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Original Articles

FOXF2 suppresses the FOXC2-mediated epithelial–mesenchymal transition and multidrug resistance of basal-like breast cancer

Jun Cai [a], Ai-Xian Tian [a], Qing-Shan Wang [a,b], Peng-Zhou Kong [a], Xin Du [a], Xiao-Qing Li [a,b], Yu-Mei Feng [a,b,*]

[a] Department of Biochemistry and Molecular Biology, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Tianjin 300060, China

[b] Key Laboratory of Breast Cancer Prevention and Treatment of the Ministry of Education, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Tianjin 300060, China

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ABSTRACT

Forkhead box (FOX) F2 and FOXC2 belong to the FOX transcription factor superfamily. FOXC2 is recognized as an inducer of epithelial–mesenchymal transition (EMT), and its overexpression promotes basal-like breast cancer (BLBC) metastasis. Our previous study demonstrated that FOXF2 functions as an EMT suppressor and that FOXF2 deficiency promotes BLBC metastasis. However, the relationship between the opposite EMT-related transcription factors FOXF2 and FOXC2 remains unknown. Here, we found that FOXF2 directly targets *FOXC2* to negatively regulate FOXC2 transcription in BLBC cells. Functionally, we observed that FOXC2 mediates the FOXF2-regulated EMT phenotype, aggressive behavior, and multiple chemotherapy drug resistance of BLBC cells. Additionally, we detected a significant negative correlation between the *FOXF2* and *FOXC2* mRNA levels in triple-negative breast cancer (TNBC) tissues. TNBC patients in the $FOXF2_{high}/FOXC2_{low}$ and $FOXF2_{low}/FOXC2_{high}$ groups exhibited the best and worst disease-free survival (DFS), respectively, whereas the patients in the $FOXF2_{high}/FOXC2_{high}$ and $FOXF2_{low}/FOXC2_{low}$ groups exhibited moderate DFS. In summary, we found that FOXF2 transcriptionally targets *FOXC2* and suppresses EMT and multidrug resistance by negatively regulating the transcription of *FOXC2* in BLBC cells. The combined expression levels of *FOXF2* and *FOXC2* mRNA might serve as an effective prognostic indicator and could guide tailored therapy for TNBC or BLBC patients.

Introduction

Breast cancer is the most frequently diagnosed cancer and is the leading cause of cancer death among women [1]. Breast cancer is a heterogeneous disease that can be classified based on gene expression profiling into biologically distinct intrinsic subtypes including luminal subtypes A and B, HER2-positive, basal-like, and normal-like breast cancer. These subtype tumors exhibit unique molecular characteristics and prognostic significance [2]. The luminal tumor subtypes maintain a more differentiated state and confer a more favorable patient prognosis than other tumor subtypes. Although basal-like breast cancer (BLBC) only accounts for 10–20% of all breast cancers, this subtype has drawn particular attention due to its poor differentiation status, aggressive phenotype and unfavorable clinical outcome [3]. BLBC lacks the expression of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2). Thus, the definition of BLBC overlaps with that of triple-negative breast cancer (TNBC), and TNBC/BLBC patients would not be expected to benefit from anti-hormonal-based or anti-HER2 molecularly targeted therapies [4]. A better understanding of the molecular regulatory mechanisms underlying the different breast cancer subtypes would contribute to the development of molecular markers for the evaluation of patient prognosis and the prediction of drug resistance, which in turn could provide effective therapeutic strategies for the breast cancer patients with aggressive biological subtypes.

Epithelial–mesenchymal transition (EMT) is an important mechanism that induces epithelial cells to lose their polarity and become migratory mesenchymal cells, and enables cancer cells to acquire the ability to complete various steps in the metastatic cascade [5]. In EMT programming, pleiotropic EMT-related transcription factors (EMT-TFs), such as TWIST1, SNAIL1, SNAIL2, ZEB1 and ZEB2, form an interaction network in concert to regulate the EMT phenotype [6]. The EMT process allows the reversion of differentiated cells to a primitive, mesenchymal stem cell-like phenotype [7,8], which is an inherent characteristic of normal basal stem cells [9]. It is known

* Corresponding author. Tel.: +86 22 23340123 ext. 6002.
E-mail address: ymfeng@tijmu.edu.cn (Y.-M. Feng).

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that the myoepithelial cells derived from differentiation of normal basal stem cells can attain a mesenchymal-like phenotype by activating mesenchymal EMT-TFs. Conversely, mesenchymal cells could acquire differentiated epithelial traits *via* a mesenchymal-to-epithelial transition (MET) [9]. However, the role and mechanism underlying myoepithelial cell transformation to a mesenchymal-like phenotype remain to be determined.

The Forkhead-box (FOX) transcription factor superfamily is characterized by a highly conserved Forkhead/winged helix DNA-binding domain. These transcription factors mediate a wide spectrum of biological processes, such as metabolism, differentiation, proliferation, apoptosis, and migration [10,11]. Emerging evidence has demonstrated that the alterative expression of FOX transcription factors participate in the onset and progression in a variety of cancers through directly triggering EMT or cross-linking with other EMT regulatory pathways [12]. Notably, several FOX transcription factors play critical roles in cell type-specific fate decisions, which are associated with the biological characteristics of breast cancer. FOXA1 is highly expressed in luminal subtype breast cancer and represses the molecular phenotypic shift from luminal to basal gene expression signatures [13]. FOXC2, a mesenchymal FOX transcription factor, is highly expressed in aggressive BLBC and promotes metastasis and resistance to paclitaxel drug treatment due to induced mesenchymal differentiation to generate EMT phenotype and cancer stem cell properties [14,15].

FOXF2, another mesenchymal FOX transcription factor, is specifically expressed in the mesenchyme adjacent to the epithelium in organs derived from the splanchnic mesoderm. FOXF2 plays an important role in tissue homeostasis by regulating the epithelium–mesenchyme interaction to maintain epithelial polarity [16]. Our previous clinical study revealed that the under-expression of *FOXF2* is associated with early-onset metastasis and poor prognosis in patients with TNBC [17]. More recent work from our group revealed that FOXF2 acts as a novel EMT-suppressing transcription factor in BLBC cells and that FOXF2 deficiency enhances the metastatic ability of BLBC cells through inducing the EMT program [18]. In this study, we found that *FOXC2* is a novel transcriptional target of FOXF2 and serves as a mediator of the FOXF2 deficiency-induced EMT phenotype, aggressive behavior and multidrug resistance of BLBC cells. The combined expression status of *FOXF2* and *FOXC2* more effectively predicts the prognosis of TNBC patients than when they were used separately.

Materials and methods

Tissue specimens and clinical data

A total of 156 primary breast cancer tissue specimens diagnosed as invasive ductal carcinoma were obtained from patients who underwent breast surgery at Tianjin Medical University Cancer Institute and Hospital (TMUCIH; Tianjin, China). The tumors were classified into luminal (ER+ and/or PR+; n = 96), HER2-positive (ER–/PR–/HER2+; n = 26) and triple-negative (ER–/PR–/HER2–; n = 34) subtypes as described previously [17]. None of the cases were subjected to preoperative chemotherapy, and all were followed up for at least 3 years. Of the 156 individuals, 149 cases were followed up over 5 years. Disease-free survival (DFS) was defined as the time interval between primary surgery and any relapse (local-regional, contra-lateral and/or distant), or the terminal time of follow-up without any relapse events. This study was approved by the Institutional Review Board of TMUCIH, and written consent was obtained from all participants.

