Caveolin-1 mediates chemoresistance in breast cancer stem cells via β-catenin/ABCG2 signaling pathway

Zhiyu Wang1,2,*, Neng Wang1,†, Wenping Li3, Pengxi Liu1, Qianjun Chen1, Honglin Situ¹, Shaowen Zhong1, Li Guo1, Yi Lin1, Jiangang Shen2 and Jianping Chen2,†

1Department of Mammary Disease, Guangdong Provincial Hospital of Chinese Medicine, the Second Clinical College of Guangzhou University of Chinese Medicine, Guangzhou 510120, China, 2School of Chinese Medicine, University of Hong Kong, Pokfulam, Hong Kong, China and 3Guangdong Women and Child Health Hospital, Guangzhou 510120, China

*To whom correspondence should be addressed. Tel: +865 95147068; Fax: +852 28725633; Email: jppchen@yahoo.com

Accumulating evidence has suggested that cancer stem cells (CSCs) are at the root of drug resistance, and recent studies have indicated that caveolin-1, a membrane transporter protein, is involved in the regulation of cancer chemoresistance and stem cell signaling. However, the current understanding of the role of caveolin-1 in breast cancer development remains controversial. Herein, we demonstrate that caveolin-1 expression was upregulated after breast cancer chemotherapy in vitro and in vivo, accompanied by co-overexpression of β-catenin and ATP-binding cassette subfamily G member 2 (ABCG2) signaling. Additionally, breast CSCs were enriched for caveolin-1 expression. Caveolin-1 silencing sensitized breast CSCs by limiting their self-renewal ability but promoting the differentiation process, β-catenin silencing prevented the enhanced chemoresistance of CSCs induced by caveolin-1 overexpression, indicating that β-catenin is an essential molecule responsible for caveolin-1-mediated action. Further mechanistic investigation revealed that caveolin-1 silencing could downregulate the β-catenin/ABCG2 pathway through glycosgen synthase kinase 3 beta activation and Akt inhibition, resulting in increased β-catenin phosphorylation and proapoptotic degradation. Clinical investigation also revealed a close correlation between caveolin-1 and β-catenin/ABCG2 signaling in breast cancer samples. Notably, caveolin-1 was highly elevated in triple-negative breast cancer, and caveolin-1 silencing significantly impaired the tumorigenicity and chemoresistance of breast CSCs in in vivo models. Overall, our study not only highlights the role of caveolin-1 in mediating the chemoresistance of breast CSCs via β-catenin/ABCG2 regulation but also provides novel approaches for future therapies targeting CSCs.

Introduction

Breast cancer is the most common malignancy and the second leading cause of cancer death in women worldwide (1). Drug resistance is considered one of the most important factors influencing the clinical outcomes of patients (2). However, the current therapeutic strategies are seldom successful in reversing drug resistance in clinical settings (3). In the past decade, numerous studies have found cancer stem cells (CSCs) to be naturally resistant to chemotherapeutics or radiotherapy in a variety of cancers (4,5). Thus, elucidating the key cellular signaling events that mediate the chemosensitivity of CSCs is critical for drug discovery and improving patient prognosis.

Membrane transporters play a critical role in regulating drug resistance. In the last decade, the ATP-binding cassette protein superfamily has received much attention, particularly the proteins ATP-binding cassette subfamily B member 1 (ABCB1), ATP-binding cassette subfamily G member 2 (ABCG2) and ATP-binding cassette subfamily C member 1 (6). These adenosine triphosphate-binding cassette transporters play a significant role in mediating drug efflux and are closely correlated with the primary or acquired drug resistance presented in clinical settings. A number of studies have attempted to develop specific inhibitors targeting ATP-binding cassette subfamily B member 1 or ABCG2 (7,8). However, current progress in finding a potent, selective agent to restore cancer chemosensitivity has been slow and challenging, especially after the disappointing results obtained from the present candidates at the clinical trial stage (3). Furthermore, the discovery of CSCs in various cancers has challenged our traditional view of drug resistance. In addition to self-renewal and multilineage differentiation abilities, numerous studies have found that breast CSCs are highly resistant to chemotherapeutics and enriched for the expression of adenosine triphosphate-binding cassette transporters such as ABCG2 (9,10). It has been reported that the proportion of CD44+CD24low cells was 9.5-fold higher in tumor samples after chemotherapy (11). Breast cancer patients with high CSC populations have a poor clinical outcome (P = 0.0003) independent of tumor size, histological grade, nodal status, hormone receptors and human epidermal growth factor receptor 2 (HER2) status (12). Although the mechanisms underlying the therapeutic resistance of CSCs have not been fully elucidated, recent evidence has suggested that β-catenin signaling is closely correlated to cancer chemosensitivity. Indeed, β-catenin expression is highly elevated in drug-resistant cancer cells, and β-catenin silencing sensitizes CSCs to chemotherapeutics (13–15). Nonetheless, the current progress is still far from elucidating the molecular networks regulating CSC drug sensitivity.

