cell apoptosis in BC cells. (a) Twelve differently expressed mRNAs in TNBC tissues compared with non-TNBC tissues were shown, and the expression level of FABP7 in 53 cases of TNBC tissues and paired non-TNBC tissues were shown. Data were obtained from the GSE62931 microarray dataset downloaded from the GEO database. (b) FABP7 mRNA expression in 50 cases of BC tissues and normal tissues was measured by qRT-PCR. (c) Pearson correlation coefficient was used to assess the correlation between circ_ZFR and FABP7 expression in 50 BC patient tissues. (d) FABP7 protein level in BC and normal tissues was detected by Western blot. (e),(f) The mRNA and protein levels of FABP7 in BC cell lines (MCF-7 and MDA-MB-231) and normal epithelial cells MCF-10A were estimated by qRT-PCR and Western blot, respectively. (g)–(o) MCF-7 and MDA-MB-231 cells were transfected with si-NC or si-FABP7. (g),(h) The mRNA and protein levels of FABP7 were measured by qRT-PCR and Western blot, respectively. (i) Fourteen days on transfection, colony numbers was examined. (j),(k) CCK-8 assay was used to detect cell viability, and the OD value of BC cells was detected at 0, 1, 2, or 3 days on transfection. (l),(m) Twenty-four hours on transfection, cell migration, and invasion were determined by Transwell assay. (n) Western blot assay was employed to measure the protein levels of snail, Twist1, and E-cadherin. (o) Forty-eight hours on transfection, cell apoptosis rate was assessed through flow cytometry assay. *$p < 0.05$

its expression was decreased more than half by anti-miR-223-3p transfection (Figure 5(c)). Furthermore, overexpression of miR-223-3p also decreased the protein levels of FABP7, whereas it was promoted by miR-223-3p knockdown (Figure 5(d)–(f)). Next, dual luciferase reporter assay was performed to determine whether miR-223-3p directly