Cells and culture

The human immortalized non-tumorigenic mammary epithelial cell line MCF-10A and breast cancer cell lines MCF-7, T47D, BT474, MDA-MB-453, SKBR-3, MDA-MB-231, and BT549 were obtained from American Type Culture Collection (Manassas, VA, USA). The culturing of all cell lines was performed as previously described [18].

Plasmids, small interfering RNA, and transfection

The pcDNA3.1-HA-FOXF2 plasmid expressing HA-tagged FOXF2 (HA-FOXF2) was obtained as described previously [18]. The *FOXC2* promoter region from –1922 to +164 (containing a candidate FOXF2-binding site) or from –1800 to +164 (lacking the candidate FOXF2-binding site) relative to the transcription start site (TSS) was amplified from human genomic DNA by PCR. The PCR products were then inserted into the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA; pGL3-FOXC2). Three small interfering RNAs (siRNAs) targeting independent sequences of the human *FOXF2* (siFOXF2) or *FOXC2* (siFOXC2) genes were synthesized (RiboBio Co., Guangzhou, China). The siRNA displaying optimal knockdown efficiency, as determined by reverse transcription–quantitative PCR (RT-QPCR) and Western blot, was selected for further experiments. Non-targeting siRNA was used as a control (siControl). The transfection of cells with plasmids or siRNAs was performed using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

Chromatin immunoprecipitation

The HA-FOXF2 plasmid was transfected into MDA-MB-231 and MCF-7 cells transfected with an HA-FOXF2-expressing plasmid for 48 hours, and a chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Millipore, Billerica, MA, USA) as previously described [18]. Anti-HA antibody (Abcam, Cambridge, MA, USA)-enriched FOXC2 promoter fragments containing (–1949 to –1774) and lacking (–1793 to –1592) a putative FOXF2 binding site were PCR amplified. Isotype IgG (Abcam) was used as a negative control.

Dual-luciferase reporter assay

Cells were seeded on 24-well plates cultured without antibiotics until growth to 80% confluence. Then, siFOXF2 or HA-FOXF2 as well as their controls were co-transfected with *FOXC2* promoter constructs and internal control pRL-TK into the cells. After 48 hours, the luciferase activities of the cells were measured using a dual-luciferase reporter assay kit (Promega). Reporter luciferase activity was normalized to Renilla luciferase activity.

RT-QPCR

Total RNA isolation, RT-QPCR, and the quantification of target gene expression were performed as previously described [17]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression was used as an internal control for the normalization of target gene expression.

Western blot

Cultured cells were collected and solubilized using protein lysis buffer. The proteins were then separated by size using SDS-PAGE and then transferred to polyvinyl difluoride membrane (Millipore). The membranes were incubated in primary antibodies followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive proteins were detected using an enhanced chemiluminescence kit (GE Healthcare, Waukesha, WI, USA). The primary antibodies were rabbit anti-HA, goat anti-FOXC2 (Abcam), mouse anti-E-cadherin, mouse anti-vimentin, mouse anti-fibronectin (BD Biosciences, Bedford, MA, USA), mouse anti-FOXF2 (Abnova, Taiwan, China), rabbit anti-integrin β3 (Cell Signaling Technology, Danvers, MA, USA), and mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence

Cells were seeded on coverslips and cultured for 48 hours, and then fixed in 4% paraformaldehyde for 20 min. After washing with PBS, the cells were permeabilized in 0.5% Triton X-100 for 15 min, washed with PBS and blocked with 2% bovine serum albumin, then incubated with primary antibodies overnight at 4 °C. The cells were then washed with PBS and incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated secondary antibodies. Nuclei were stained using DAPI. The protein labeling was visualized using a fluorescence microscope.

Drug treatment and MTT assay

Cells were seeded on a 96-well plate at a density of 1×10^4 cells/well and cells subjected to treatment with 0.1 μmol/L paclitaxel, 1.0 μmol/L epirubicin, 25 μmol/L 5-Fu or 1.0 μmol/L cisplatin for 1–3 days. Then, 10 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL in PBS) was added to each well and incubated for 4 hours. The cell culture medium was replaced with 100 μL of dimethyl sulfoxide and the optical density was measured at 570 nm. The quantification cell viability was determined according to the optical density values for each culture time point.

Cell migration and invasion assays

The migration and invasion abilities of cells were assessed using non-Matrigel-coated and Matrigel-coated Transwell inserts (BD Biosciences, San Diego, CA, USA),

respectively. The assays and counting of migrating or invading cells were performed as described previously [19].

Cell-fibronectin adhesion assay

Cells were seeded on fibronectin-coated 96-well plates at 1×10^5 cells/well and were incubated in medium for 1.5 hours (MDA-MB-231) or 2 hours (MCF-10A). Then, the non-adherent cells were removed, and the adherence of cells was estimated by MTT assay.

Statistical analysis

Data from *in vitro* experiments are presented as mean ± standard deviation (SD). Student's *t*-test was used to compare the differences between experimental and control groups. Pearson's correlation coefficient was calculated to analyze the correlation between *FOXF2* and *FOXC2* mRNA expression in breast cancer tissues. Survival plots were generated by Kaplan–Meier analysis, and the log-rank test was used to assess the significance of the differences. $P < 0.05$ was considered to be significant.

Results

FOXF2 and FOXC2 present distinct expression patterns in different breast cancer subtypes

To determine the relationship between FOXF2 and FOXC2 expression, the mRNA levels of *FOXF2* and *FOXC2* in 156 primary breast cancer tissues were measured by RT-QPCR. Pearson's correlation was used to analyze the correlation between *FOXF2* and *FOXC2* mRNA levels in all cases and in the cases with different molecular subtypes. The results for all cases revealed no correlation between *FOXF2* and *FOXC2* mRNA levels (Pearson's $r = 0.064$, $P = 0.446$). Further stratification analysis showed that the correlation of *FOXF2* and *FOXC2* mRNA levels was distinct in different subtypes: a positive correlation in luminal subtype cases (n = 96; $r = 0.385$, $P < 0.001$), no significant correlation in HER2-positive subtype cases (n = 26; $r = 0.29$, $P = 0.169$), and a negative correlation in TNBC cases (n = 34; $r = –0.79$, $P < 0.001$; Fig. 1A). The comparison of *FOXF2* or *FOXC2* mRNA levels among the groups of luminal, HER2-positive and triple-negative subtype cases revealed that *FOXC2* mRNA levels in the triple-negative group were significant higher than that in the luminal and HER2-positive group; however, no significant differences of *FOXF2* mRNA levels among the three groups were observed (Fig. 1B). *FOXF2* and *FOXC2* mRNA levels were also detected in different subtype breast cancer cell lines. The results showed that both *FOXF2* and *FOXC2* mRNA levels are significantly high in the triple-negative/basal-like subtype cell lines, but low in other non-basal-like subtype cell lines (Fig. 1C). In addition, *FOXF2* and *FOXC2* mRNA levels presented a reverse relevant trend in the triple-negative/basal-like subtype cell lines (Fig. 1C), which is consistent with the result obtained from TNBC tissues. The negative correlation between *FOXF2* and *FOXC2* expression in TNBC is consistent with their opposite roles in the EMT and metastasis of TNBC/BLBC. The co-expression of *FOXF2* and *FOXC2* in luminal tumors implicates that these two transcription factors play distinct roles in TNBC/BLBC and non-TNBC/BLBC subtypes. Interestingly, *FOXC2* mRNA levels were higher than *FOXF2* mRNA levels either in breast cancer tissues or breast cancer cell

