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Original Research

Hsa_circ_0092276 promotes doxorubicin resistance in breast cancer cells by regulating autophagy via miR-348/ATG7 axis

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ABSTRACT

Previous study has confirmed that hsa_circ_0092276 is highly expressed in doxorubicin (DOX)-resistant breast cancer cells, indicating that hsa_circ_0092276 may be involved in regulating the chemotherapy resistance of breast cancer. Here we attempted to investigate the biological role of hsa_circ_0092276 in breast cancer. We first constructed DOX-resistant breast cancer cells (MCF-7/DOX and MDA-MB-468/DOX). The 50% inhibiting concentration of MCF-7/DOX and MDA-MB-468/DOX cells was significantly higher than that of their parental breast cancer cells, MCF-7 and MDA-MB-46. MCF-7/DOX and MDA-MB-468/DOX cells also exhibited an up-regulation of drug resistance-related protein MDR1. Compared with MCF-7 and MDA-MB-46 cells, hsa_circ_0092276 was highly expressed in MCF-7/DOX and MDA-MB-468/DOX cells. Hsa_circ_0092276 overexpression enhanced proliferation and the expression of LC3-II/LC3-I and Beclin-1, and repressed apoptosis of breast cancer cells. The effect of hsa_circ_0092276 up-regulation on breast cancer cells was abolished by 3-methyladenine (autophagy inhibitor). Hsa_circ_0092276 modulated autophagy-related gene 7 (ATG7) expression via sponging miR-384. Hsa_circ_0092276 up-regulation promoted autophagy and proliferation, and repressed apoptosis of breast cancer cells, which was abolished by miR-384 overexpression or ATG7 knockdown. In addition, LV-circ_0092276 transfected MCF-7 cell transplantation promoted autophagy and tumor growth of breast cancer in mice. In conclusion, our data demonstrate that hsa_circ_0092276 promotes autophagy and DOX resistance in breast cancer by regulating miR-348/ATG7 axis. Thus, this article highlights a novel competing endogenous RNA circuitry involved in DOX resistance in breast cancer.

Introduction

Breast cancer is a malignant tumor that starts from cells of the breast [1]. Breast cancer is the second cause of death from cancer and poses a serious threat to the physical and mental health of contemporary women [2]. In recent years, the prognosis of breast cancer has been greatly improved with the development of comprehensive treatment models for breast cancer [3]. Chemotherapy plays a pivotal role in the treatment of breast cancer. While, there are also many serious problems in the application, the most important of which is the drug resistance [4]. Doxorubicin (DOX) is considered to be the most effective and commonly used chemotherapeutic drug. It is often used in the chemotherapy of breast cancer alone or in combination with some other drugs, such as docetaxel. Drug resistance often appears during the chemotherapy process, then leading to chemotherapy failure. Chemotherapy resistance of breast cancer is closely associated with the recurrence and metastasis of breast cancer [5].

More and more studies have confirmed that circular RNA (circRNA) is closely associated with the occurrence, development and chemotherapy resistance of breast cancer [6,7]. CircRNA regulates the proliferation, invasion and apoptosis of breast cancer cells by sponging microRNAs (miRNAs) and interacting with biomolecules [8]. The study of Yang et al. has demonstrated that circAGFG1 functions as an endogenous competitive RNA to regulate the expression of CCNE1 through sponging miR-19–5p, thereby affecting the progression of triple-negative breast cancer [9]. Hsa_circ_001783 is up-regulated in breast cancer, and knockdown of hsa_circ_001783 significantly inhibits the proliferation, migration and invasion of breast cancer cells [10]. Sang et al. have found that hsa_circ_0025202 is significantly down-regulated in tamoxifen-resistant breast cancer cells. Hsa_circ_0025202 overexpression increases the sensitivity of tamoxifen-resistant breast cancer cells and promotes apoptosis of breast cancer cells by regulating miR-182–5p/FOXO3a axis [11]. Breast cancer cells display a decrease in the expression of circKDM4C. Exogenous overexpression of circKDM4C significantly inhibits proliferation, metastasis and DOX resistance of breast cancer, and circKDM4C promotes the expression of tumor suppressor gene PBLD via competitively binding miR-548p [12].

Survey conducted by Gao et al. has confirmed that hsa_circ_0092276 is highly expressed in DOX-resistant breast cancer cells, suggesting

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that hsa_circ_0092276 may be involved in the regulation of breast cancer chemotherapy resistance [13]. Bioinformatics software (Circular RNA Interactome) predictive analysis of hsa_circ_0092276 has revealed that there are binding sites between hsa_circ_0092276 and miR-384, suggesting that hsa_circ_0092276 can regulate the downstream gene expression by binding with miR-384. Bioinformatics software (Targetscan) predictive analysis of the target gene of miR-384 has found that miR-384 may play a role by targeting the autophagy-related gene 7 (ATG7). Recent study has shown that autophagy is the main cause of resistance to chemotherapy drugs [14]. Therefore, we speculated that hsa_circ_0092276 may release the inhibitory effect of miR-384 on ATG7 by competitively binding with miR-384, and promote the expression of ATG7, thereby promoting autophagy and chemotherapy resistance of breast cancer cells.

Materials and methods

Cell culture

Human breast cancer cell lines, MCF-7 and MDA-MB-468, were purchased from ATCC (Manassas, VA, USA). MCF-7 and MDA-MB-468 were treated with step wise increasing concentrations of DOX (up to 8 μg/mL) for 8 months to induce DOX-resistant human breast cancer cells. Each DOX treatment lasts for two weeks, and the surviving cells are collected and treated with a higher concentration of DOX. After 8 months of DOX induction, the surviving cells are were considered as DOX-resistant human breast cancer cells, MCF-7/DOX and MDA-MB-468/DOX. MCF-7, MCF-7/DOX, MDA-MB-468 and MDA-MB-468/DOX cells were cultured in dulbecco's modified eagle medium (DMEM) (Senbeijia, Nanjing, China) at 37 ˚C and 5% CO_2. The medium supplemented with 10% fetal bovine serum (FBS, Gibco, Middleton, WI, USA) and 1% penicillin/streptomycin (Senbeijia). MCF-7 and MDA-MB-468 cells were incubated with 0.5 μg/mL of DOX (Aladdin, Shanghai, China) or phosphate buffer saline (PBS). MCF-7/DOX and MDA-MB-468/DOX were incubated with 3 μg/mL of DOX or PBS. MCF-7, MCF-7/DOX, MDA-MB-468 and MDA-MB-468/DOX cells were treated with 2 mM of 3-methyladenine (3-MA, autophagy inhibitor, dissolved in DMSO, Aladdin) or 0.1% DMSO, respectively.