Caveolin-1 is an essential constituent protein of specialized membrane invaginations called caveolae. Studies have demonstrated that caveolin-1 acts as a molecular hub, integrating the transduction of multiple signaling molecules, including Src, epidermal growth factor receptor (EGFR), HER2 and the mitogen-activated protein kinase cascade (16). Most of these signals are highlighted in cancer development. Regarding drug response analyses, caveolin-1 was found to be upregulated in multidrug-resistant colon cancer cells, adriamycin-resistant breast cancer cells, and Taxol- and gemcitabine-resistant lung cancer cells (17–19). In addition, a recent study has identified caveolin-1 as a potential causative factor in mediating trastuzumab resistance via an effect on endocytosis (20). With regard to clinical significance, a highly significant positive correlation between caveolin-1 and the ABCB1 gene was revealed in acute myeloid leukemia patients (21). Another lung cancer study also strongly indicated that patients with caveolin-1 expression had a significantly lower chemotherapy response rate and poor progression-free survival and overall survival rates (17). Moreover, emerging evidence has implied that caveolin-1 is involved in regulating stem cell signaling networks. It was found that caveolin-1 could promote fibroblast-induced mouse embryonic stem cell proliferation via the RhoA-PI3K/Akt-ERK1/2 pathway (22). One study demonstrated that mutated caveolin-1 (P132L) promoted breast cancer cells metastasis, and gene expression profiling assays showed that mammary tumors with this caveolin-1 (P132L) mutation exhibited a stem cell-associated signature, indicating that caveolin-1 might be an important regulator of breast CSCs (23). However, the relationships between caveolin-1, drug resistance and breast CSCs are still largely unknown. Elucidation of these relationships might have evolutionary significance, a highly significant positive correlation between caveolin-1 and the ABCB1 gene was revealed in acute myeloid leukemia patients (21). Another lung cancer study also strongly indicated that patients with caveolin-1 expression had a significantly lower chemotherapy response rate and poor progression-free survival and overall survival rates (17). Moreover, emerging evidence has implied that caveolin-1 is involved in regulating stem cell signaling networks. It was found that caveolin-1 could promote fibroblast-induced mouse embryonic stem cell proliferation via the RhoA-PI3K/Akt-ERK1/2 pathway (22). One study demonstrated that mutated caveolin-1 (P132L) promoted breast cancer cells metastasis, and gene expression profiling assays showed that mammary tumors with this caveolin-1 (P132L) mutation exhibited a stem cell-associated signature, indicating that caveolin-1 might be an important regulator of breast CSCs (23). 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Abbreviations: ABCG2, ATP-binding cassette subfamily G member 2; ABCB1, ATP binding cassette subfamily B member 1; CSCs, cancer stem cells; CCND1, cyclin D1; CK14, cytokeratin 14; HER2, human epidermal growth factor receptor 2; MDC, multiprotein destruction complex; MUC1, mucin 1; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; SP, side population; siRNA, small interfering RNA; TUNEL, TdT-mediated deoxyuridine triphosphate nick end labeling.

These authors contributed equally to this work.

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impacts to the current understanding of drug resistance, leading to the development of prognostic techniques, novel drug discovery, and an improved patient survival times.

In this study, we investigated the role of caveolin-1 in mediating the inherent chemoresistance of breast CSCs. Our findings illustrated that caveolin-1 expression was highly elevated after chemotherapy in vitro and in vivo. Caveolin-1 also exhibited enriched expression in breast cancer side population (SP) cells and in CSCs. Caveolin-1 silencing could sensitize breast CSCs by limiting their self-renewal ability and promoting their differentiation. Mechanistic investigation found that caveolin-1 expression had a positive correlation with ABCG2 expression via the modulation of the β-catenin proapotasmal degradation pathway, and clinical investigation further demonstrated a positive regulatory correlation between caveolin-1 and the β-catenin/ABC2 pathway. Notably, caveolin-1 was highly elevated in triple-negative breast cancer, and caveolin-1 silencing significantly impaired the tumorigenic and chemoresistance capability of breast CSCs in in vivo models. Taken together, our study not only sheds new light on the mechanisms for drug resistance in CSCs but also advances the current understanding of the significance of caveolin-1 during breast cancer therapy.

Materials and methods

Cell culture

MCF-7 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection. MCF-7/ADR cells were derived from MCF-7 cells via treatment with a gradually increasing concentration of epirubicin for 6 months. The cells were cultured in medium (RPMI 1640 for MCF-7 and MCF-7/ADR; L-15 for MDA-MB-231) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Gibco Life Technologies, Lofer, Austria) at 37°C in a humidified incubator with 5% CO₂ or without CO₂.

Human breast cancer xenografts

All animal studies were approved by the animal ethics committee of the University of Hong Kong. Six-week-old female nude mice were obtained from the laboratory animal unit at the University of Hong Kong. The mice were maintained under pathogen-free conditions and a 12 h light/dark cycle, with free access to food and water. All mice were cared for in accordance with the institutional guidelines. For the establishment of breast cancer xenografts, 5 x 10⁵ MCF-7 or MDA-MB-231 cells were resuspended in phosphate-buffered saline (PBS) and injected into the fourth mammary fat pads of mice under anesthetics. For each cell line, a total number of 12 mice were randomly divided into vehicle control and epirubicin treatment groups. For the MCF-7 xenografts, 17β-estradiol (Sigma, St. Louis, MO) was delivered subcutaneously at a dose of 1.5 mg per animal at 3-day intervals to support cancer growth. After the tumors reached approximately 5 mm x 5 mm, epirubicin was administered by intraperitoneal injection at 2.5 mg/kg/week (W) for 4 weeks. The tumors were measured at 3-day intervals with calipers, and the tumor volume was calculated using the following formula: volume (mm³) = width² x length/2. At the end of the experiment, the tumor tissues were removed and fixed in 4% paraformaldehyde overnight. After washing for 8 h in distilled water, the tissues were dehydrated with increasing concentrations of ethanol, cleared with xylene, and finally infiltrated and embedded in paraffin. The prepared tissues were sectioned (4 µm) and subjected to immunohistochemical examination for caveolin-1, β-catenin (Cell Signaling Technology, Danvers, MA), and ABCG2 (Santa Cruz, CA). The detailed procedures are described in the methods section ‘Immunohistochemistry analysis’.

Flow cytometry and sorting

For SP analysis, breast cancer cells were detached with trypsin, washed once with PBS containing 2% fetal bovine serum, and stained with 5 µg/ml Hoechst 33342 (Sigma) in medium at 37°C for 2 h. For CSC population analysis and sorting, breast cancer cells were stained with CD44-fluorescein isothiocyanate and CD24-PE antibodies (BD Biosciences, San Jose, CA); fluorescein isothiocyanate- or PE-labeled isotype IgG (BD biosciences) as control. The fluorescence assays were performed using a 10 µg/ml epirubicin (Sigma) for 60min at 37°C. After drug exposure and washing, the cells were released in drug-free medium for 90min and subjected to flow cytometry.