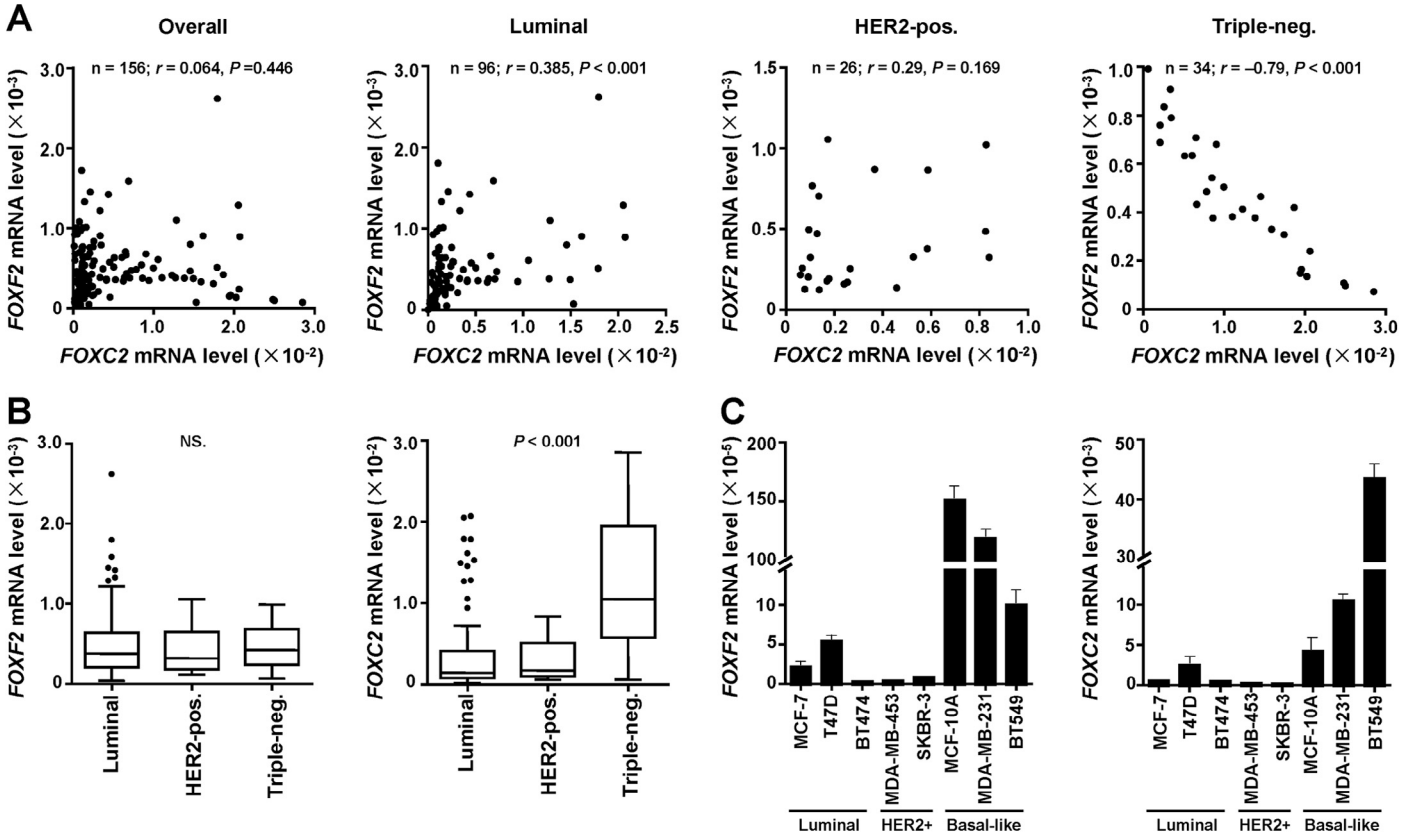

Fig. 1. The distinct expression patterns of *FOXF2* and *FOXC2* in different breast cancer subtypes. The *FOXF2* and *FOXC2* mRNA levels in primary breast cancer tissues were measured by RT-QPCR. The assay was performed in triplicate for each sample, and the data quantification was normalized to the internal control *GAPDH*. (A) The correlation between *FOXF2* and *FOXC2* mRNA levels in overall cases (n = 156), as well as in luminal (n = 96), HER2-positive (n = 26) or triple-negative (n = 34) subtype cases, was analyzed by Pearson's correlation analysis. (B) The comparison of *FOXF2* or *FOXC2* mRNA levels among the groups of luminal, HER2-positive and triple-negative subtype cases. NS, no significant difference among the three groups; $P < 0.001$, the one-way ANOVA significance for triple-negative group compared with luminal and HER2-positive group. (C) *FOXF2* and *FOXC2* mRNA levels in different subtype breast cancer cell lines.

lines, which implies that FOXF2, similar to other multiple EMT signaling and EMT-TFs [14], is an upstream regulator of FOXC2.

FOXF2 binds to the FOXC2 promoter and uniquely negatively regulates FOXC2 transcription in BLBC

To investigate whether FOXF2 and FOXC2 transcription factors transcriptionally regulate each other, we searched the sequences of the *FOXF2* and *FOXC2* promoter regions for potential binding sites of the opposite protein. A conservative FOXF2 binding site (5′-AATAAACA-3′) was found in the *FOXC2* promoter region located –1845 to –1838 bp from the TSS (Fig. 2A). However, no potential FOXC2 binding site was found in the *FOXF2* promoter region. To determine the binding of FOXF2 to the *FOXC2* promoter region, we performed a ChIP assay in the BLBC cell line MDA-MB-231 and the luminal breast cancer cell line MCF-7 transfected with an HA-FOXF2-expressing plasmid using an anti-HA antibody or isotype IgG. FOXF2-enriched promoter fragments of *FOXC2* containing (–1949/–1774 bp) or lacking (–1793/–1592 bp) the putative FOXF2-binding element were then amplified by PCR. The results revealed the binding of FOXF2 to the *FOXC2* promoter region containing the FOXF2-binding element in both the basal-like (MDA-MB-231) and luminal (MCF-7) breast cancer cells. However, no binding of FOXF2 to the *FOXC2* promoter region in the absence of the FOXF2-binding site was observed in either cell line (Fig. 2B).