MTT assay

The 50% inhibiting concentration (IC50) of cells was examined by MTT assay using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Cells were seeded into 96-well plate (2000 cells/well) and incubated with 10 μL MTT solution (5 mg/mL) at 37 ˚C for 4 h. Subsequently, cells were stained with 10 μL formazan. Finally, the absorbance of each well at 570 nm was detected using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Cell transfection

Full-length of hsa_circ_0092276 was cloned into pcDNA3.1 vector to generate pcDNA3.1-circ_0092276 vector (RiboBio, Guanghzou, China). Empty vector pcDNA3.1-NC served as control (Ctrl). The miR-348 mimic, small interference RNA (siRNA) specifically targeting hsa_circ_0092276 (si-circ_0092276) or ATG7 (si-ATG7), and the corresponding negative (NC) (mimic NC, si-Ctrl or si-NC) were synthesized by RiboBio. Lentivirus harboring hsa_circ_0092276 (LV-circ_0092276) and the corresponding NC (LV-Ctrl) were generated by RiboBio. Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as the protocol described. After 48 h of transfection, the modified cells were collected and stored at − 20 ˚C for further use.

Cell proliferation

Cell proliferation of breast cancer cells was assessed using Yefluor 488 EdU Imaging Kit (YEASEN, Shanghai, China) according to the manufacturer's protocol. Cells were seeded into 24-well plate and incubated with 50 μM of EdU at 37 ˚C. After 4 h of incubation, cells were fixed with 4% paraformaldehyde solution at room temperature for 15 min. The cells were washed with PBS, and then permeabilized with 0.5% Triton X-100 at room temperature for 20 min. After washing with PBS, the cells were reacted with 1 mL of 1 mL Click-iT reaction cocktail in darkness for 30 min. Subsequently, cells were stained with 1 μg/mL of DAPI for 30 min and visualized under a fluorescence microscope (Nikon, Tokyo, Japan).

Cell apoptosis

The apoptosis of breast cancer cells was examined using Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN) following the manufacturer's instruction. Breast cancer cells were collected and washed with pre-cooling PBS for 2 times. Cells were then resuspended with 100 μL of 1 × Binding Buffer. The cell suspension was mixed with 5 μL of Annexin V-FITC and 10 μL of PI Staining Solution, and incubated at darkness for 15 min. Then, the cell suspension was mixed with 400 μL of 1 × Binding Buffer and put on ice. Cell apoptosis was determined by flow cytometry in an hour.

Luciferase reporter assay

The wild-type (WT) or mutant type (Mut) of hsa_circ_0092276 or ATG7 containing the predicted miR-348 binding sites were cloned into pGL3 luciferase reporter vector to generate pGL3-hsa_circ_0092276-WT, pGL3-hsa_circ_0092276-Mut, pGL3-ATG7-WT and pGL3-ATG7-Mut vectors (RiboBio). The WT/Mut hsa_circ_0092276 vector, WT/Mut 3′ untranslated region (UTR) of ATG7 vector and miR-348 mimic or mimic NC were co-transfected into 293T cells. The vector pRL-TK was transfected into 293T cells as a reference for normalization. After 48 h of transfection, Dual luciferase assay kit (Promega, Madison, USA) was used to measure the activities of firefly and renilla luciferase on luciferase assay system (Ambion, Austin, TX, USA). The relative Rluc/Luc ratio was calculated to analyze the relationship among hsa_circ_0092276, miR-348 and ATG7.

Quantitative real-time PCR (qRT-PCR)

RNA isolation from breast cancer cells or tissues was performed using TRIzol reagent (Invitrogen). The integrity of RNA was examined by 1.5% agarose gel electrophoresis. Total RNA was reverse transcribed into complementary DNA using PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Tokyo, Japan). QRT-PCR was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara). GAPDH was used as an internal control. Primer sequences (5′−3′) were as follows: hsa_circ_0092276-F: GAA GAG GCT CCG ACT CCA GAG G; hsa_circ_0092276-R: TGG CAG GCA AGC ACA AGG TTC; miR-384-F: TGT TAA ATC AGG AAT TTT AA; miR-384-R: TGT TAC AGG CAT TAT GAA; ATG7-F: GCT CCT TCT GGA GCA GTC AGC CAA; ATG7-R: AAG CCC ACA GGT CCC CGG ATT-3′; GAPDH-F: GGG CTG CTT TTA ACT CTG GTA AAG; GAPDH-R: CCA TGG GTG GAA TCA TAT TGG. PCR reactions were performed as the following conditions: step 1 (denaturation): 95 ˚C, 5 min; step 2 (amplification): 95 ˚C for 10 s and 60 ˚C for 30 s, 45 cycles; step 3 (cooling): 40 ˚C, 30 s. The results were analyzed using $2^{-\Delta\Delta CT}$ method for quantification.

Western blot (WB)

Total protein was extracted from breast cancer cells or tissues using Total Protein Extraction Kit (Solarbio, Beijing, China). BCA Protein Assay Kit (Solarbio) was used to detect the concentration of proteins. Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto polyvinylidene

fluoride membranes (Merck Millipore, Billerica, MA, USA). After immersed into blocking buffer, the membranes were incubated with the primary antibodies, LC3 (1:1000, #14,600–1-AP, Proteintech, Wuhan, China), Beclin-1 (1:1000, #11,306–1-AP, Proteintech), ATG7 (1:1000, #10,088–2-AP, Proteintech), MDR1 (1:1000, #ab235954, Abcam, Cambridge, MA, USA) or GAPDH (1:20,000, #10,494–1-AP, Proteintech) at 4 ˚C overnight. Subsequently, horseradish peroxidase-conjugated second antibody (1:5000, #SA00001–2, Proteintech) was incubated with the membranes for 1 h at room temperature. GAPDH was used as a reference protein for normalization. The data were analyzed by Image J software.

Mouse xenograft models

BALB/c nude male mice with 4–6 weeks old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). The mice were housed under SPF conditions. BALB/c nude mice were injected subcutaneously with MCF-7 cells (1×10^6 cells/200 μL) through the right armpit of mice to induce tumor xenografts. After 2 weeks of tumor transplantation, the mice with MCF-7-derived tumors were injected intraperitoneally with DOX (5 mg/kg) or PBS once every 3 days. BALB/c nude mice were randomly divided into 4 groups ($n = 30$): (1) LV-Ctrl + PBS: mice were received inoculation of LV-Ctrl-transfected MCF-7 cells and injection of PBS; (2) LV-Ctrl + DOX: mice were received inoculation of LV-Ctrl-transfected MCF-7 cells and injection of DOX; (3) LV-circ_0092276 + PBS: mice were received inoculation of LV-circ_0092276-transfected MCF-7 cells and injection of PBS; (4) LV-circ_0092276 + DOX: mice were received inoculation of LV-circ_0092276-transfected MCF-7 cells and injection of DOX. The mice failing to have tumor in tumor assessment, and mice died from other unexpected causes were excluded from the samples. A total of 15 mice were excluded from the samples, including 3 in the LV-Ctrl + PBS group, 4 in the LV-Ctrl + DOX, 3 in the LV-circ_0092276 + PBS group; 5 in the LV-circ_0092276 + DOX group. All protocols were authorized by the Ethics Committee of Henan Provincial People's Hospital.