Plasmids and siRNA transfection

The pDNA 3.1(+)-Cav-1 plasmid was purchased from Addgene company (Cambridge, MA). The pDNA 3.1(+)-β-catenin plasmid was a gift from Dr Yu Wang at the University of Hong Kong. The plasmids were transfected into MCF-7 and MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24h, the transfected cells were passaged and selected for 2 weeks with 200 µg/ml G418 (Invitrogen). Human Cav-1 shRNA was purchased from Origene (Rockville, MD) and transfected into MDA-MB-231 cells using Lipofectamine 2000. The transfected cells were passaged after 24h and selected for 1 µg/ml puromycin (Origene). Pools of populations of positive cells, obtained at 2 weeks after drug selection without subcloning, were subjected to in vitro experiments. The negative control cells were generated by infecting the cells with scrambled plasmids. The Cav-1 small interfering RNA (siRNA), β-catenin siRNA and scrambled siRNA were purchased from Invitrogen and transfected into MCF-7 and MDA-MB-231 cells using the X-tremeGENE siRNA transfection reagent (Roche Diagnostics, Mannheim, Germany).

Cell proliferation assay

Cells were seeded at 3000 cells per well in 96-well plates after siRNA transfection and cultured for 3 days with or without epirubicin (0.5 µg/ml), 3-(4,5-Dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (Sigma) was added, and the plates were incubated for 4 h at 37°C. The supernatants were removed, and the formazan crystals were dissolved in 150 µl/well of dimethyl sulfoxide. The absorbance at 490 nm of each sample was measured using a multilabel plate reader (Bio-Rad, Hercules, CA).

Mammosphere cultures and differentiation assays

The sorted breast CSCs were plated and cultured in ultralow attachment plates in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 1% penicillin and streptomycin, B27 (Invitrogen), 20µg/ml HEGF (BD bioscience), 5 µg/ml insulin, and 0.4% bovine serum albumin (Sigma). To quantify the effect of caveolin-1 on mammospheres, primary- and secondary-passage mammospheres were used and microscopically analyzed by evaluating the mammosphere number and size. For the differentiation assay, mammospheres from the third day of culture were suspended in Matrigel (BD bioscience) and loaded into 12-well plates that were previously coated with Matrigel. The mammospheres were then cultured in Dulbecco’s modified Eagle’s medium medium containing 1% fetal bovine serum in a 37°C incubator. After 3 days, the Matrigel was cut out of the well and embedded in OCT for cryosectioning. The sections were mounted on slides and fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100. After blocking in 10% goat serum for 1 h, the slides were incubated with secondary antibodies against Cytokeratin 14 (CK14) and Mucin 1 (MUC1) (Santa Cruz, CA) overnight at 4°C, followed by secondary fluorescence-labeled antibodies (Santa Cruz, CA) for 2h at room temperature. Finally, the samples were incubated with 4',6-diamidino-2-phenylindole (Sigma) for nuclear staining, and the signal was detected with a confocal microscope.

Western blotting analysis

To determine the protein concentration, cells were lysed in RIPA buffer (Sigma) containing a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). The protein concentration was measured with the bichinchoninic acid assay (Thermo Fisher Scientific, Bonn, Germany). Quantified protein lysates (15 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and resolved on 12% polyacrylamide gels. The proteins were then transferred onto a PVDF membrane (GE Healthcare, Freiburg, Germany). The membrane was probed with primary antibodies against caveolin-1, β-catenin, P-β-catenin (Ser33/37/Thr41), Akt, P-Akt (Ser473), glycogen synthase kinase 3 beta (GSK3β), P-GSK3β (Ser9), β-actin (Cell Signaling Technology) and ABCG2 (Santa Cruz, CA) at 4°C overnight. After three washes with Tris-buffered saline with 0.05% Tween-20, the membrane was incubated with secondary antibody and horseradish peroxidase (Roche) and the signal was detected using chemiluminescent ECL detection reagent (Roche Diagnostics, Mannheim, Germany).

Real-time PCR analysis

Total RNA from breast CSCs or human blood was extracted using the TRIzol reagent (Invitrogen), and reverse transcription was carried out using the first-strand cDNA synthesis kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The real-time PCR analysis was performed using a SYBR Green kit (Roche) on a Roche Lightcycler 480 detector. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 1 min. The primers for caveolin-1, β-catenin, c-Myc, survivin, Cyclin D1 (CCND1), ABCG2 and β-actin were designed as follows: caveolin-1 forward primer (F), 5′-AACACGTAGTCTGGCCTTCTAG-3′; reverse primer (R), 5′-GGATGGGACAGTTGTAGAGAT-3′; β-catenin (F), 5′-GCTTTCGAGTTGAGCTGACCA-3′; (R), 5′-CAAGTCCAAGATCCAGCTCCT-3′; c-Myc (F), 5′-GCTTCACTTAGCCGTGATTT-3′; (R), 5′-TAAAGGTGAGG.
The data are expressed as the mean ± SD. A two-tailed Student’s t-test was used to examine the significance of the data between two groups. Pearson’s χ² test was applied to study the relationship between caveolin-1 and other clinicopathological features of breast cancer patients. Statistical significance was achieved when the P value was < 0.05. A linear regression was applied to study the relationship between caveolin-1 and ABCG2 or β-catenin in breast cancer patients.