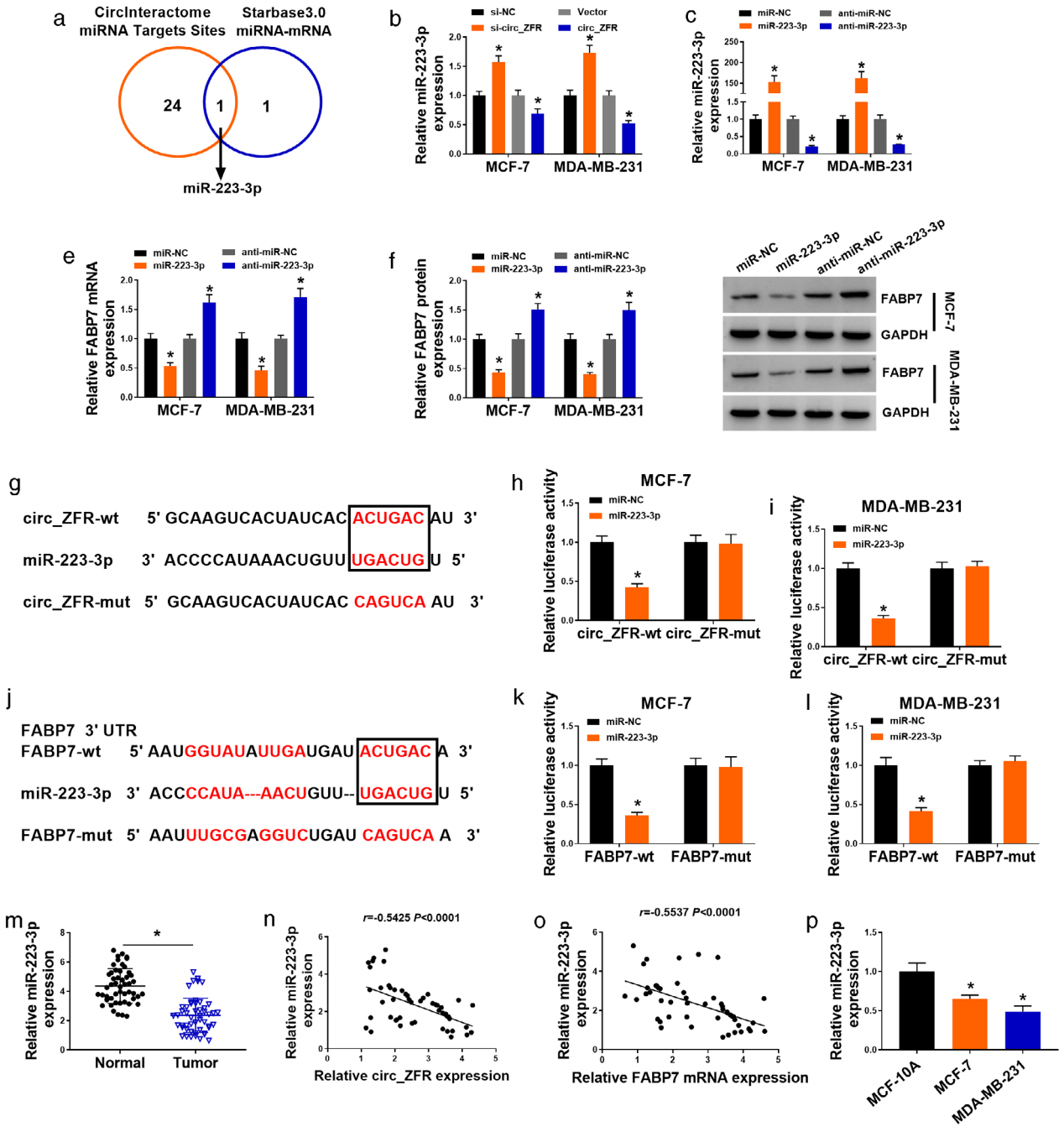

FIGURE 5 Circ_ZFR directly targeted miR-223-3p and FABP7 was a target of miR-223-3p. (a) Circinteractome database was used to predict the target miRNAs of circ_ZFR, and Starbase v3.0 was used to predict the target miRNAs of FABP7. (b) The expression of miR-223-3p in MCF-7 and MDA-MB-231 cells transfected with si-NC, si-circ_ZFR, vector, or circ_ZFR was measured by qRT-PCR. (c)–(f) MCF-7 and MDA-MB-231 cells were transfected with miR-NC, miR-223-3p, anti-miR-NC, or anti-miR-223-3p. (c)–(e) the expression levels of miR-223-3p and FABP7 in transfected cells were detected by qRT-PCR. (f) FABP7 protein level was determined using Western blot assay. (g) Circ_ZFR sequences contained the wild-type or mutant type miR-223-3p binding sites were shown. (h),(i) dual-luciferase reporter assay was performed in MCF-7 and MDA-MB-231 cells to confirm the interaction between miR-223-3p and circ_ZFR. (j) FABP7 3′UTR sequences contained the wild-type or mutant type miR-223-3p binding sites were shown. (k),(l) the correlation between miR-223-3p and FABP7 was verified using dual-luciferase reporter assay in MCF-7 and MDA-MB-231 cells. (m) Relative expression of miR-223-3p in BC and normal tissues was examined by qRT-PCR. (n),(o) Pearson correlation coefficient was used to evaluate the correlation between miR-223-3p expression and circ_ZFR or FABP7 expression in 50 BC tissues. (p) Relative expression of miR-223-3p in BC cells (MCF-7 and MDA-MB-231) and MCF-10A cells was determined by qRT-PCR. *$p < 0.05$

targeted circ_ZFR or FABP7. Full length of circ_ZFR sequences contained wild-type or mutant type miR-223-3p binding sites were used to construct dual-luciferase reporter vectors (Figure 5(g)). The results indicated that over-expression of miR-223-3p reduced the luciferase activity of circ_ZFR-wt group in contrast with the cells with miR-NC