Detection of tumor volume

The tumor volume of breast cancer was measured from the 7th day of tumor transplantation. The mice were sacrificed by cervical dislocation, and the tumor tissues of breast cancer were rapidly excised. The long and short diameters of the isolated tumor tissues were measured using vernier caliper. Tumor volume (mm^3) = Volume = (length × $width^2$)/2. After 5 weeks of tumor transplantation, the tumor tissues of breast cancer were excised and weighed. Subsequently, the tumor tissues were snap-frozen in liquid nitrogen and stored at −80 ˚C for further use.

Statistical analysis

Each assay was performed at least 3 times. Data were reported as mean ± standard deviation and analyzed using SPSS 22.0 statistical software (IBM, Armonk, NY, USA). Two-tailed Student's t, one-way or two-way ANOVA was used to analyze the statistical difference. $P < 0.05$ was considered as a significant difference.

Results

Hsa_circ_0092276 promoted DOX resistance in breast cancer cells

We initially treated MCF-7 and MDA-MB-468 cells with step wise increasing concentrations of DOX to induce DOX-resistant human breast cancer cell lines. After DOX exposure, the IC50 value was 0.52 ± 0.09 μg/mL for the MCF-7 cells and 8.22 ± 0.27 μg/mL for the MCF-7/DOX cells, showing a 15.80-fold increase. Additionally, the IC50 value following DOX treatment was 0.37 ± 0.07 μg/mL for the MDA-MB-468 cells and 3.52 ± 0.12 μg/mL for the MDA-MB-468/DOX cells, showing a 9.51-fold increase (Supplementary figure 1). We also examined the expression of drug resistance-related protein MDR1 in the drug resistant cells, showing that MDR1 was highly expressed in the drug resistant cells, MCF-7/DOX and MDA-MB-468/DOX (Supplementary figure 2). Thus, we successfully established the drug resistant cell lines, MCF-7/DOX and MDA-MB-468/DOX. In order to investigate the biological role of hsa_circ_0092276 in drug resistance of breast cancer, we assessed the expression of hsa_circ_0092276 in the breast cancer cell lines by qRT-PCR. Compared with MCF-7 or MDA-MB-468 cells, MCF-7/DOX and MDA-MB-468/DOX cells exhibited an increase of hsa_circ_0092276 expression (Fig. 1A). Moreover, hsa_circ_0092276 was highly expressed in MCF-7 or MDA-MB-468 cells after hsa_circ_0092276 overexpression (Fig. 1B). The expression of hsa_circ_0092276 was severely decreased in MCF-7/DOX or MDA-MB-468/DOX cells in the presence of si-circ_0092276 (Fig. 1C). Subsequently, under DOX treatment, we assessed cell proliferation and apoptosis by Edu assay and flow cytometry. DOX treatment significantly repressed proliferation of the wild and modified MCF-7 and MDA-MB-468 cells. Hsa_circ_0092276 overexpression promoted proliferation of MCF-7 and MDA-MB-468 cells. Hsa_circ_0092276 up-regulation led to an increase in proliferation of DOX-treated MCF-7 and MDA-MB-468 cells (Fig. 1D and E). The proliferation of the wild and modified MCF-7/DOX and MDA-MB-468/DOX cells was repressed by DOX treatment. Hsa_circ_0092276 knockdown suppressed proliferation of MCF-7/DOX and MDA-MB-468/DOX cells with or without DOX treatment (Fig. 1F and G). In addition, DOX treatment enhanced apoptosis of the wild and modified MCF-7 and MDA-MB-468 cells. Hsa_circ_0092276 overexpression reduced apoptosis of MCF-7 and MDA-MB-468 cells in the presence of PBS or DOX (Fig. 1H and I). DOX treatment caused an increase in apoptosis of the wild and hsa_circ_0092276-silenced MCF-7/DOX and MDA-MB-468/DOX cells. Hsa_circ_0092276 silencing enhanced apoptosis of MCF-7/DOX and MDA-MB-468/DOX cells after treatment of PBS or DOX (Fig. 1J and K). Thus, these data confirmed that hsa_circ_0092276 promoted DOX resistance in breast cancer cells.

Hsa_circ_0092276 enhanced DOX resistance in breast cancer cells by promoting autophagy

Next, we explored the mechanism of action of hsa_circ_0092276 in promoting DOX resistance in breast cancer. We estimated the influence of hsa_circ_0092276 on the expression of autophagy-related proteins (LC3-I, LC3-II and Beclin-1) in MCF-7, MDA-MB-468, MCF-7/DOX and MDA-MB-468/DOX cells. WB data revealed that hsa_circ_0092276 overexpression enhanced the expression of LC3-II/LC3-I and Beclin-1 in MCF-7 and MDA-MB-468 cells (Fig. 2A-C). Moreover, Hsa_circ_0092276 silencing led to a decrease in the expression of LC3-II/LC3-I and Beclin-1 in MCF-7/DOX and MDA-MB-468/DOX cells (Fig. 2D-F). Subsequently, we used 3-MA to trest breast cancer cells for autophagy inhibition. The data obtained from Edu assay showed that hsa_circ_0092276 overexpression enhanced proliferation of MCF-7 and MDA-MB-468 cells. Hsa_circ_0092276 up-regulation led to an increase of proliferation of 3-MA treated MCF-7 and MDA-MB-468 cells. However, 3-MA treatment abolished the promoting effect of hsa_circ_0092276 up-regulation on proliferation of MCF-7 and MDA-MB-468 cells (Fig. 2G and H). In addition, hsa_circ_0092276 overexpression reduced apoptosis of MCF-7 and MDA-MB-468 cells after DMSO or 3-MA treatment. 3-MA-medicated inhibition of autophagy impaired the impact of hsa_circ_0092276 overexpression on apoptosis of MCF-7 and MDA-MB-468 cells (Fig. 2I and J). In the early stage of tumorigenesis, autophagy plays an anti-tumor effect by removing the abnormally folded proteins [15]. In contrast, autophagy in the formed tumor tissues resists tumor metabolic stress by degrading and reusing the damaged proteins, thereby promoting the tumor development [16]. The excessive autophagy is an important factor in the drug resistance of tumor cells. Thus, these data indicated that hsa_circ_0092276 enhanced DOX resistance in breast cancer cells by activating autophagy.