To further determine whether FOXF2 regulates *FOXC2* promoter activity in both BLBC and luminal subtype breast cancer cells, the BLBC cell lines MDA-MB-231 (with high FOXF2 expression) and

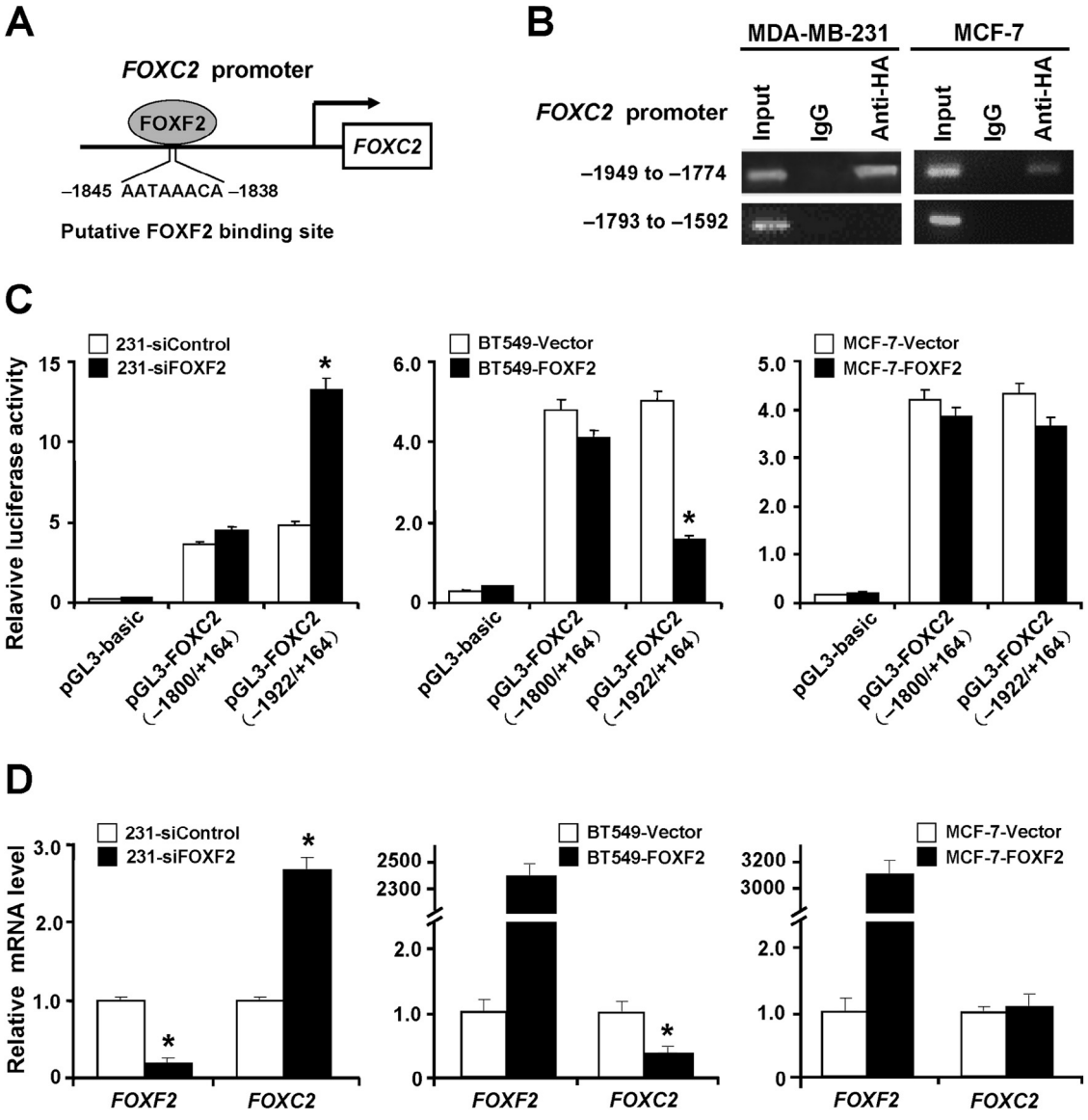

Fig. 2. FOXF2 binds to the *FOXC2* promoter and negatively regulates *FOXC2* transcription in BLBC cells. (A) Schematic representation of a conservative FOXF2 binding site (5′-AATAAACA-3′) in the *FOXC2* promoter region located at –1845 to –1838 bp from the TSS. (B) The binding of FOXF2 to the *FOXC2* promoter region in MDA-MB-231 and MCF-7 cells transfected with the HA-FOXF2 expression plasmid was assessed by ChIP assay using an anti-HA antibody. FOXF2-enriched the promoter regions of *FOXC2* containing (–1949/–1774) or lacking (–1793/–1592) the putative FOXF2 binding element were amplified by PCR. Isotype IgG was used as a negative control, and the total input was used as a positive control. (C) The luciferase activity of the reporters in the indicated cells was assessed by a dual-luciferase reporter assay. The experiment was repeated twice in duplicate. The relative luciferase activity is the ratio of the luciferase activity in each test cells to that in the control cells. The data are expressed as mean ± SD. $^*P < 0.05$ compared with the control cells. (D) The mRNA levels of *FOXF2* and *FOXC2* in the indicated cells were measured by RT-QPCR. The assays were performed independently repeated three times in triplicate. The relative mRNA level of each gene is the ratio of the mRNA level in each test cell to that in the control cells, and the data are expressed as mean ± SD. $^*P < 0.05$ compared to the control cells.

BT-549 (with low FOXF2 expression) and the luminal cell line MCF-7 (less FOXF2 expression) [18] were subjected to the dual-luciferase reporter assay. The cells were transiently transfected with siFOXF2 (MDA-MB-231) or FOXF2 plasmid (BT-549 and MCF-7) or the corresponding controls. Then, these cells were co-transfected with the internal control pRL-TK and the *FOXC2* promoter-luciferase reporter construct pGL3-FOXC2 (−1922/+164 bp; containing the FOXF2-binding element) or pGL3-FOXC2 (−1800/+164 bp; lacking the FOXF2-binding element). The results demonstrated that the reporter activity of pGL3-FOXC2 (−1922/+164) was significantly increased in the FOXF2-depleted MDA-MB-231 cells and decreased in the FOXF2-overexpressed BT-549 cells compared to the corresponding controls. In contrast, the reporter activity of pGL3-FOXC2 (−1800/+164) was not significantly changed by either FOXF2 depletion or FOXF2 overexpression. Surprisingly, the reporter activity of pGL3-FOXC2 (−1922/+164 bp) was not significantly altered in the FOXF2-overexpressed MCF-7 cells (Fig. 2C). These results indicated that FOXF2 represses the transcriptional activity of *FOXC2* by binding to the *FOXC2* promoter element in BLBC cells, but does not regulate *FOXC2* promoter activity in luminal breast cancer cells.