Results

Chemotherapy induces caveolin-1 up-regulation in breast cancer cells and tissue

To determine the correlation between caveolin-1 and the chemotherapeutic response, we initially treated breast cancer cells with chemotherapeutic agents including epirubicin, Taxol and 5-Fluorouracil purchased from Sigma. Three drugs are generally used as first-line candidates for breast cancer therapy. After drug administration for 24h, the caveolin-1 expression in both MCF-7 and MDA-MB-231 cells was significantly elevated in a dose-dependent manner, accompanied by the up-regulation of ABCG2 and β-catenin (Figure 1A and B). These results not only indicate that caveolin-1 might be closely correlated to drug response, independent of cell type or drug type, but also might impact chemosensitivity via β-catenin/ABCG2 signaling. To confirm whether the in vivo results are consistent with in vitro findings, we implanted breast cancer cells in nude mice and administered chemotherapy with epirubicin. The results showed that epirubicin treatment significantly limited cancer growth, especially in the MDA-MB-231 model (Figure 1C). In addition, immunohistochemistry results revealed a significant upregulation of all three proteins in the tumor tissues after chemotherapy (Figure 1D). Because chemotherapy was reported to be an effective tool for enriching CSCs, we detected the CSC population in both the control and chemotherapy groups and found that the CSC population was increased 3- and 1.14-folds in the MCF-7 and MDA-MB-231 xenografts, respectively (Figure 1E), indicating a potential correlation between CSCs and caveolin-1.

Caveolin-1 expression is enriched in breast cancer SpS and CSCs

To confirm the correlation between caveolin-1 and CSCs, we first isolated the SP of MCF-7 cells and its drug-resistant lineage MCF-7/ADR cells. SP cells are described as a subpopulation with stem-like characteristics and a strong ability to efflux chemotherapeutics, thus SP cells are usually applied to reflect the drug-resistant property of cells or tissues. We found that the ratio of the SP in MCF-7/ADR cells was much higher than that in MCF-7 cells (22.7 ± 5.7% versus 2.38 ± 0.65%) (Figure 2A). A drug efflux assay also demonstrated that the MCF-7/ADR cells have a strong ability to efflux epirubicin, as illustrated by their lower intracellular drug intensity (Figure 2B). Therefore, it is worthwhile to further detect difference in caveolin-1 expression between these two cell lines. Immunoblotting results indicated that compared with MCF-7 cells, there was a significant upregulation of caveolin-1, ABCG2, and β-catenin signaling in MCF-7/ADR cells, suggesting that caveolin-1 is closely correlated to cellular drug resistance and stem-like characteristics (Figure 2C). Therefore, we further sorted SP and CSCs from MCF-7 and MDA-MB-231 cells to detect the caveolin-1 expression status and found enriched caveolin-1 expression on SP and CSCs from both cell lines compared with non-SP and non-CSCs. In addition, the expression status of ABCG2 and β-catenin was synchronous with caveolin-1, indicating that caveolin-1 might also be involved in regulating the chemosensitivity of CSCs (Figure 2D and E).

Caveolin-1 is closely correlated to the chemosensitivity and cellular decision of CSCs

We next evaluated the influence of caveolin-1 on breast CSC functions. MCF-7 and MDA-MB-231 cells were transfected with Cav-1 siRNA and then subjected to CSC analysis. Compared with the scrambled group, Cav-1 siRNA significantly limited the CSC.
Caveolin-1 mediates breast CSC chemoresistance

Regardless, Caveolin-1 overexpression rescued the epirubicin-induced inhibition of cell viability, suggesting that caveolin-1 is a critical molecule influencing breast cancer chemosensitivity (Figure 3C). Immunoblotting assays revealed that after caveolin-1 silencing in breast MDA-MB-231 CSCs, the expression of ABCG2 and β-catenin were both significantly downregulated, which was accompanied by an increased epirubicin concentration in breast CSCs, as detected by flow cytometry (Figure 3D). In contrast, caveolin-1 overexpression stimulated enhanced expression of β-catenin and ABCG2. A drug efflux assay also demonstrated that caveolin-1 upregulation promoted the drug efflux ability of breast CSCs (Figure 3D). Similar findings were also observed in the CSCs sorted from the MCF-7 cells (Supplementary Figure 1, available at Carcinogenesis Online). These results confirmed a significant role for caveolin-1 in regulating the chemosensitivity of breast CSCs, which is closely correlated to β-catenin/ABCG2 signaling.

Breast CSCs are enriched in non-adherent spherical clusters of cells, termed mammospheres, and these cells are capable of yielding secondary spheres and differentiating along multiple lineages. To evaluate whether caveolin-1 could suppress the formation of mammospheres in vitro, we sorted CSCs from MDA-MB-231 cells in the presence or absence of Cav-1 siRNA treatment. Compared with the scrambled control groups, caveolin-1 silencing greatly limited not only the number of mammospheres but also the size of the spheres, indicating a reduced self-renewal capacity of breast CSCs (Figure 4A). In MDA-MB-231/Cav-1(+)+ cells, the sorted CSCs demonstrated a stronger ability to form mammospheres, with increased numbers and sizes (Figure 4B). Immunofluorescence results further indicated that caveolin-1 silencing inhibited ABCG2 signaling in mammospheres, whereas caveolin-1 overexpression activated the signaling pathway (Figure 4C). Similar findings were also observed

Fig. 1. Chemotherapy induced the upregulation of caveolin-1 both in vitro and in vivo, accompanied by the increased expression of β-catenin/ABCG2 signaling. (A) MCF-7 and MDA-MB-231 breast cancer cells were treated with the chemotherapeutics epirubicin, Taxol and 5-fluorouracil for 24h. The expression of caveolin-1 in both cell lines was significantly increased after chemotherapy and was accompanied by the co-upregulation of β-catenin/ABCG2 signaling. (B) Quantification of caveolin-1 expression changes before and after chemotherapy in both breast cancer cell lines. (C) Epirubicin treatment significantly limited tumor growth in breast cancer xenografts introduced in nude mice (values are presented as the mean ± SD, n = 6, *P < 0.01 versus normal controls). (D) Immunohistochemistry results revealed that after chemotherapy, the expression of caveolin-1, β-catenin, and ABCG2 was highly elevated for both cancer samples, particularly the MDA-MB-231 xenografts. (E) Flow cytometry results revealed that chemotherapy also induced enriched populations of CSCs in both breast cancer xenografts (values are presented as the mean ± SD, n = 3, *P < 0.05 versus normal controls).