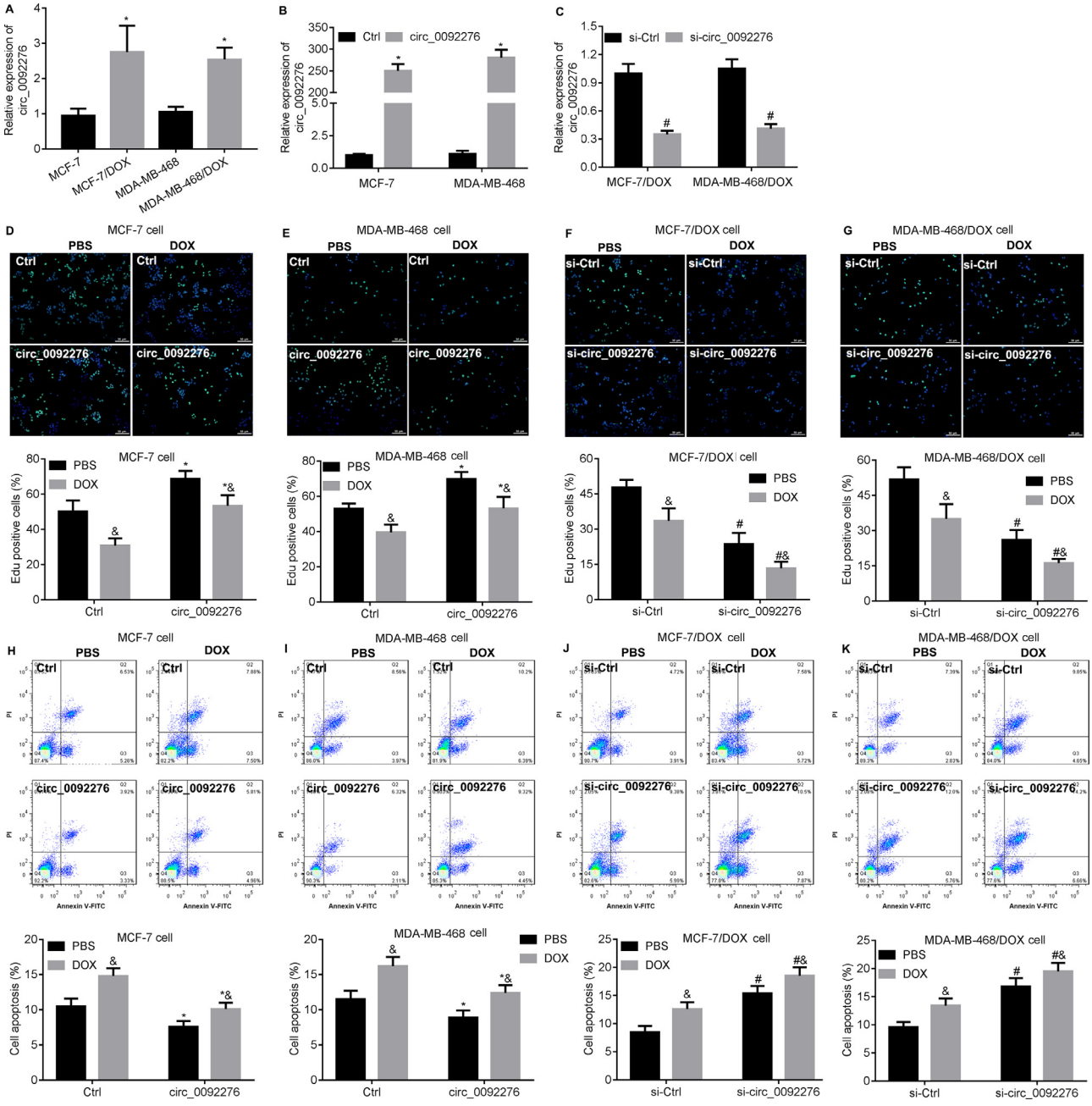

Fig. 1. Hsa_circ_0092276 enhances DOX resistance in breast cancer cells.
(A) The expression of hsa_circ_0092276 in MCF-7, MCF-7/DOX, MDA-MB-468 and MDA-MB-468/DOX cells was detected using qRT-PCR. MCF-7 or MDA-MB-468 cells were transfected with pcDNA3.1-circ_0092276 or pcDNA3.1-NC. (B) QRT-PCR was performed to examine the expression of hsa_circ_0092276 in modified MCF-7 or MDA-MB-468 cells. MCF-7/DOX or MDA-MB-468/DOX cells were transfected with si-circ_0092276 or si-Ctrl. (C) QRT-PCR was performed to estimate the expression of hsa_circ_0092276 in modified MCF-7/DOX or MDA-MB-468/DOX cells. The wild or modified MCF-7 and MDA-MB-468 cells were treated with DOX (0.5 μg/mL) or PBS. The wild or modified MCF-7/DOX and MDA-MB-468/DOX cells were treated with DOX (3 μg/mL) or PBS. (D-G) Edu assay was performed to examine cell proliferation. (H-K) Flow cytometry was performed to examine cell apoptosis. $^{*}P < 0.05$ vs. the corresponding parent cells or Ctrl; $^{\#}P < 0.05$ vs. si-Ctrl; $^{\&}P < 0.05$ vs. PBS.

Hsa_circ_0092276 modulated ATG7 expression via sponging miR-384

Our data confirmed the involvement of hsa_circ_0092276 in DOX resistance in breast cancer cells. To further determine the molecular mechanism of hsa_circ_0092276 in regulating DOX resistance in breast cancer cells, we performed predictive analysis (Circular RNA Interactome) on hsa_circ_0092276 and found that it had binding sites with miR-384. Further software predictive analysis (Targetscan) found that there were binding sites between miR-384 and ATG7. Thus, we speculated that hsa_circ_0092276 may regulate autophagy through regulating miR-384/ATG7 axis in breast cancer cells. To verify this hypothesis, we first examined the expression of miR-384 in breast cancer cells by qRT-PCR. Compared with MCF-7 or MDA-MB-468 cells, MCF-7/DOX and MDA-MB-468/DOX cells exhibited a decrease of miR-384 expression (Fig. 3A). The data of luciferase reporter assay showed that hsa_circ_0092276 interacted with miR-384 (Fig. 3B and C). Moreover, we estimated the ex-