transfection, whereas it has little effect on the luciferase activity of circ_ZFR-mut (Figure 5(h),(i)). Next, we constructed dual-luciferase reporter vectors containing the 3′UTR of FABP7 sequences that contained complementary binding sites with miR-223-3p or mutant miR-223-3p binding sites (Figure 5(j)). A significant reduction in luciferase reporter activity was detected in cells co-transfected with miR-223-3p and FABP7-wt, but not with the FABP7-mut (Figure 5(k),(l)). These results suggested that miR-223-3p was the target miRNA of circ_ZFR and directly targeted FABP7. Next, miR-223-3p expression in BC tissues was detected. As shown in Figure 5(m), miR-223-3p expression

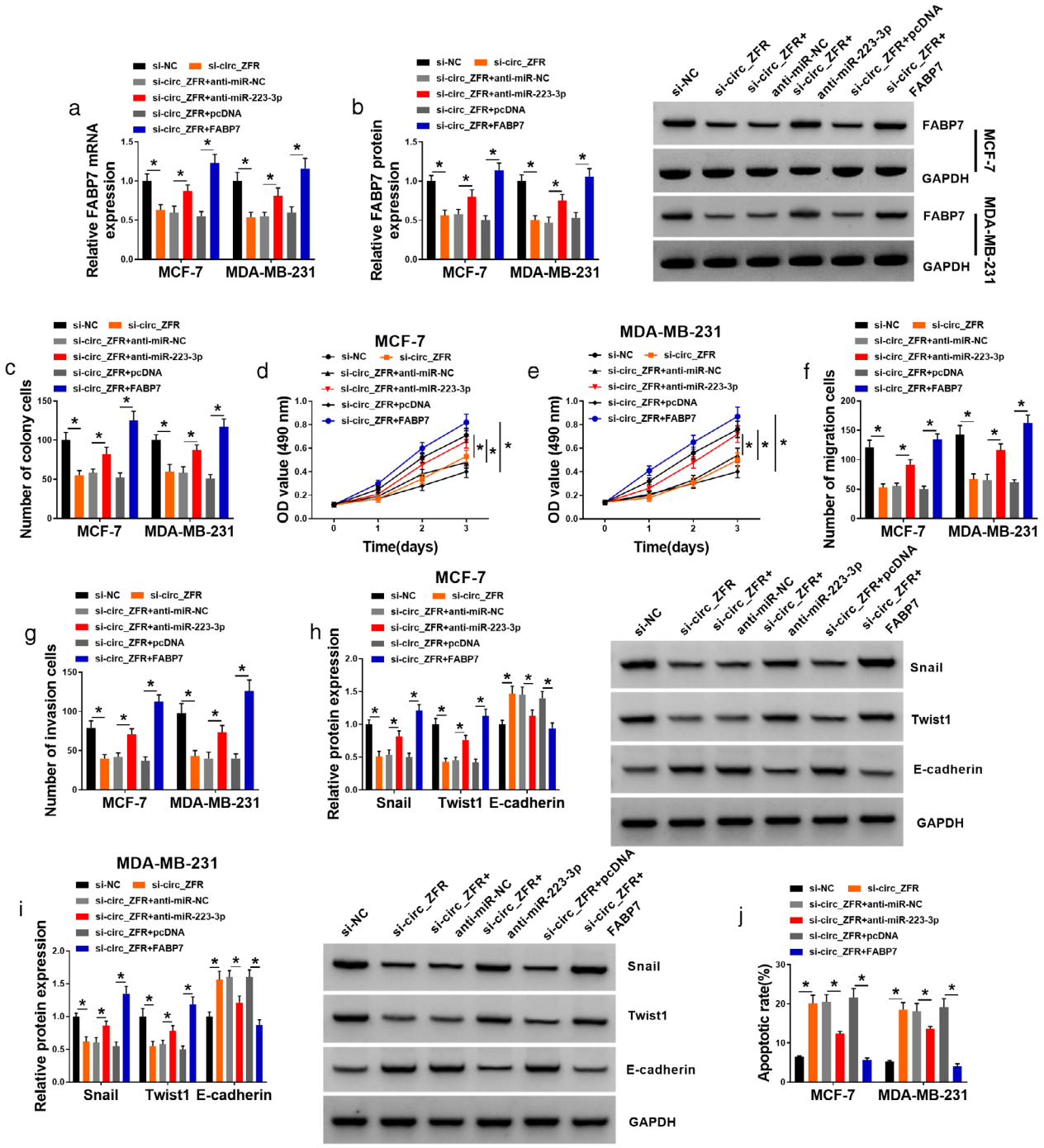

FIGURE 6 Circ_ZFR depletion repressed the progression of BC cells by regulating the miR-223-3p/FABP7 axis. (a)–(j) MCF-7 and MDA-MB-231 cells were transfected with si-NC, si-circ_ZFR, si-circ_ZFR + anti-miR-NC, si-circ_ZFR + anti-miR-223-3p, si-circ_ZFR + pcDNA or si-circ_ZFR + FABP7, respectively. (a) FABP7 mRNA level was measured by qRT-PCR. (b) FABP7 protein level was detected by Western blot. (c) Fourteen days on transfection, colony numbers was detected. (d),(e) CCK-8 assay was used to detect cell viability, and the OD value of BC cells was detected at 0, 1, 2, or 3 days on transfection. (f),(g) Twenty-four hours on transfection, cell migration and invasion ability were assessed by Transwell assay. (h),(i) Western blot assay was used to assess the protein levels of snail, Twist1, and E-cadherin. (j) Forty-eight hours on transfection, cell apoptosis was analyzed by flow cytometry assay. $^*p < 0.05$