Population from 6.22 ± 1.31% to 1.64 ± 0.42% in MCF-7 cells, and CSCs in the MDA-MB-231 cell line were also reduced from the original 86.5 ± 3.26% to 65.2 ± 2.88% after caveolin-1 silencing (Figure 3A). To further confirm the role of caveolin-1 in regulating breast CSCs, we upregulated caveolin-1 expression in MCF-7 and MDA-MB-231 cells by transfecting the recombined Cav-1 plasmid. Flow cytometry results showed that the CSC population was increased from 6.35 ± 1.28% to 14.8 ± 3.72% and 84.8 ± 4.66% to 95.8 ± 2.35%, respectively, in MCF-7/Cav-1 (+) and MDA-MB-231/Cav-1 (+) cells (Figure 3B). These results indicate that caveolin-1 might be a critical modulator in regulating the population of breast CSCs.

To investigate whether the regulation of caveolin-1 specifically influences breast cancer cell proliferation and chemosensitivity, the impact of caveolin-1 modulation on the adherent proliferation, β-catenin/ABCG2 expression, and drug efflux ability of CSCs was determined. However, the inhibition or up-regulation of caveolin-1 had no impact on the cell viability of MCF-7 or MDA-MB-231 cells, illustrating that caveolin-1 is largely not an essential gene for the bulk of both breast cancer cell lines (Figure 3C). Regardless, the inhibition of caveolin-1 did lead to a decrease in cell viability under epirubicin treatment when compared with the scrambled control, and caveolin-1 overexpression rescued the epirubicin-induced inhibition of cell viability, suggesting that caveolin-1 is a critical molecule influencing breast cancer chemosensitivity (Figure 3C). Immunoblotting assays revealed that after caveolin-1 silencing in breast MDA-MB-231 CSCs, the expression of ABCG2 and β-catenin were both significantly downregulated, which was accompanied by an increased epirubicin concentration in breast CSCs, as detected by flow cytometry (Figure 3D). In contrast, caveolin-1 overexpression stimulated enhanced expression of β-catenin and ABCG2. A drug efflux assay also demonstrated that caveolin-1 upregulation promoted the drug efflux ability of breast CSCs (Figure 3D). Similar findings were also observed in the CSCs sorted from the MCF-7 cells (Supplementary Figure 1, available at Carcinogenesis Online). These results confirmed a significant role for caveolin-1 in regulating the chemosensitivity of breast CSCs, which is closely correlated to β-catenin/ABCG2 signaling.
in the CSCs sorted from MCF-7 cells (Supplementary Figure 2A–C, available at Carcinogenesis Online).

Because β-catenin has been reported to participate in the regulation of differentiation, we next evaluated the influence of caveolin-1 on the differentiation state of CSCs. The formed mammospheres were embedded in Matrigel to induce differentiation, and the Matrigel was then subjected to freezing for sectioning and immunostaining for differentiation markers. In our study, the basal-like marker CK14 and the luminal-like marker MUC1 were used to determine the differentiation status. The results showed that after caveolin-1 silencing, the expression of CK14 and MUC1 on mammospheres was enhanced. In Cav-1(+) CSCs, the fluorescence intensity of the differentiation markers was inhibited compared with the control group, indicating that caveolin-1 could maintain the self-renewal ability of CSCs while limiting their differentiation capacity (Figure 4D and Supplementary Figure 2D, available at Carcinogenesis Online). Because CSCs exhibit a greater drug resistance than normal cancer cells, the enhanced chemosensitivity after caveolin-1 silencing might also be attributed to accelerated differentiation.

**Caveolin-1 silencing promotes CSC chemosensitivity by inducing β-catenin degradation**

Based on the clarification regarding caveolin-1 and CSC chemosensitivity, it is necessary to elucidate the molecular mechanisms involved. There is considerable evidence supporting a role for β-catenin-mediated signaling regulating the self-renewal, differentiation and chemosensitivity of CSCs. In addition, studies have shown previously that the ABCG2 gene is a downstream target of β-catenin based on its binding to T-cell factor/lymphoid-enhancer-factor motifs in the ABCG2 promoter. Therefore, we determined the effect of caveolin-1 on β-catenin signaling in breast CSCs sorted from MDA-MB-231 cells. First, we transfected β-catenin siRNA into breast CSCs over-expressing caveolin-1; the upregulation of ABCG2 induced by caveolin-1 was blocked after β-catenin silencing. Conversely, when we administered Cav-1 siRNA to CSCs overexpressing β-catenin, the inhibition of ABCG2 expression was relieved, indicating that β-catenin is a necessary signal in mediating caveolin-1-regulated chemosensitivity (Figure 5A).

Numerous studies since the 1980s have highly implicated the β-catenin signal in mammary development and tumorigenesis (24,25). Under normal circumstances, β-catenin is maintained at a normalized level by the multiprotein destruction complex (MDC), which induces excessive β-catenin degradation via the ubiquitination and proteasome pathway (26). However, the β-catenin pathway is continuously activated in breast CSCs due to Wnt stimulation and MDC inactivation (27). Therefore, we first studied whether caveolin-1 affects β-catenin mRNA transcription. Our real-time PCR results revealed that caveolin-1 silencing had no inhibitory effects on β-catenin mRNA levels but reduced the transcription of the genes downstream of β-catenin, including c-myc, ABCG2, CCND1 and survivin, indicating that caveolin-1 loss might decrease β-catenin accumulation in the nucleus of CSCs (Figure 5B). In other words, the proteasomal degradation pathway might be activated following caveolin-1 silencing. Therefore, we added cycloheximide (Sigma), a protein synthesis inhibitor, to the CSCs treated with Cav-1 siRNA and found that the degradation rate of β-catenin was accelerated in the caveolin-1-silenced group. In contrast, when we added the proteasome inhibitor MG132 (Sigma) to Cav-1 siRNA-treated cells, β-catenin degradation was blocked (Figure 5C). These results indicated that caveolin-1 regulates the intracellular β-catenin level mainly via the proteasome degradation pathway.