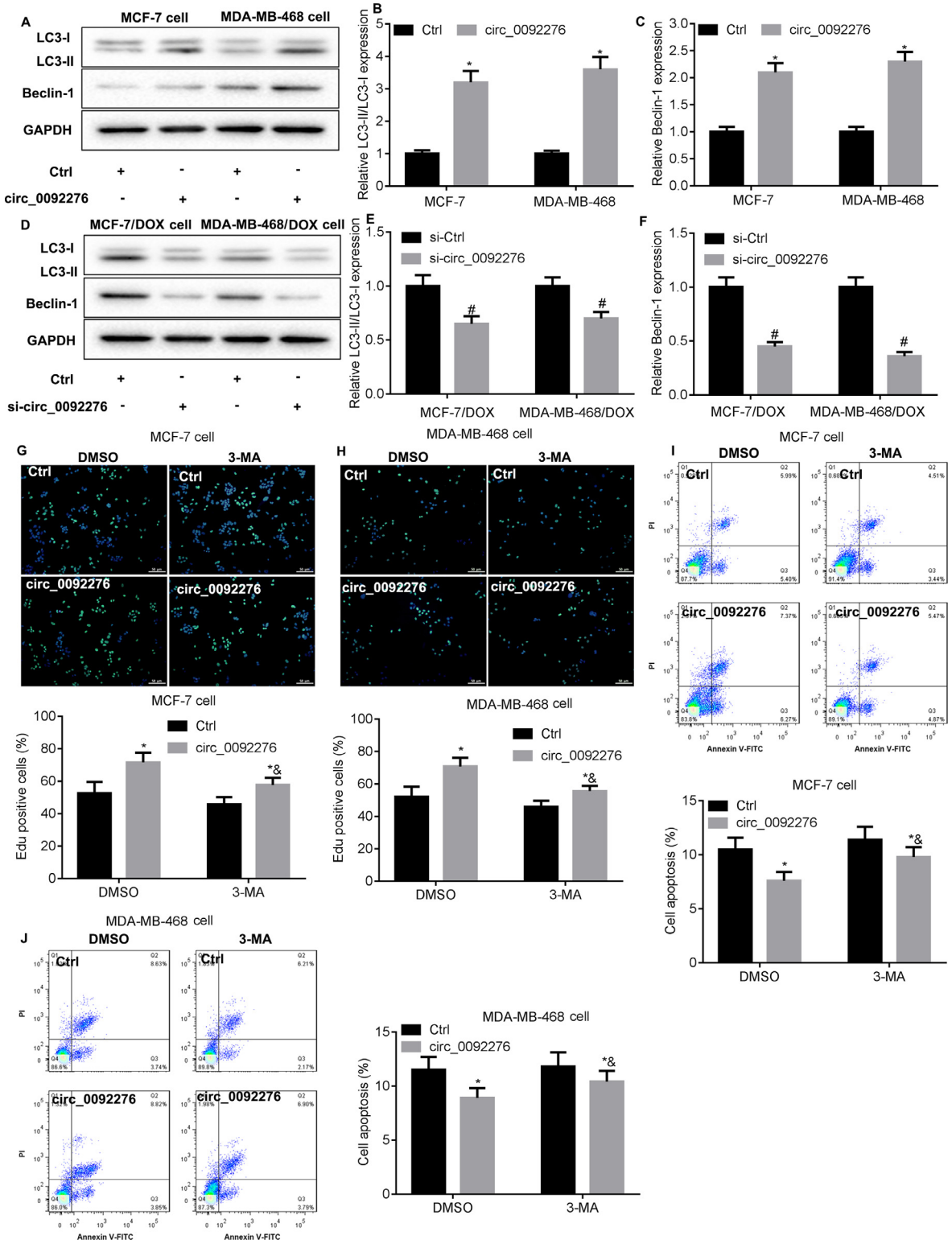

Fig. 2. Hsa_circ_0092276 enhances DOX resistance in breast cancer cells by promoting autophagy.
MCF-7 or MDA-MB-468 cells were transfected with pcDNA3.1-circ_0092276 or pcDNA3.1-NC. (A-C) WB was performed to examine the expression of LC3-I, LC3-II and Beclin-1 in the modified MCF-7 or MDA-MB-468 cells. MCF-7/DOX or MDA-MB-468/DOX cells were transfected with si-circ_0092276 or si-Ctrl. (D-F) WB was performed to detect the expression of LC3-I, LC3-II and Beclin-1 in the modified MCF-7/DOX or MDA-MB-468/DOX cells. MCF-7 or MDA-MB-468 cells were transfected with pcDNA3.1-circ_0092276 or pcDNA3.1-NC, and the modified cells were treated with 3-MA and DOX (0.5 μg/mL). The modified cells were treated with DMSO and DOX (0.5 μg/mL) as control. (G and H) Edu assay was performed to examine cell proliferation. (I and J) Flow cytometry was performed to examine cell apoptosis. $^{*}P < 0.05$ vs. the corresponding parent cells or Ctrl; $^{\#}P < 0.05$ vs. si-Ctrl; $^{\&}P < 0.05$ vs. DMSO.