To verify the regulatory role of FOXF2 on *FOXC2* expression in BLBC and luminal breast cancer cells, the *FOXF2* and *FOXC2* mRNA levels were assessed in MDA-MB-231, BT-549 and MCF-7 cells with RNAi-mediated FOXF2 knockdown or FOXF2 overexpression, as well as in the corresponding control cells. The results showed that FOXF2 negatively regulated *FOXC2* expression in MDA-MB-231 and BT-549 BLBC cells but did not affect *FOXC2* expression in MCF-7 luminal breast cancer cells (Fig. 2D). Taken together, these results suggested that *FOXC2* acts as a direct transcriptional target of FOXF2, and FOXF2 negatively regulates *FOXC2* transcription in BLBC cells.

FOXF2 negatively regulates the FOXC2-mediated EMT phenotype and programming of BLBC cells

To determine the role of FOXC2 in the FOXF2-regulated EMT of BLBC cells, we examined the alteration of the EMT phenotype in basal-like mammary epithelial cell line MCF-10A and BLBC cell line MDA-MB-231 with the RNAi-mediated FOXF2- or/and FOXC2 knockdown and the corresponding controls. As expected, FOXF2 depletion substantially converted MCF-10A and MDA-MB-231 cells to a more mesenchymal/fibroblast-like morphology with a spindle-like cell shape and cell scattering (Fig. 3A). The FOXF2-depleted cells displayed decreased expression of epithelial marker E-cadherin (*CDH1*) and elevated the expression of mesenchymal markers vimentin (*VIM*) and fibronectin 1 (*FN1*), as detected by immunofluorescence (Fig. 3A), RT-QPCR (Fig. 3B) and immunoblotting (Fig. 3C). Conversely, the inhibition of FOXC2 resulted in the reduction of this fibroblast-like morphology, which presents increased epithelial-like cell clustering, prominent cell–cell contacts, and decreased expression of mesenchymal markers vimentin and fibronectin (Fig. 3A–C). Importantly, the FOXF2 depletion-induced EMT phenotype was rescued by FOXC2 knockdown (Fig. 3A–C). In addition, FOXF2 negatively regulated the expression of the FOXC2 target integrin β3 through suppressing FOXC2 transcription (Fig. 3C). These results indicated that FOXC2 mediates the FOXF2-regulated EMT phenotype of BLBC cells.

In EMT programming, pleiotropic EMT-TFs form an interaction network and act in concert to regulate the EMT phenotype. To further investigate whether FOXC2 mediates FOXF2-regulated EMT programming in the EMT-TF interaction network, we examined the mRNA expression of EMT-TFs *TWIST1*, *SNAIL1*, *SNAIL2*, *ZEB1* and *ZEB2* in the above cells. The results revealed that FOXF2 depletion significantly upregulated *TWIST1*, *SNAIL2*, *ZEB1*, and *ZEB2* expression in MCF-10A and MDA-MB-231 cells, while FOXC2 knockdown downregulated the mRNA expression of these EMT-TFs. Consistent with the EMT phenotype of the MCF-10A and MDA-MB-231 cells with FOXF2 and FOXC2 knockdown, FOXF2 depletion-induced EMT-TF expression was reversed by FOXC2 knockdown (Fig. 3D). These results indicated that FOXC2 mediates the FOXF2-regulated EMT programming in BLBC cells.

FOXC2 mediates the FOXF2-regulated aggressive behavior of BLBC cells

Since FOXF2 negatively regulates FOXC2-mediated EMT and expression of adhesion molecule integrin β3 in BLBC cells, we further investigated whether FOXC2 mediates the FOXF2-regulated aggressive phenotype of BLBC cells. The migration, invasion and adhesion abilities of the MCF-10A-siFOXF2 and MDA-MB-231-siFOXF2 cells were assessed by Transwell and cell–fibronectin (a ligand of integrin β3 [20]) adhesion assays. The transwell assays showed that FOXF2 depletion induced a dramatic increase of the migration and invasion abilities of MCF-10A and MDA-MB-231 cells, and this increase was partially rescued by the inhibition of FOXC2 expression (Fig. 4A and B). Consistently, the adhesion of cells to fibronectin was induced by FOXF2 knockdown, and this interaction was abolished by FOXC2 depletion (Fig. 4C). These results indicated that FOXC2 mediates the FOXF2-regulated aggressive behavior of BLBC cells.

FOXF2 depletion enhances the multidrug resistance of BLBC cells through targeting FOXC2

Since the EMT generates cells exhibiting the properties of stem cells [8] and the acquisition of stem cell properties increases their resistance to chemotherapeutic agents [21], we further tested whether FOXF2 and FOXC2 contribute to the drug resistance of breast cancer cells. The chemotherapy drugs commonly used for the clinical treatment of breast cancer, including paclitaxel, epirubicin, 5-fluorouracil (5-Fu) and cisplatin, were used to treat MDA-MB-231 cells transfected with siFOXF2 or/and siFOXC2. MTT assays revealed that FOXF2 depletion led these cells to become more resistant to these drugs, while FOXC2 knockdown resulted in significantly increased sensitization compared with the control cells. Consistently, the chemosensitivity of cells co-transfected with siFOXF2 and siFOXC2 was between that of the cells transfected with siFOXF2 or siFOXC2 alone (Fig. 5). These results suggested that FOXF2 and FOXC2 play opposite roles in the multidrug resistance of BLBC cells.

The combination of FOXF2 and FOXC2 mRNA levels effectively predicts the prognosis of TNBC patients

Based on our novel findings that *FOXF2* and *FOXC2* are inversely expressed in TNBC tissues and that these transcription factors play opposite roles in regulating the EMT phenotype and aggressive behavior of BLBC cells, we next addressed whether the combined detection of *FOXF2* and *FOXC2* mRNA levels effectively predicts TNBC patient prognosis. Thus, we grouped the 34 cases of TNBC and 122 cases of non-TNBC based on their *FOXF2* and *FOXC2* mRNA levels: $FOXF2_{high}/FOXC2_{low}$, $FOXF2_{high}/FOXC2_{high}$, $FOXF2_{low}/FOXC2_{low}$, and $FOXF2_{low}/FOXC2_{high}$. Kaplan–Meier analysis revealed that in TNBC cases, patients in the $FOXF2_{high}/FOXC2_{low}$ and $FOXF2_{low}/FOXC2_{high}$ groups exhibited the best and worst DFS, respectively, while patients in the $FOXF2_{high}/FOXC2_{high}$ and $FOXF2_{low}/FOXC2_{low}$ groups exhibited moderate DFS (Fig. 6A). In contrast, *FOXF2* and *FOXC2* mRNA levels did not distinguish DFS in the non-TNBC cases (Fig. 6B). These results suggested that $FOXC2_{low}$ may contribute to the $FOXF2_{high}$-suppressed recurrence and metastasis of TNBC and that $FOXC2_{high}$ may facilitate the $FOXF2_{low}$-resulted unfavorable prognosis in TNBC. FOXC2 functionally mediates the effect of FOXF2 on BLBC/TNBC metastasis,