To determine how the proteasomal degradation pathway is activated after caveolin-1 silencing, lithium chloride (LiCl, Sigma) was
Caveolin-1 mediates breast CSC chemoresistance

administered to inactivate GSK3β, a major component of MDC. LiCl promotes GSK3β phosphorylation at Ser9, which in turn reduces the phosphorylation of β-catenin at Ser33/Ser37/Thr41 and thus its degradation. In our study, the enhancement of caveolin-1 silencing-mediated β-catenin phosphorylation was reversed upon LiCl treatment (Figure 5D). Because Akt is reported to be an upstream regulator of GSK3β (28), we sought to determine whether the Akt inhibitor LY294002 (Sigma) could also influence ABCG2 expression via β-catenin modulation. The results showed that after LY294002 administration, ABCG2 expression was inhibited, accompanied by a reduction of GSK3β phosphorylation and increased phosphorylated β-catenin (Figure 5E). We also detected the levels of phosphorylated Akt in CSCs with caveolin-1 silencing and overexpression. The results showed that caveolin-1 silencing inhibited Akt phosphorylation at the Ser 473 site, whereas caveolin-1 overexpression significantly increased the phosphorylation level (Figure 5E). More importantly, the caveolin-1-mediated up-regulation of the ABCG2 level could be blocked by LY294002 treatment, indicating that the accelerated β-catenin proteasome degradation following caveolin-1 silencing might be due to the inhibition of Akt phosphorylation (Figure 5F).

Fig. 3. Caveolin-1 was closely correlated to the breast CSC population and drug resistance. (A) Caveolin-1 silencing significantly limited the CSC population in both MCF-7 and MDA-MB-231 breast cancer cells (values are presented as the mean ± SD, n = 3, *P < 0.01 versus scrambled controls). (B) The pcDNA 3.1(+) /Cav-1 plasmid was stably transfected into MCF-7 and MDA-MB-231 cells and subjected to flow cytometry analysis. The results showed that caveolin-1 overexpression significantly increased the CSC population in both breast cancer cell lines (values are presented as the mean ± SD, n = 3, *P < 0.01 versus empty vector controls). (C) 3-(4,5-Dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide assays revealed that caveolin-1 silencing or overexpression did not influence the proliferation of MCF-7 and MDA-MB-231 cells. However, caveolin-1 silencing did increase the chemosensitivity of both breast cancer cell lines to epirubicin, whereas caveolin-1 overexpression significantly restored the cell viability inhibited by epirubicin, indicating that caveolin-1 affects cancer chemosensitivity independent of cell proliferation (values are presented as the mean ± SD, n = 6, *P < 0.01 versus scrambled controls). (D) Western blotting results revealed that caveolin-1 silencing led to the decreased expression of β-catenin and ABCG2 in MDA-MB-231 CSCs, whereas caveolin-1 overexpression resulted in the enhanced expression of β-catenin and ABCG2. Flow cytometry also demonstrated that caveolin-1 silencing greatly elevated the concentration of epirubicin in breast CSCs, whereas caveolin-1 overexpression significantly promoted epirubicin efflux from breast CSCs, indicating that caveolin-1 expression was closely correlated to the chemoresistance property of breast CSCs.
Caveolin-1 silencing promotes the chemosensitivity of breast CSCs in vivo

To obtain direct evidence demonstrating that caveolin-1 is closely correlated to clinical chemosensitivity, we detected caveolin-1 mRNA expression in ten breast cancer patients before and after chemotherapy completion. The results showed that caveolin-1 mRNA expression was elevated in all patients, indicating that caveolin-1 might be critical in mediating drug resistance during breast cancer chemotherapy (Figure 6A). To further determine the clinical significance of the correlation between caveolin-1 and β-catenin/ABCG2 signaling, we collected 46 breast cancer clinical samples to characterize by immunohistochemical detection. The results showed that among breast cancer patients, caveolin-1 expression is highly elevated in the triple-negative subtype (Figure 6A). Western blotting also revealed increased expression levels of caveolin-1, β-catenin, and ABCG2 in MDA-MB-231 cells relative to MCF-7 cells (Supplementary Figure 3A, available at Carcinogenesis Online). Triple-negative breast cancer has low chemosensitivity and a high metastatic risk in the clinical setting, and some studies have reported that triple-negative breast cancer patients have a relatively larger CSC population (29–31). Our results provide indirect evidence that caveolin-1 overexpression might be closely correlated to the poor chemotherapeutic response in the clinical setting. Furthermore, we carried out linear regression analyses between caveolin-1 and other pathological parameters of breast cancer (Figure 6B and C). Detailed information regarding caveolin-1 and other pathological parameters of breast cancer is listed in Supplementary Table 1, available at Carcinogenesis Online.