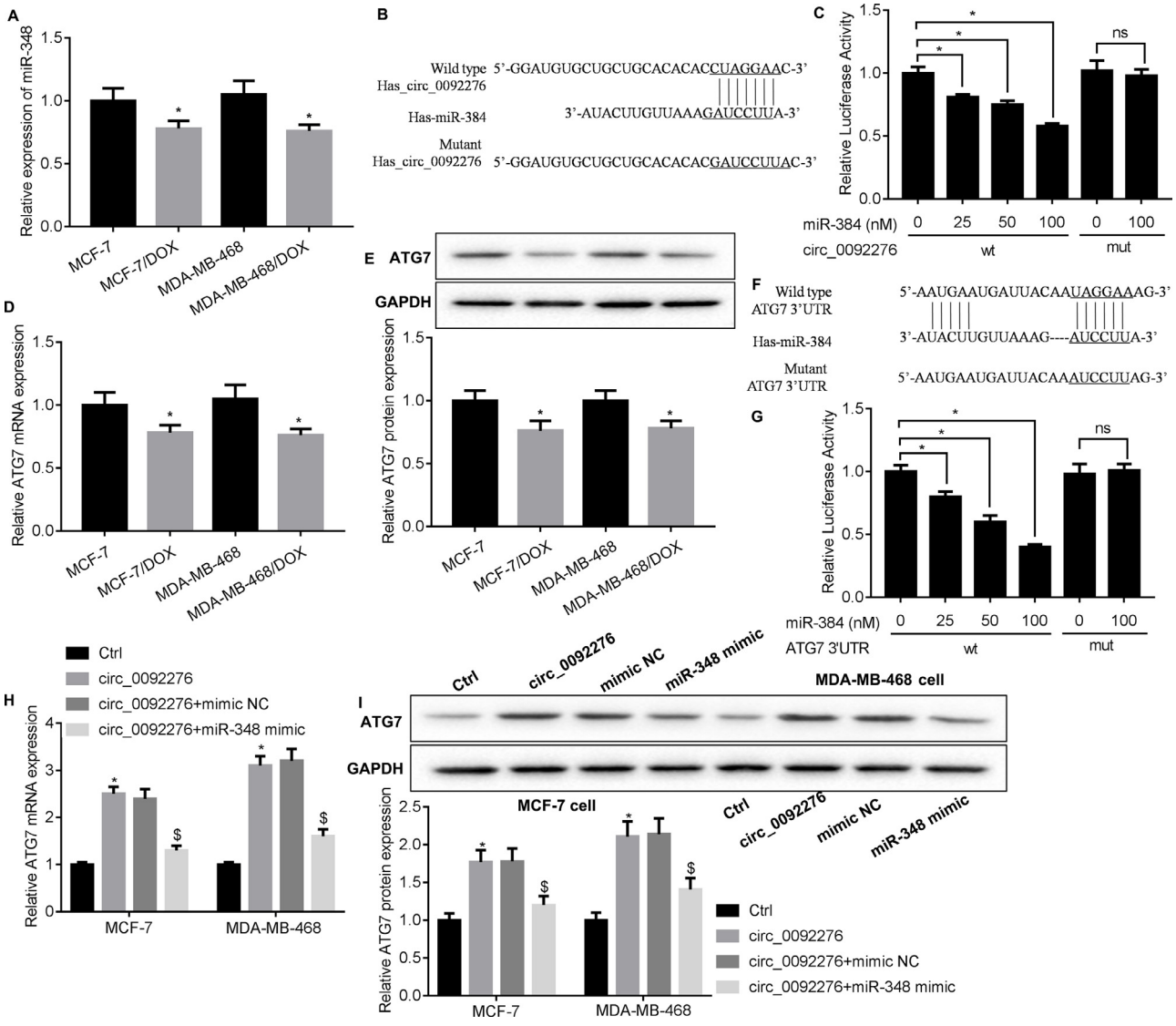

Fig. 3. Hsa_circ_0092276 modulates ATG7 expression via competitively binding miR-384.
(A) The expression of miR-384 in MCF-7, MCF-7/DOX, MDA-MB-468 and MDA-MB-468/DOX cells was detected using qRT-PCR. (B and C) Luciferase reporter assay was performed to estimate the relationship between hsa_circ_0092276 and miR-384. (D and E) The expression of ATG7 in MCF-7, MCF-7/DOX, MDA-MB-468 and MDA-MB-468/DOX cells was examined using qRT-PCR and WB. (F and G) Luciferase reporter assay was performed to estimate the relationship between ATG7 and miR-384. MCF-7 or MDA-MB-468 cells were co-transfected with pcDNA3.1-circ_0092276 or pcDNA3.1-NC and miR-384 mimic or mimic NC. (H and I) The expression of ATG7 in the modified MCF-7 or MDA-MB-468 cells was examined using qRT-PCR and WB. $^{*}P < 0.05$ vs. the corresponding parent cells or Ctrl; $^{\$}P < 0.05$ vs. circ_0092276 + mimic NC.

pression of ATG7 in breast cancer cells by qRT-PCR and WB. The gene and protein expression of ATG7 was down-regulated in MCF-7/DOX and MDA-MB-468/DOX cells as compared with MCF-7 and MDA-MB-468 cells (Fig. 3D and E). We also found that ATG7 was the target of miR-384 (Fig. 3F and G). Furthermore, MCF-7 or MDA-MB-468 cells were co-transfected with pcDNA3.1-circ_0092276 and miR-384 mimic, and the expression of ATG7 in the modified MCF-7 or MDA-MB-468 cells was examined using qRT-PCR and WB. Hsa_circ_0092276 overexpression enhanced the gene and protein expression of ATG7 in MCF-7 or MDA-MB-468 cells. The influence conferred by hsa_circ_0092276 up-regulation was abolished by miR-384 overexpression (Fig. 3H and I). Therefore, these results verified that hsa_circ_0092276 functioned as a competing endogenous RNA to repress miR-384, which controlled its down-stream target ATG7.

Hsa_circ_0092276 promoted autophagy and DOX resistance in breast cancer cells via miR-384/ATG7 axis

Here, we investigated the molecular mechanism of hsa_circ_0092276/miR-384/ATG7 axis in breast cancer. WB data revealed that hsa_circ_0092276 up-regulation caused an increase in the expression of LC3-II/LC3-I and Beclin-1 in MCF-7 and MDA-MB-468 cells. The influence conferred by hsa_circ_0092276 overexpression was abrogated by miR-384 overexpression or ATG7 knockdown (Fig. 4A-C). Moreover, we assessed the proliferation and apoptosis abilities by Edu assay and flow cytometry. Hsa_circ_0092276 overexpression enhanced proliferation of MCF-7 and MDA-MB-468 cells. The promoting effect of hsa_circ_0092276 overexpression on proliferation of MCF-7 and MDA-MB-468 cells was abolished by miR-384 up-regulation or ATG7

