

## Downregulation of CXXC Finger Protein 4 Leads to a Tamoxifen-resistant Phenotype in Breast Cancer Cells Through Activation of the Wnt/ $\beta$ -catenin Pathway



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### Abstract

Tamoxifen is a successful endocrine therapy drug for estrogen receptor–positive (ER+) breast cancer. However, resistance to tamoxifen compromises the efficacy of endocrine treatment. In the present study, we identified potential tamoxifen resistance–related gene markers and investigated their mechanistic details. First, we established two ER + breast cancer cell lines resistant to tamoxifen, named MCF-7/TMR and BT474/TMR. Gene expression profiling showed that CXXC finger protein 4 (CXXC4) expression is lower in MCF-7/TMR cells than in MCF-7 cells. Furthermore, CXXC4 mRNA and protein expression are lower in the resistant cell lines than in the corresponding parental cell lines. We also investigated the correlation between CXXC4 and endocrine resistance in ER + breast cancer cells. CXXC4 knockdown accelerates cell proliferation *in vitro* and *in vivo* and renders breast cancer cells insensitive to tamoxifen, whereas CXXC4 overexpression inhibits cancer cell growth and increases tamoxifen sensitivity of resistant cells. In addition, we demonstrated that CXXC4 inhibits Wnt/ $\beta$ -catenin signaling in cancer cells by modulating the phosphorylation of GSK-3 $\beta$ , influencing the integrity of the  $\beta$ -catenin degradation complex. Silencing the CXXC4 gene upregulates expression of cyclinD1 and c-myc (the downstream targets of Wnt signaling) and promotes cell cycle progression. Conversely, ectopic expression of CXXC4 downregulates the expression of these proteins and arrests the cell cycle in the G0/G1 phase. Finally, the small-molecule inhibitor XAV939 suppresses Wnt signaling and sensitizes resistant cells to tamoxifen. These results indicate that components of Wnt pathway that are early in response to tamoxifen could be involved as an intrinsic factor of the transition to endocrine resistance, and inhibition of Wnt signaling may be an effective therapeutic strategy to overcome tamoxifen resistance.

*Translational Oncology* (2020) 13, 423–440

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Received 18 August 2019; Revised 2 December 2019; Accepted 3 December 2019

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1936-5233/19  
<https://doi.org/10.1016/j.tranon.2019.12.005>

## Introduction

Approximately 50%–70% of patients with breast cancer express estrogen receptor alpha (ER $\alpha$ ) [1,2]. These cases are more responsive to endocrine therapy agents, including selective ER modulators (SERMs), selective ER down modulators (SERDs), and aromatase inhibitors (AIs) [3,4]. Tamoxifen, a representative SERM, is recognized as the most successful targeted therapy agent for ER + breast cancer. Tamoxifen is similar to estrogen in structure and inhibits estrogen-responsive element (ERE)-mediated gene transcription through competitive combination with ERs, resulting in breast cancer cell stagnation in the G0/G1 phase and tumor growth inhibition [5–7]. Clinically, long-term tamoxifen treatment can reduce the recurrence and mortality of ER + breast cancer [8,9]. However, primary or acquired tamoxifen resistance greatly influences the effectiveness of endocrine therapy [10–12].

The factors resulting in failure of endocrine treatment include loss of ER expression [13], alterations in ER-coregulatory factor [14,15], and dysregulation of certain signaling pathways [16–20]. Specifically, abnormalities in the Wnt signaling pathway are common in several human cancers, including breast cancer. This pathway is important for cell proliferation, survival, invasion, and migration, as well as the cell cycle and apoptosis, epithelial-mesenchymal transition (EMT), and stemness [21,22]. Aberrance in Wnt signaling is also related to drug resistance in tumors. Activation of the Wnt/ $\beta$ -catenin pathway promotes transcription of the downstream multidrug resistance gene (MDR1), causing a decrease of intracellular drug concentration and impairing its efficacy [23,24]. Accordingly, inhibition of the Wnt pathway leads to a reduction of MDR1 expression and reversal of multidrug resistance [25,26].