Based on the findings that caveolin-1 is highly expressed in triple-negative breast cancer patients and is closely correlated to the chemosensitivity and self-renewal properties of breast CSCs, we therefore assessed whether caveolin-1 silencing would affect the tumorigenesis and chemoresistance of breast CSCs sorted from the triple-negative cell line MDA-MB-231 (Supplementary Figure 3B, available at Carcinogenesis Online). As shown in Figure 6D, injection of $1 \times 10^3$ breast CSCs generated tumors in five out of eight mice, whereas no tumors developed in the mice injected with MDA-MB-231 cells that were not the CD44$^+$/CD24$^-$ form. Increasing the number of injected breast CSCs to $5 \times 10^3$ resulted in 100% tumor incidence, but no tumors developed in the mice that received cells lacking this phenotype. However, Cav-1 shRNA transduction resulted in limited tumorigenic ability of breast CSCs, with only 25% tumor incidence ratio when $5 \times 10^3$ breast CSCs were injected. Therefore, caveolin-1 silencing significantly interferes with tumor initiation and the in vivo self-renewal of breast CSCs. We further evaluated whether caveolin-1 silencing could improve chemosensitivity in breast CSCs of triple-negative xenografts. The chemoresponse to epirubicin was measured for breast CSCs or CSCs/Cav-1(−), which were sorted from parental or caveolin-1-silenced MDA-MB-231 cells and inoculated into the mammary pads of NOD/SCID mice. The results showed that epirubicin alone only resulted in a 28.5±4.6% chemotherapeutic response in CSC xenografts, whereas caveolin-1 silencing significantly limited breast cancer growth, with an inhibition ratio of 44.8±5.7% (Figure 6D). More importantly, when epirubicin was administered to the CSCs/Cav-1(−) xenografts, the chemoresponse reached as high as 85.3±6.2% (Figure 6E), suggesting that caveolin-1 silencing could significantly improve breast cancer chemosensitivity. This is consistent with our in vitro findings. Immunohistochemistry demonstrated that caveolin-1 silencing led to decreased β-catenin/ABCG2 expression, though it had little effect on Ki67 expression and TUNEL staining (Figure 6F and Supplementary Figure 3C, available at Carcinogenesis Online). However, epirubicin treatment not only resulted in a further
Caveolin-1 mediates breast CSC chemoresistance

Fig. 5. Caveolin-1 regulated the ß-catenin/ABCG2 pathway via the proteasome degradation pathway and Akt signaling. (A) ß-catenin silencing inhibited the upregulation of ABCG2 induced by caveolin-1 overexpression; in breast CSCs overexpressing ß-catenin, the inhibitory effect of Cav-1 siRNA on ABCG2 was blocked, indicating that ß-catenin was a necessary mediator between caveolin-1 and ABCG2. (B) After Cav-1 siRNA administration, qPCR revealed little influence on the ß-catenin mRNA level, whereas the transcription of genes downstream of ß-catenin were down-regulated, including c-Myc, Survivin, CCND1 and ABCG2 (values are presented as the mean ± SD, n = 3, *P < 0.01 versus control). (C) The protein synthesis inhibitor cycloheximide was administered to breast CSCs with or without Cav-1 siRNA. ß-catenin was degraded faster in the Cav-1 siRNA group compared with the scrambled group. However, when the proteasomal inhibitor MG132 was administered, the degradation-inducing effects of Cav-1 siRNA on ß-catenin were blocked, indicating that Cav-1 siRNA could inhibit ß-catenin expression via the proteasomal degradation pathway (values are presented as the mean ± SD, n = 3, *P < 0.01 versus scrambled control). (D) The GSK3ß inhibitor LiCl was administered to breast CSCs with or without Cav-1 siRNA. Western blotting results revealed that Cav-1 siRNA inhibited the level of p-GSK3ß, therefore inducing ß-catenin phosphorylation and inhibiting ABCG2 expression. However, LiCl activated GSK3ß phosphorylation and blocked ß-catenin phosphorylation, resulting in the increased expression of ABCG2 compared with the Cav-1 siRNA alone group. These results indicated that GSK3ß might be a critical molecule for mediating the caveolin-1-regulated proteasomal degradation pathway. (E) The Akt inhibitor LY294002 inhibited the ß-catenin/ABCG2 pathway. Cav-1 siRNA inhibited p-Akt, whereas caveolin-1 overexpression increased the level of p-Akt, indicating that caveolin-1 might regulate the ß-catenin proteasome degradation pathway via Akt signaling. (F) When the Akt inhibitor LY294002 was administered to breast CSCs overexpressing caveolin-1, the caveolin-1-induced up-regulation of ß-catenin/ABCG2 signaling was blocked through the inhibition of p-GSK3ß.

Discussion

Chemotherapy plays a significant role in improving the survival and decreasing mortality in breast cancer patients. However, chemoresistance, whether inherent or acquired, greatly threatens the clinical outcomes and lives of breast cancer patients. At present, approximately 40% of all breast cancer patients suffer local recurrence or distant metastasis after chemotherapy, which are both closely correlated to poor chemosensitivity (32). Although numerous molecular markers correlate with chemosensitivity, they do not appear to be causative factors and thus are not ideal targets for developing sensitizing drugs; furthermore, the phenotypic diversity of cancer cells changes significantly between pre- and post-treatment samples (33). In recent years, CSCs have emerged as the most likely determining factor for cancer chemosensitivity. The CSC theory holds that cancer is initiated from or driven by a small population of stem-like cells that are capable of continuous self-renewal and differentiation (34). The hypothesis is supported by increasing evidence of the existence of CSCs in various types of malignancies. Moreover, accumulating studies suggest that classical chemotherapy does not effectively eradicate and in fact may even increase the relative proportion of CSCs in cancer (35,36). At present, the mechanisms underlying the therapeutic resistance of CSCs have not been fully elucidated. However, several mechanisms have been suggested, including the following: (i) stem cells remain quiescent, making them resistant to chemotherapeutics that rely on an active cell cycle; (ii) CSCs have a high DNA repair ability; (iii) CSCs have a high expression of drug efflux proteins such as ABCG2 and (iv) most current therapies do not target the signaling pathways that regulating CSC self-renewal (5,37). In consideration of these important biological properties of CSCs, the elucidation of the molecular networks regulating the chemosensitivity of CSCs might be more critical than killing the rapidly proliferating cells.