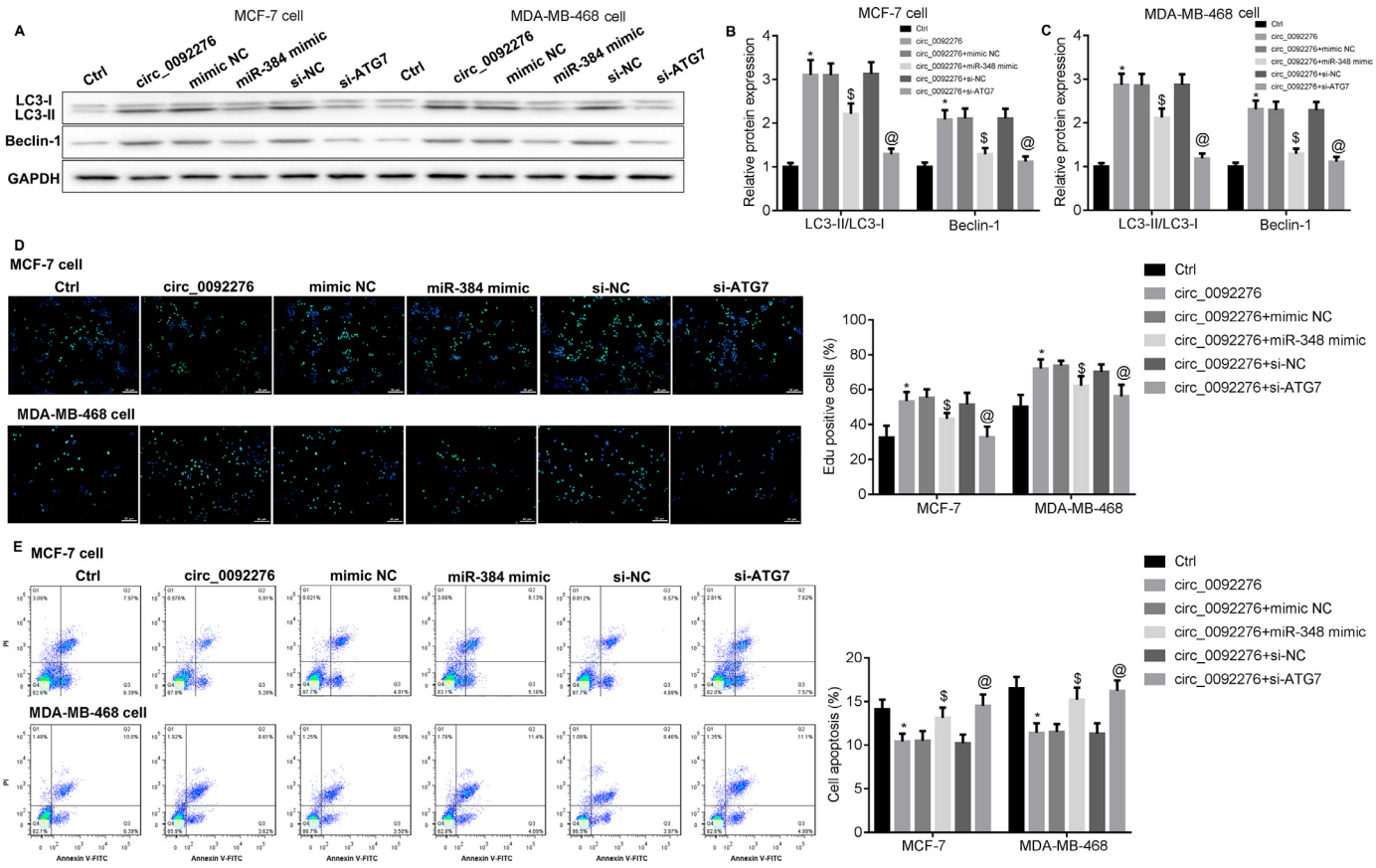

Fig. 4. Hsa_circ_0092276 promotes autophagy and DOX resistance in breast cancer cells by regulating miR-384/ATG7 axis.
MCF-7 or MDA-MB-468 cells were co-transfected with pcDNA3.1-circ_0092276 or pcDNA3.1-NC and miR-384 mimic, mimic NC, si-circ_0092276 or si-NC, and then the modified cells were treated with DOX (0.5 μg/mL). (A-C) WB was performed to examine the expression of LC3-I, LC3-II and Beclin-1 in the modified MCF-7 or MDA-MB-468 cells. (D) Edu assay was performed to examine cell proliferation. (E) Flow cytometry was performed to examine cell apoptosis. $^{*}P < 0.05$ vs. Ctrl; $^{\$}P < 0.05$ vs. circ_0092276 + mimic NC; $^{@}P < 0.05$ vs. circ_0092276 + si-NC.

silencing (Fig. 4D). The apoptosis of MCF-7 and MDA-MB-468 cells was repressed by hsa_circ_0092276 overexpression. However, miR-384 up-regulation or ATG7 deficiency enhanced apoptosis of MCF-7 and MDA-MB-468 cells after hsa_circ_0092276 overexpression (Fig. 4E). Taken together, these data confirmed that hsa_circ_0092276 promoted autophagy and DOX resistance in breast cancer cells by regulating miR-384/ATG7 axis.

Hsa_circ_0092276 enhanced DOX resistance in breast cancer mice

We further verified the biological role of hsa_circ_0092276 *in vivo*. We constructed mouse xenograft models, and observed the tumor growth. Compared with the volume (0.65 ± 0.07 mm^3) and weight (0.32 ± 0.08 g) of tumor in LV-Ctrl + PBS group, DOX treatment notably reduced the volume (0.07 ± 0.01 mm^3) and weight (0.11 ± 0.05 g) of tumor tissues in the transplanted mice. LV-circ_0092276 + PBS group displayed a boost in the volume (1.43 ± 0.12 mm^3) and weight (0.54 ± 0.05 g) of tumor tissues with respect to LV-Ctrl + PBS group. There were no difference between LV-circ_0092276 + PBS and LV-circ_0092276 + DOX groups (tumor volume: 1.22 ± 0.11 mm^3, and weight: 0.50 ± 0.06 g) (Fig. 5A-C). Subsequently, the expression of hsa_circ_0092276 and miR-384 in tumor tissues was detected by qRT-PCR. After PBS or DOX treatment, LV-circ_0092276 group enhanced the expression of hsa_circ_0092276, and repressed the expression of miR-384 in tumor tissues (Fig. 5D and E). We also found that compared with LV-Ctrl + PBS group, LV-circ_0092276 + PBS group exhibited an up-regulation of ATG7, LC3-II/LC3-I and Beclin-1 in tumor tissues. The expression of ATG7, LC3-II/LC3-I and Beclin-1 in LV-circ_0092276 + DOX group was up-regulated as compared with LV-Ctrl + DOX group (Fig. 5F and G). Thus, these findings demonstrated that hsa_circ_0092276 enhanced DOX resistance in breast cancer mice.

Discussion

CircRNAs play a crucial role in the progression of breast cancer. CircRNAs are highly conserved and have tissue-specificity, so it has the potential to become a marker for breast cancer screening [17]. The expression of circRNAs is different between normal breast tissues and breast cancer tissues [18]. Yin et al. have found that there are 41 differentially expressed circRNAs between normal breast and tumor tissues, indicating that these circRNAs are related to breast cancer [19]. The study of Dou et al. has confirmed that CircUBE2D2 is highly expressed in triple-negative breast cancer. CircUBE2D2 promotes CDCA3 expression by sponging miR-512–3p, thereby promoting proliferation, invasion, migration and tumor growth of triple-negative breast cancer [20]. CircZFR is up-regulated in breast cancer cells and tissues, and the up-regulation of circZFR indicates a poor prognosis for breast cancer patients [21]. Gao et al. has screened the circRNA related to DOX resistance in MCF-7/DOX cells, showing that hsa_circ_0092276 may be involved in the DOX resistance of breast cancer [13]. In addition, there is no report on the research of hsa_circ_0092276 in any benign cell lines and tumors. Our work first demonstrated the molecular mechanism of hsa_circ_0092276 in breast cancer. Hsa_circ_0092276 was highly expressed in MCF-7/DOX and MDA-MB-468/DOX cells, suggesting that hsa_circ_0092276 was associated with DOX resistance in breast cancer cells. DOX treatment inhibited proliferation and promoted apoptosis of