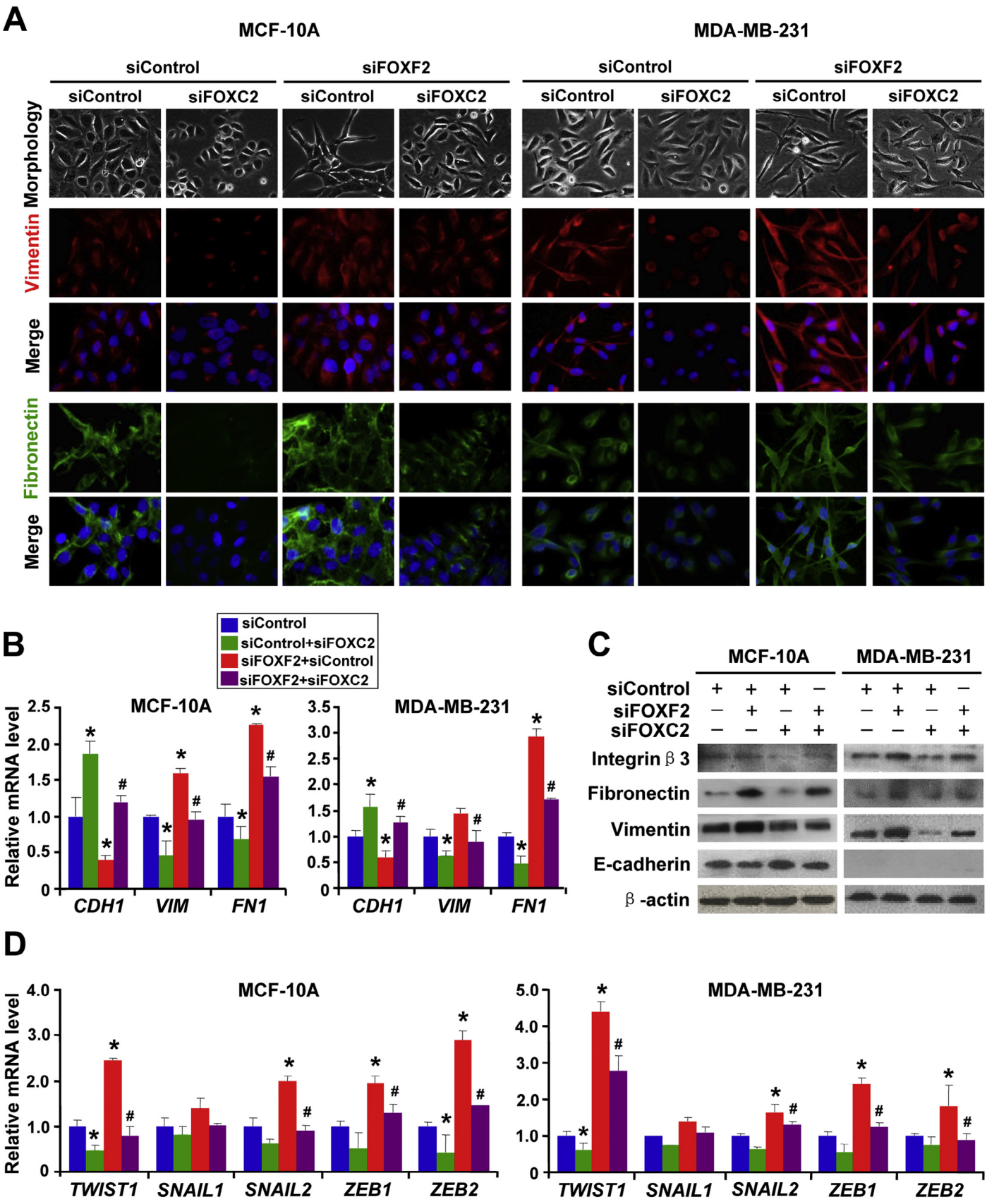

Fig. 3. FOXF2 negatively regulates the FOXC2-mediated EMT phenotype and programming of BLBC cells. (A) The morphology of MCF-10A and MDA-MB-231 cells transfected with siFOXF2 or/and siFOXC2 as well as their controls. (B) The protein expression levels of mesenchymal markers vimentin (red) and fibronectin 1 (green) in the indicated cells were detected by immunofluorescence. DAPI (blue) was used to reveal the nucleus. (C) The mRNA expression levels of *VIM* and *FN1* in the indicated cells were measured by RT-QPCR. (D) The protein expression levels of E-cadherin, vimentin, fibronectin 1 and integrin β3 in the indicated cells were detected by immunoblotting. β-actin was used as the internal control. (E) The mRNA expression levels of the EMT-TFs *TWIST1*, *SNAIL1*, *SNAIL2*, *ZEB1* and *ZEB2* in the indicated cells were measured by RT-QPCR. $^{*}P < 0.05$, transfection with siFOXC2 or siFOXF2 *versus* the control treatment; $^{\#}P < 0.05$, co-transfection with siFOXC2 and siFOXF2 *versus* transfection with siFOXF2 alone.