Several recent studies have indicated that caveolin-1, a membrane transporter protein, is involved in mediating cancer drug resistance...
and stem cell signaling transduction (17–21). Accordingly, it is worthwhile to further explore the role of caveolin-1 in regulating the chemosensitivity of CSCs. We used in vitro and in vivo systems to validate whether caveolin-1 is closely correlated to breast cancer chemosensitivity. Our findings suggested that caveolin-1 expression was significantly elevated after chemotherapy treatment and in drug-resistant breast cancer cells, accompanied by an enrichment of CSCs. In particular, we sorted breast CSCs and SPs and observed that caveolin-1 was preferentially expressed on these stem-like cells, indicating that caveolin-1 might be a signaling mechanism that is essential for the chemosensitivity modulation of CSCs. However, current studies on the role of caveolin-1 in breast cancer occurrence and development remain contentious. It was reported that caveolin-1 was negatively correlated with breast cancer transformation and oncogene activation (38,39). Caveolin-1 was also found to be reduced or absent in mammary tumors from MMTV-c-Myc, -Her2,
Caveolin-1 mediates breast CSC chemoresistance

-Src, -Ha-Ras and p53-null transgenic mice (40). Several clinical studies further demonstrated that caveolin-1 is reduced in breast cancer compared with normal tissues (41,42). Consistent with the above findings, our study also supported the notion that caveolin-1 is lost in non-CSCs. Nevertheless, we identified breast CSCs with caveolin-1 enrichment, which was not previously studied. We found that caveolin-1 silencing could significantly limit breast CSC self-renewal and enhance their chemosensitivity. In contrast, caveolin-1 overexpression was found to promote the self-renewal and drug-resistant property of CSCs. As these observations suggested that caveolin-1 plays a significant role in maintaining CSC survival, it is necessary to reconsider the role of caveolin-1 in breast cancer development. Because CSC differentiation is reported to be significant in maintaining cancer cell proliferation, angiogenesis and metastasis (43,44), we performed experiments to determine the effects of caveolin-1 on CSC differentiation. Interestingly, we found that caveolin-1 silencing could promote breast CSC differentiation to basal-like or luminal-like cells, indicating that caveolin-1 loss may promote CSC differentiation to normal cancer cells. These results provide a logical explanation for the previous findings that caveolin-1 is lost during breast cancer occurrence and development. Additionally, we also found that the expression of ABCG2 was also inhibited during the differentiation induced by the loss of caveolin-1. These results not only suggested that caveolin-1 might regulate breast cancer chemosensitivity by modulating differentiation but also implied that the induction of differentiation might become a novel approach for the chemosensitization of breast CSCs.

Compared with the Notch and Hedgehog pathways, which are involved in regulating CSC maintenance, the β-catenin pathway is highly implicated in mammary development and tumorigenesis (24). In addition to controlling self-renewal and differentiation, recent evidence further suggests that the pathway is closely correlated to chemoresistance. Yeung et al. found that β-catenin silencing re-sensitized leukemic stem cells to GSK3β inhibitors (15). Another study carried out by Heidel et al. demonstrated that the genetic and pharmacologic inhibition of β-catenin could target Imatinib-resistant leukemic stem cells in chronic myeloid leukemia (13). Chau et al. further demonstrated that ABCG2 is transcriptionally controlled by β-catenin (45). Clinical studies have also indicated that β-catenin accumulation and ABCG2 expression are significantly associated with a poor prognosis in breast cancer, especially in the triple-negative basal-like type (46,47). In addition, several studies have suggested that caveolin-1 silencing is necessary in mediating β-catenin accumulation in colon cancer and osteocyte mechanotransduction (48,49). However, the detailed molecular mechanisms are still unclear. Here, we demonstrate that β-catenin is a molecule that is essential for mediating caveolin-1-induced drug resistance. β-catenin overexpression blocked the enhanced chemosensitivity of CSCs after caveolin-1 silencing, whereas β-catenin silencing decreased the drug resistance caused by caveolin-1 overexpression. Because the β-catenin pathway is aberrantly activated in breast CSCs due to MDC dysfunction and continuous Wnt stimulation, we further investigated its changes at the transcriptional and post-translational levels after caveolin-1 intervention. Our findings revealed that caveolin-1 silencing could down-regulate the β-catenin/ABCG2 pathway through the activation of GSK3β and Akt inhibition, resulting in increased β-catenin phosphorylation and proteasomal degradation. Similar effects of caveolin-1 on Akt signaling were also observed in renal and prostate cancer cells, further confirming that caveolin-1 might be an upstream regulator of β-catenin. The in vitro modulation of caveolin-1 on β-catenin/ABCG2 signaling was also confirmed in clinical breast cancer specimens. We found that caveolin-1 mRNA was significantly elevated in breast cancer patients after chemotherapy, which was in agreement with our in vitro results. Immunohistochemistry further indicated that caveolin-1 was particularly elevated in triple-negative breast cancer and closely correlated to β-catenin/ABCG2 expression. Elsheikh et al. also found that caveolin-1 and -2 were associated with basal-like breast cancer and the triple-negative phenotype (50). Because studies have shown previously that the triple-negative breast cancer subtype has an elevated CSC population, low chemosensitivity, and high metastasis risk (29–31), we further applied a tumorigenesis assay to validate the significance of caveolin-1 in mediating the tumorigenic and chemoresistant properties of CSCs sorted from MDA-MB-231 cells. The in vivo results revealed that in MDA-MB-231 cells, caveolin-1 silencing significantly impaired CSC tumorigenic ability and enhanced their response to epirubicin, indicating that caveolin-1 might be a crucial molecular target for improving the chemosensitivity of triple-negative breast cancer.

In recent years, CSCs have emerged as factors that are essential to determine cancer chemosensitivity. Nonetheless, the mechanisms involved in chemoresistance are multifaceted and largely undefined. Our findings suggest that caveolin-1 may be an upstream factor mediating drug resistance in breast CSCs via the regulation of β-catenin/ABCG2. The results advance our current understanding of the role of caveolin-1 in mediating breast cancer drug resistance and shed new light on the future development of chemosensitizing strategies directed at breast CSCs. However, further investigation is still needed to validate the role of caveolin-1 and differentiation in mediating chemoresistance among other cancer types.

Supplementary material

Supplementary Table 1 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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Z. Wang et al.


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