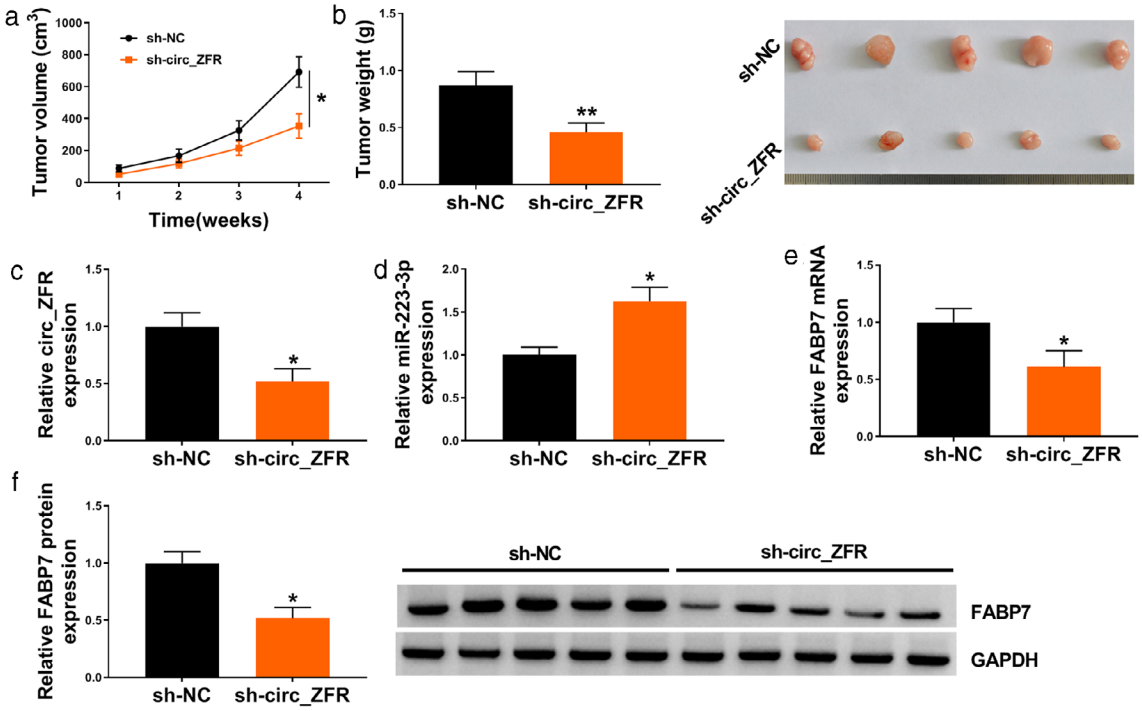

FIGURE 7 Circ_ZFR depletion restrained BC cell growth in vivo. MDA-MB-231 cells stably transfected with sh-NC or sh-circ_ZFR were injected into the flanks of nude mice. Tumor volume was measured every week for 4 weeks. Four weeks on injection, the mice were euthanized and the tumor tissues were weighted and stored. (a),(b) the volume and weight of xenograft tumors from nude mice were detected. The most representative photos of xenograft tumor tissues were shown. (c)–(e) the expression levels of circ_ZFR, miR-223-3p, and FABP7 in that tumor tissue were examined by qRT-PCR. (f) FABP7 protein expression in that tumor tissue was determined using Western blot assay. $^*p < 0.05$

was downregulated in BC tissues compared to normal tissues. Pearson correlation coefficient indicated that miR-223-3p expression was negatively correlated with FABP7 or circ_ZFR expression in BC tissues (Figure 5(n),(o)). MiR-223-3p expression was declined in BC cell lines (Figure 5(p)). The above data demonstrated that circ_ZFR function as a miR-223-3p sponge to regulate FABP7 expression in BC cell lines.

Circ_ZFR deficiency repressed the progression of BC cells by regulating the miR-223-3p/FABP7 axis

To investigate whether knockdown of circ_ZFR played a tumor-inhibiting role by regulating miR-223-3p and FABP7 in BC cells, we co-transfected with si-circ_ZFR and anti-miR-223-3p or FABP7 into MCF-7 and MDA-MB-231 cells. As demonstrated in Figure 6(a),(b), the inhibition of circ_ZFR knockdown on the mRNA and protein expression of FABP7 could be reversed by silencing miR-223-3p or overexpressing FABP7. More than that, the inhibitory effects of circ_ZFR inhibition on colony formation (Figure 6(c)), proliferation (Figure 6(d),(e)), migration (Figure 6(f)), and invasion (Figure 6(g)) of MCF-7 and MDA-MB-231 cells were attenuated by miR-223-3p inhibitor or elevated FABP7. Meanwhile, miR-223-3p inhibitor or FABP7 overexpression also reversed the effects of si-circ_ZFR on protein levels of Snail, Twist1, and E-cadherin (Figure 6(h),(i)), as well as on cell apoptosis (Figure 6(j)). These results revealed that circ_ZFR regulated BC cell progression by regulating the miR-223-3p/FABP7 axis in vitro.

Circ_ZFR inhibition restrained BC cell growth in vivo

To evaluate whether circ_ZFR regulated tumor growth in vivo, MDA-MB-231 cells stably transfected with sh-circ_ZFR or sh-NC were subcutaneously injected into nude mice. Tumor volume and weight in sh-circ_ZFR group were markedly decreased compared to sh-NC group (Figure 7(a),(b)). qRT-PCR results manifested that circ_ZFR expression was reduced in circ_ZFR-silenced group, whereas miR-223-3p expression was induced (Figure 7(c),(d)). Moreover, the mRNA and protein expression of FABP7 were obviously decreased in sh-circ_ZFR group (Figure 7(e),(f)). Therefore, knockdown of circ_ZFR could inhibit BC cell growth in vivo by regulating the miR-223-3p/FABP7 axis.