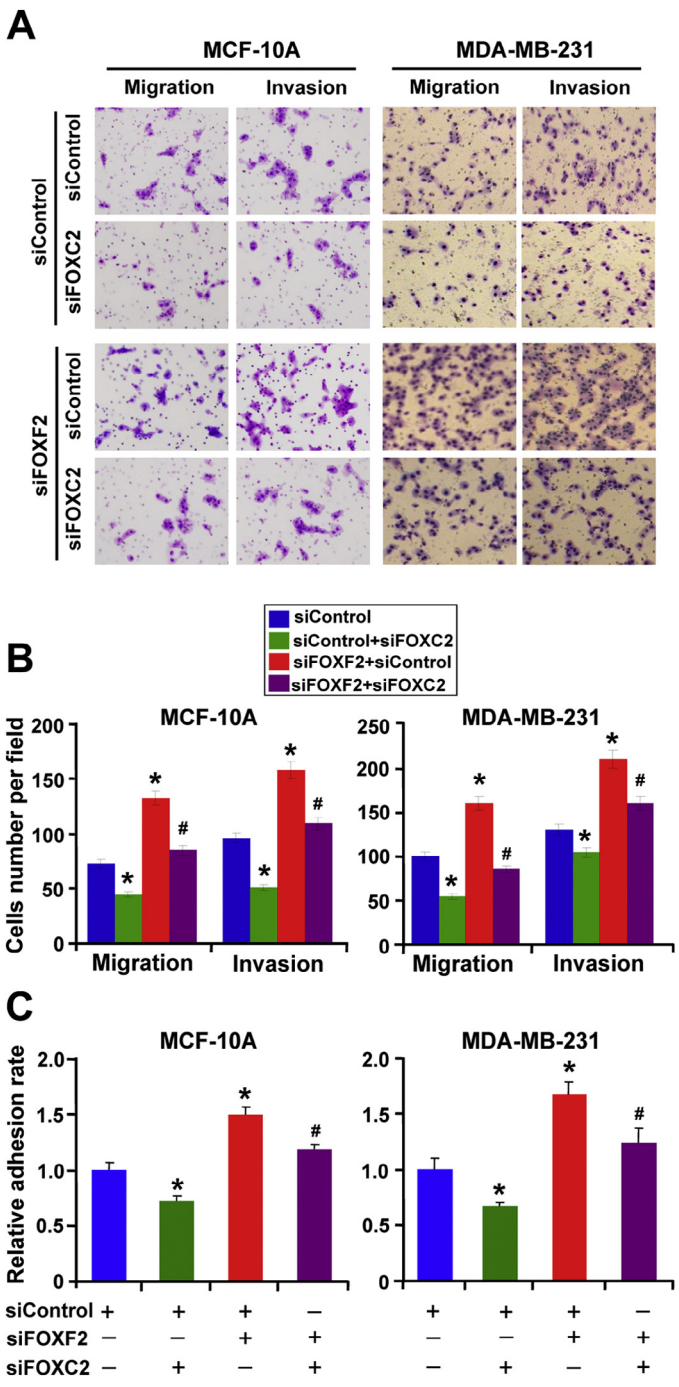

Fig. 4. FOXC2 mediates the FOXF2-regulated aggressive behavior of BLBC cells. Images (A) and quantification (B) of the migration and invasion abilities of the indicated cells were assessed by Transwell assays. (C) The adhesion ability of the indicated cells was assessed by cell–fibronectin adhesion assay. Three independent experiments were performed in triplicate. $^{*}P < 0.05$, transfection with siFOXF2 or siFOXC2 alone *versus* the control treatment; $^{\#}P < 0.05$, co-transfection with siFOXF2 and siFOXC2 *versus* transfection with siFOXF2 alone.

and the combined detection of *FOXF2* and *FOXC2* mRNA levels effectively predicts the prognosis of TNBC patients.

Discussion

During acquirement of the EMT phenotypes to complete the cascade of metastatic spread, embryonic and mesenchymal transcription factors are pleiotropically activated in cancer cells. Mesenchymal FOXC2 has been reported to be induced in response to multiple signaling (e.g. TGFβ1) and EMT-TFs (e.g. SNAIL, TWIST, and Goosecoid) and acts as a central mediator that orchestrates the mesenchymal component of the EMT program and promotes metastasis in BLBC. FOXC2 expression is correlated with highly aggressive BLBC and considered to be a highly specific molecular marker for BLBC [14]. Our previous study demonstrated that FOXF2 is highly expressed in most basal-like breast cells and functions as an EMT-suppressing transcription factor. FOXF2 deficiency promotes metastasis of BLBC cells by activating the EMT program. We also demonstrated that FOXF2 negatively targets *TWIST1* in the EMT programming and metastasis progress of BLBC [18]. On the basis of our finding, we proposed that FOXF2, as a mesenchymal FOX transcription factor, acts cooperatively with TWIST1 to maintain tissue homeostasis by balancing the differentiation or dedifferentiation of mesenchymal/myoepithelial cells. In the current study, we demonstrated that FOXC2, which was similar with TWIST1, functions as a transcriptional target of FOXF2 in BLBC cells but not in luminal breast cancer cells. FOXC2 is a novel mediator of FOXF2 deficiency-induced mesenchymal differentiation during the EMT. This finding provided a novel regulatory pathway of the FOXC2-mediated EMT-TF interaction network during the EMT programming and metastatic cascade of BLBC cells. The FOXF2/FOXC2-regulated EMT may serve as a therapeutic target for the treatment of aggressive BLBC.

Integrins are heterodimeric transmembrane receptors for components of the extracellular matrix (ECM), such as fibronectin, laminin, collagen, fibrinogen, and vitronectin. Through bidirectional "outside-in" and "inside-out" signaling, integrins regulate multiple biological processes, such as adhesion, apoptosis, proliferation, differentiation, migration, invasion, and metastasis [22]. The integrin β3 subunit and its integrin heterodimers are associated with the metastatic processes of breast cancer cells [23]. Fibronectin is a ubiquitous and abundant ECM protein. αvβ3 integrin could high-affinity bind fibronectin by interaction with the N-terminal of the FN-I type module [20]. Fibronectin–integrin interactions play an important role in cell adhesion, migration and invasion by activating integrin-linked signaling [24]. Hayashi et al. [25] reported that FOXC2 induces the expression of the integrin β3 subunit by directly binding to the *ITGB3* promoter, thereby regulating integrin β3-mediated endothelial cell adhesion and migration. In our study, we found that FOXC2 mediates FOXF2-regulated integrin β3 expression and cell-fibronectin adhesion in BLBC cells, suggesting that integrin β3 is regulated by the FOXF2/FOXC2 pathway and contributes to the acquisition of the aggressive properties of BLBC cells.

TNBC/BLBC is a subtype of breast cancer associated with poor differentiation status, aggressive phenotype and unfavorable clinical outcome. TNBC/BLBC could not benefit from anti-hormone endocrine therapies and anti-HER2 molecularly targeted therapies due to the lack of ER, PR and HER2 expressions [26]. Chemotherapy is the only systemic treatment option for TNBC/BLBC patients [27]. However, cells undergoing EMT often acquire resistance to chemotherapeutic agents due to the generation of stem cell-like properties [21]. Hollier et al. reported that FOXC2 is a critical determinant of mesenchymal and stem cell-like properties in cells that undergo EMT and that the suppression of FOXC2 expression sensitizes these cells to paclitaxel [15]. However, with the exception of paclitaxel, no evidence is available to demonstrate the effect of FOXC2 expression on the efficacy of other chemotherapy drugs routinely used in breast cancer treatment. Furthermore, no report to date has investigated the role of FOXF2 in the chemosensitivity of breast cancers. In this study, we determined the response of BLBC cells subjected to FOXF2 or/and FOXC2 inhibition to the following chemotherapy drugs commonly used in the clinical treatment of breast cancer: paclitaxel, epirubicin, 5-Fu and cisplatin [27]. We found that FOXF2 and FOXC2 play opposite roles in the treatment response to all of these chemotherapy drugs. FOXF2 inhibition