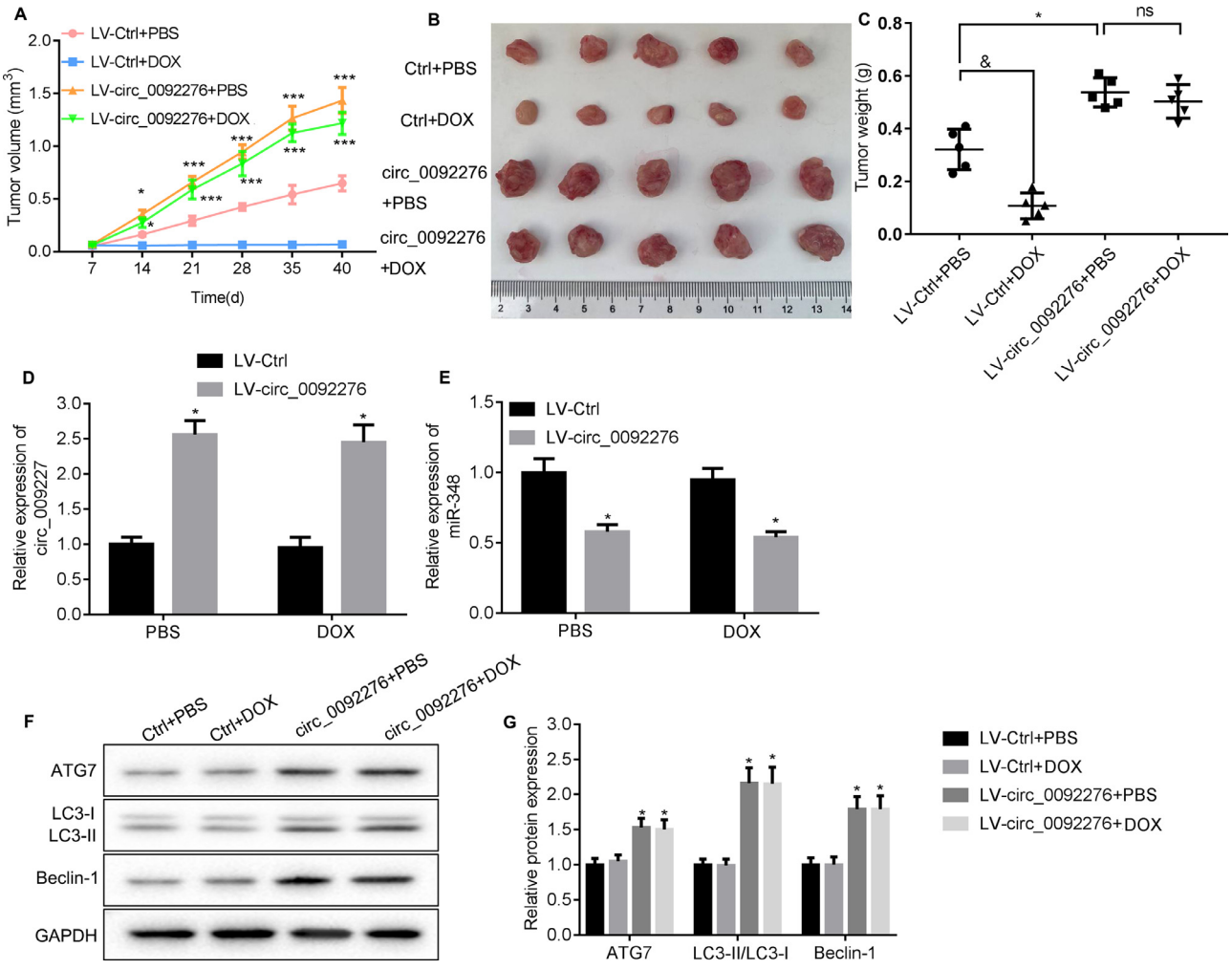

Fig. 5. Hsa_circ_0092276 enhances DOX resistance in breast cancer mice.
MCF-7 cells were transfected with LV-circ_0092276 or LV-Ctrl. BALB/c nude mice were injected subcutaneously with the modified MCF-7 cells through the right armpit of mice. After 2 weeks of tumor transplantation, the mice with MCF-7-derived tumors were injected intraperitoneally with DOX or PBS. (A) The tumor volume of breast cancer in mice was measured. (B and C) The weight of tumor tissues in mice was assessed. (D and E) The expression of hsa_circ_0092276 and miR-384 in tumor tissues was detected using qRT-PCR. (F and G) WB was performed to examine the expression of ATG7, LC3-I, LC3-II and Beclin-1 in the tumor tissues. $^{*}P$ <0.05, $^{***}P$ <0.001, *vs.* LV-Ctrl; $^{\&}P$ < 0.05 vs. PBS.

MCF-7, MCF-7/DOX, MDA-MB-468 and MDA-MB-468/DOX cells. Moreover, hsa_circ_0092276 overexpression enhanced proliferation and suppressed apoptosis of breast cancer cells. Hsa_circ_0092276 up-regulation abrogated the effect of DOX treatment on proliferation and apoptosis of breast cancer cells. Thus, these data indicated that hsa_circ_0092276 enhanced DOX resistance in breast cancer cells.

Autophagy has been reported to be associated with drug resistance of breast cancer. Lee et al. have confirmed that MTA1 activates autophagy that could contribute to tamoxifen resistance in breast cancer [22]. The long noncoding RNA H19 induces autophagy activation via SAHH/DNMT3B axis, which accelerates tamoxifen resistance in breast cancer [23]. In the present study, we found that hsa_circ_0092276 overexpression enhanced the expression of apoptosis-related proteins, LC3-II/LC3-I and Beclin-1 in MCF-7 and MDA-MB-468 cells. However, hsa_circ_0092276 silencing repressed the expression of LC3-II/LC3-I and Beclin-1 in MCF-7/DOX and MDA-MB-468/DOX cells. Moreover, hsa_circ_0092276 overexpression promoted proliferation and inhibited apoptosis of MCF-7 and MDA-MB-468 cells. The influence conferred by hsa_circ_0092276 up-regulation was abolished by 3-MA treatment, autophagy inhibitor. Taken together, these findings confirmed that hsa_circ_0092276 enhanced DOX resistance in breast cancer cells by activating autophagy.

MiR-348 is associated with various cancers, such as colorectal cancer and gastric cancer [24,25]. MiR-348 also has been reported to participate in the development of breast cancer. For example, miR-384 is down-regulated in breast cancer, especially in triple-negative breast cancer. MiR-384 overexpression represses proliferation and migration of breast cancer by inhibiting ACVR1 expression, thereby inhibiting breast cancer progression [26]. The study of Ma et al. has reported that miR-384 is a target of lncRNA SNHG3, and miR-384 interacts with HDGF. SNHG3 acts as an oncogene in breast cancer and enhances proliferation and invasion of breast cancer cells through miR-384/HDGF signaling pathway [27]. The present work also found the involvement of miR-384 in breast cancer. We first found that miR-384 was a target of hsa_circ_0092276, and there were binding sites between miR-384 and ATG7. Hsa_circ_0092276 enhanced ATG7 expression, which was abolished by miR-384 overexpression. Thus, hsa_circ_0092276 functioned as a competing endogenous RNA to repress miR-384, which controlled its down-stream target ATG7.

The study of Li et al. has reported that ATG7 is down-regulated in triple-negative breast cancer, and up-regulation of ATG7 is associated with good prognosis of triple-negative breast cancer patients [28]. ATG7-dependent autophagy protects breast cancer cells against mitoquinone-induced oxidative stress [29]. In our study,

hsa_circ_0092276 overexpression enhanced autophagy and proliferation, and repressed apoptosis of MCF-7 and MDA-MB-468 cells by regulating miR-384/ATG7 axis. In addition, LV-circ_0092276 transfected MCF-7 cell transplantation promoted autophagy and tumor growth of breast cancer in mice. Therefore, hsa_circ_0092276 enhanced DOX resistance in breast cancer by regulating miR-384/ATG7 axis *in vivo* and *in vitro*. Thus, our research provided a theoretical basis for the mechanism of chemoresistance in breast cancer. The mechanism of action of hsa_circ_0092276 in the drug resistance in breast cancer was preliminarily explained for the first time. This work highlighted a novel competing endogenous RNA circuitry (hsa_circ_0092276/miR-384/ATG7 axis) involved in the drug resistance of breast cancer, which may be the target of drug resistance in breast cancer. We will further explore the signaling pathway regulated by hsa_circ_0092276/miR-384/ATG7 axis in the mechanism of breast cancer resistance.

Conclusions

In conclusion, our work reveals the biological role of hsa_circ_0092276 in breast cancer. Hsa_circ_0092276 promotes autophagy and doxorubicin resistance in breast cancer by regulating miR-348/ATG7 axis. Thus, this article connects hsa_circ_0092276 with doxorubicin resistance in breast cancer, and highlights a novel competing endogenous RNA circuitry involved in the occurrence and development of breast cancer.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgement

Not applicable.

Author Contributions

Q. Wang designed the study; Q. Wang, D. Liang, P. Shen, Y. Yu, Y. Yan, W. You performed the study; Q. Wang, W. You analyzed the data; Q. Wang drafted the paper. All authors approved the paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101045.

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