DISCUSSION

The role of circRNAs has been demonstrated in a variety of human cancers, including BC.[30] In this research, we found that circ_ZFR was distinctly raised in BC tissues through the

GSE101124 database. To date, the function of circ_ZFR has been studied in gastric cancer[31] and PTC,[11] whereas the effect of circ_ZFR in BC has not been investigated. In results of qRT-PCR, circ_ZFR was shown to be evidently induced in BC samples and cells, suggesting that circ_ZFR might play a pivotal role in the development of BC.

The regulation of cancer progression by circRNAs is often reflected in the biological behavior regulation of tumor cell proliferation, migration, invasion, and EMT.[32] As for circ_ZFR, previous studies showed that it decelerated gastric cancer progression via miR-130a/PTEN axis and miR-107/PTEN axis,[31] and repressed colorectal cancer malignancy by miR-532-3p/FOXO4 axis.[33] Circ_ZFR aggravated PTC growth and invasion via miR-1261/C80rf4 axis,[11] facilitated the development of hepatocellular carcinoma via miR-3619-5p/CTNNB1 axis and Wnt/β-catenin pathway,[34] promoted non–small-cell lung carcinoma progression via miR-101-3p/CUL4B axis.[35] Moreover, circ_ZFR could promote the progression of human renal carcinoma,[36] bladder cancer,[37,38] and thyroid cancer.[39] These reports indicated that circ_ZFR played different roles in human cancer progression. In this study, our data showed that knockdown of circ_ZFR suppressed proliferation, migration, invasion, and EMT of BC cells, and promoted cell apoptosis. However, overexpression of circ_ZFR has the opposite effects on BC cells. These findings were in accordance with previous data by Chen et al.[40] Circ_ZFR might act as a tumor promoter in BC.

FABP7, a member of the FABP intracellular lipid chaperone family, is aberrantly expressed in diverse types of cancers, including BC.[13,41] Liu et al.[14] reported that FABP7 was highly expressed in TNBC, and FABP7 overexpression inhibited cell proliferation and survival. In our study, FABP7 was upregulated in BC tissues and cells, and FABP7 inhibition impeded cell proliferation, migration, invasion, and EMT, whereas it elevated cell apoptosis. These results implied that FABP7 might be a carcinogen in BC. It has been found that circRNAs could act as miRNA sponges, thereby regulating downstream mRNA expression, therefore, regulating the progression of many cancers.[42] We speculated whether circ_ZFR could regulate FABP7 expression through regulating miRNAs. Fortunately, we found that circ_ZFR could combine with miR-223-3p, and miR-223-3p directly targeted FABP7 through bioinformatics prediction and dual-luciferase reporter assay. Moreover, miR-223-3p expression was downregulated in BC tissues and cell lines. Previous studies have enunciated that miR-223-3p was involved in the progression of several cancers, including gastric cancer,[43] lung cancer,[44] and colon cancer.[45] The effects of miR-223-3p depend on the tumor cell type. Wang et al.[46] have shown that miR-223-3p could hinder activity, migration, and invasion of BC cells. In line with the results, interference of miR-223-3p reversed the inhibition of circ_ZFR knockdown on BC cells. Moreover, FABP7 overexpression could weaken the inhibitory impact of si-circ_ZFR on BC cells. Taken together, our results revealed that circ_ZFR knockdown inhibited BC cell progression by regulating the miR-223-3p/FABP7 axis. Circ_ZFR depletion also retarded BC tumor growth in vivo by increasing miR-223-3p and reducing FABP7.

However, there are some limitations in our research. First, we have confirmed the regulatory functions of circ_ZFR/miR-223-3p/FABP7 axis in BC. However, whether this axis exerted regulatory function in clinical cases is largely unknown. Second, the limited sample size is another limitation in this study. Hence, a larger tumor cohort is needed to further confirm our results in the future.

CONCLUSION

Our study demonstrated that circ_ZFR could facilitate BC cell proliferation, migration, invasion, and EMT, and impair cell apoptosis by sponging miR-223-3p and regulating FABP7. These findings indicated the important role of circ_ZFR/FABP7 axis in BC tumorigenesis.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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