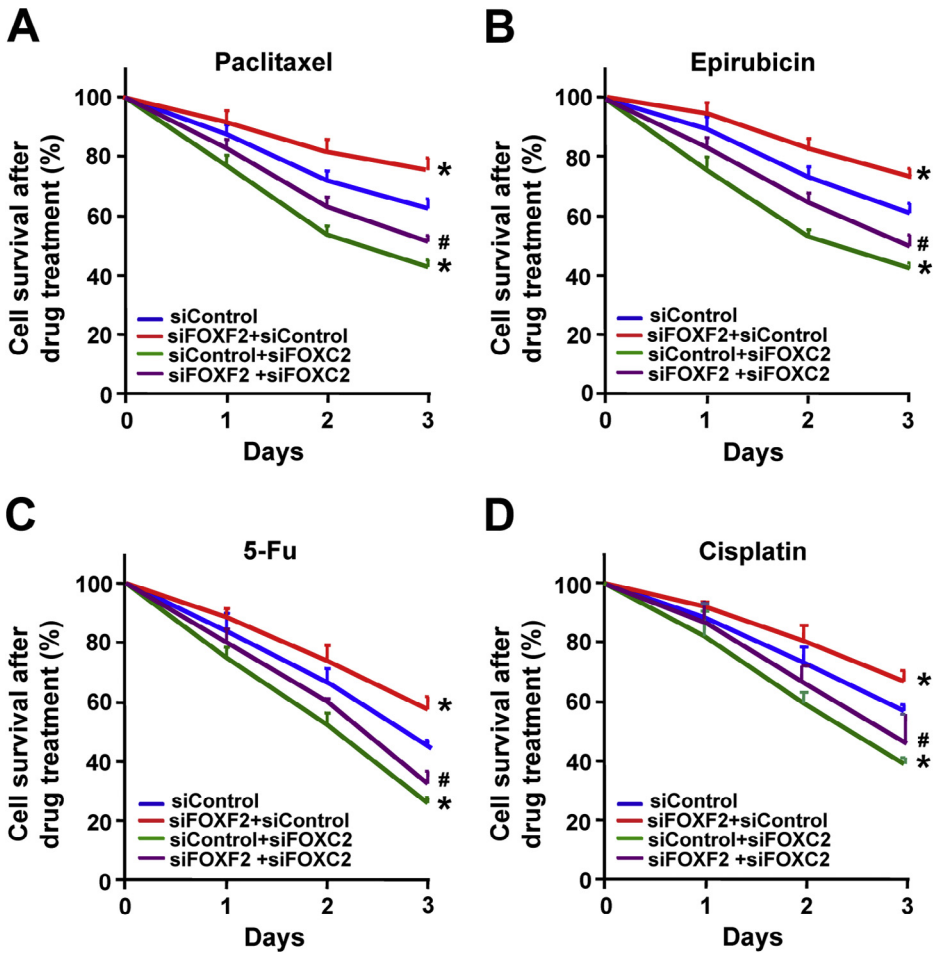

Fig. 5. FOXF2 depletion enhances the multidrug resistance of BLBC cells through targeting FOXC2. The cell viability was assessed by MTT assay. The MDA-MB-231 cells were transfected with siFOXF2 or/and siFOXC2 as well as their corresponding control cells subjected to treatment with (A) 0.1 μmol/L paclitaxel, (B) 1.0 μmol/L epirubicin, (C) 25 μmol/L 5-Fu or (D) 1.0 μmol/L cisplatin for the indicated times. Three independent experiments were performed in triplicate. $^{*}P < 0.05$, transfection with siFOXF2 or siFOXC2 alone *versus* the control treatment; $^{\#}P < 0.05$, co-transfection with siFOXF2 and siFOXC2 *versus* transfection with siFOXF2 alone.

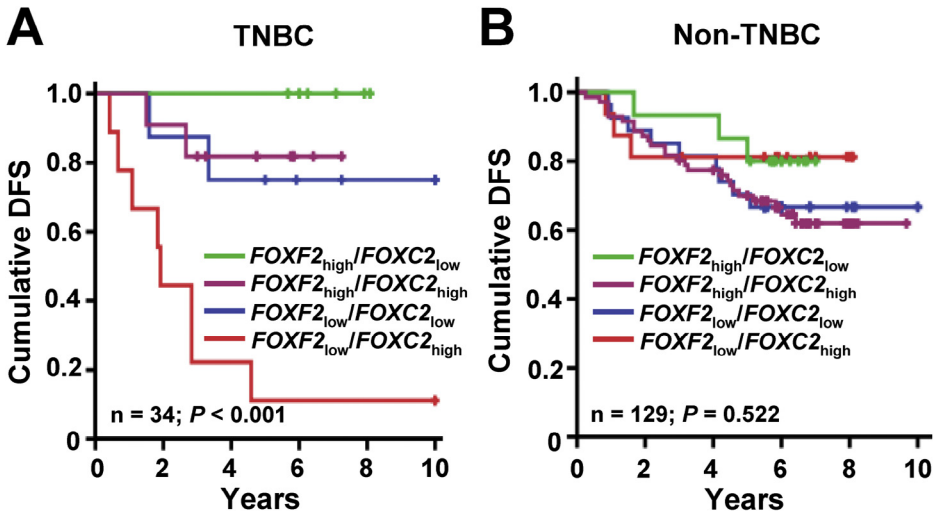

Fig. 6. The combination of *FOXF2* and *FOXC2* mRNA levels effectively predicts the prognosis of TNBC patients. Kaplan–Meier survival analysis based on patient's DFS and combined *FOXF2* and *FOXC2* mRNA expression levels in primary TNBC tissues (n = 34; A) or non-TNBC tissues (n = 122; B). The optimal cutoff value of *FOXF2* or *FOXC2* mRNA level was determined based on receiver operating characteristic curves at a mutually maximized sensitivity and specificity to stratify the patients into $FOXF2_{high}$ and $FOXF2_{low}$ or $FOXC2_{high}$ and $FOXC2_{low}$ groups with distinct DFS status. Log–rank test was used to assess the significance of DFS differences among groups with different *FOXF2*/*FOXC2* status.

enhanced multidrug resistance, while FOXC2 inhibition reversed FOXF2 depletion-induced drug resistance. Thus, exogenous administration of FOXF2 or interference of FOXC2, as well as FOXF2/FOXC2 pathway, might be efficient strategies for improving the therapeutic outcomes of chemotherapy for the treatment of TNBC/BLBC patients.

TNBC/BLBC is heterogeneous with different differentiation characteristics [28]. Therefore, patients with tumors of the TNBC/BLBC subtype might have distinct prognosis. There is an urgent need for powerful biological markers to further distinguish the outcome of breast cancer patients with this tumor subtype. FOXC2 is recognized as a marker of aggressive BLBC [14], and our previous study found that *FOXF2* mRNA levels positively correlate with the prognosis of TNBC patients [17]. In the present study, we further demonstrated that *FOXF2* and *FOXC2* mRNA levels are uniquely and inversely expressed in TNBC, and FOXF2 and FOXC2 play opposite roles in TNBC/BLBC metastasis. Thus, we assessed the clinical value of the combined detection of *FOXF2* and *FOXC2* mRNA levels for the prognosis of TNBC. We found that TNBC cases could be divided into distinct DFS subgroups based on the combined *FOXF2* and *FOXC2* mRNA levels: the patients in the $FOXF2_{high}/FOXC2_{low}$ and $FOXF2_{low}/FOXC2_{high}$ groups had the best and worst DFS, respectively, while the patients in the $FOXF2_{high}/FOXC2_{high}$ and $FOXF2_{low}/FOXC2_{low}$ groups had moderate DFS. The combined detection of *FOXF2* and *FOXC2* mRNA levels more effectively predicted the prognosis of TNBC patients than the detection of either factor alone. Although our results provide a potential powerful gene-based diagnosis for distinguishing the outcome of TNBC subtype patients, the prognostic significance of these two markers for TNBC/BLBC patients requires further validation by expanding the number of clinical cases from multiple centers.

In conclusion, here, we identified a novel molecular mechanism of FOXF2 in controlling BLBC metastasis and multidrug resistance. We found that *FOXC2* is a transcriptional target of FOXF2. FOXF2 suppresses the EMT and multidrug resistance through negatively regulating FOXC2 expression in BLBC. The combined *FOXF2* and *FOXC2* mRNA levels in primary breast cancers might serve as an effective prognostic indicator and guide tailored therapy for TNBC patients. Moreover, targeting the FOXF2/FOXC2 pathway might be a potential therapeutic strategy for TNBC/BLBC patients.

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Conflict of interest

The authors declare no conflicts of interest.

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