The Wnt signaling pathway is considered to be of significance in the acquisition of tamoxifen resistance. Studies have demonstrated that acquired tamoxifen resistance may be associated with an increase in Wnt signaling and EMT [27] and that Sox2-dependent activation of Wnt signaling increases the proportion of breast cancer stem/progenitor cells, actuating the acquisition of tamoxifen resistance [28]. However, other than these rare examples, no further studies on whether Wnt signaling affects the acquisition of tamoxifen resistance in other ways have been reported. Thus, the ways in which the Wnt signaling pathway is activated in the development of tamoxifen resistance remain largely unclear.

CXXC finger protein 4 (CXXC4), also known as Idax, is encoded by the gene mapping to human chromosome 4q24 [29,30]. This protein contains a CXXC-type zinc finger domain by which CXXC4 binds to promoters and CpG islands and modifies the methylation status of DNA [31]. It has been demonstrated in gastric cancer (GC) that CXXC4 interacts directly with the PDZ domain of Dvl1 through a special “KTXXXI” motif, suppressing Dvl, recruiting Axin-GSK-3 $\beta$ , and thus stabilizing the  $\beta$ -catenin degradation complex [32]. When CXXC4 inactivates Wnt signaling, there is no accumulation of  $\beta$ -catenin in the cytoplasm and no activation of T-cell factor/lymphoid enhancing factor in the nucleus. A survey of the literature has revealed that CXXC4 might also have an inhibitory effect on the Wnt/ $\beta$ -catenin pathway in renal cell carcinoma (RCC) [33]. However, to the best of the authors' knowledge, no studies on the functional role of CXXC4 in breast cancer, let alone its relationship to tamoxifen resistance, have been reported.

Accordingly, in the present study, we adapted the ER + breast cancer cell lines MCF-7 and BT474 to establish tamoxifen-resistant

cell models and found that CXXC4 expression is clearly lower in the resistant cell lines than in their corresponding parental cell lines. Furthermore, we have demonstrated for the first time that CXXC4 is a negative regulator of Wnt/ $\beta$ -catenin signaling in breast cancer and that knockdown of CXXC4 increases expression of cyclinD1 and c-myc (the downstream targets of Wnt signaling), resulting in acceleration of G1/S transition, cell proliferation, and a reduced susceptibility to tamoxifen. Conversely, CXXC4 overexpression raises tamoxifen sensitivity to a degree by blocking the cell cycle. These findings imply that CXXC4 might be a potential biomarker for acquisition of tamoxifen resistance and that the Wnt/ $\beta$ -catenin pathway may provide a new therapeutic target for overcoming tamoxifen resistance.

## Materials and Methods

### Cell culture

ER + breast cancer cell lines MCF-7 and BT474, purchased from the Cell Bank of the Chinese Academy of Sciences (Kunming, China), were grown in RPMI1640 and Dulbecco's Modified Eagle Medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), respectively, containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 100 IU/mL penicillin-streptomycin. In addition, the culture medium for BT474 was supplemented with 0.1 IU/mL insulin. The tamoxifen-resistant sublines were isolated by continuous exposure of high-density MCF-7 or BT474 cells to 4-hydroxytamoxifen (4-OHT, 20  $\mu$ M) (Sigma-Aldrich, St. Louis, MO, USA) for approximately three weeks. After a further seven-day drug-free culturing, cells were sequentially exposed to increasing doses of 4-OHT up to 12.5  $\mu$ M and then maintained in the presence of 12.5  $\mu$ M 4-OHT for at least six months. Before subsequent experiments, cells were transferred to tamoxifen-free medium for three days to avoid drug interference. All cells were cultured at 37 °C in a 5% CO<sub>2</sub> humidified incubator.

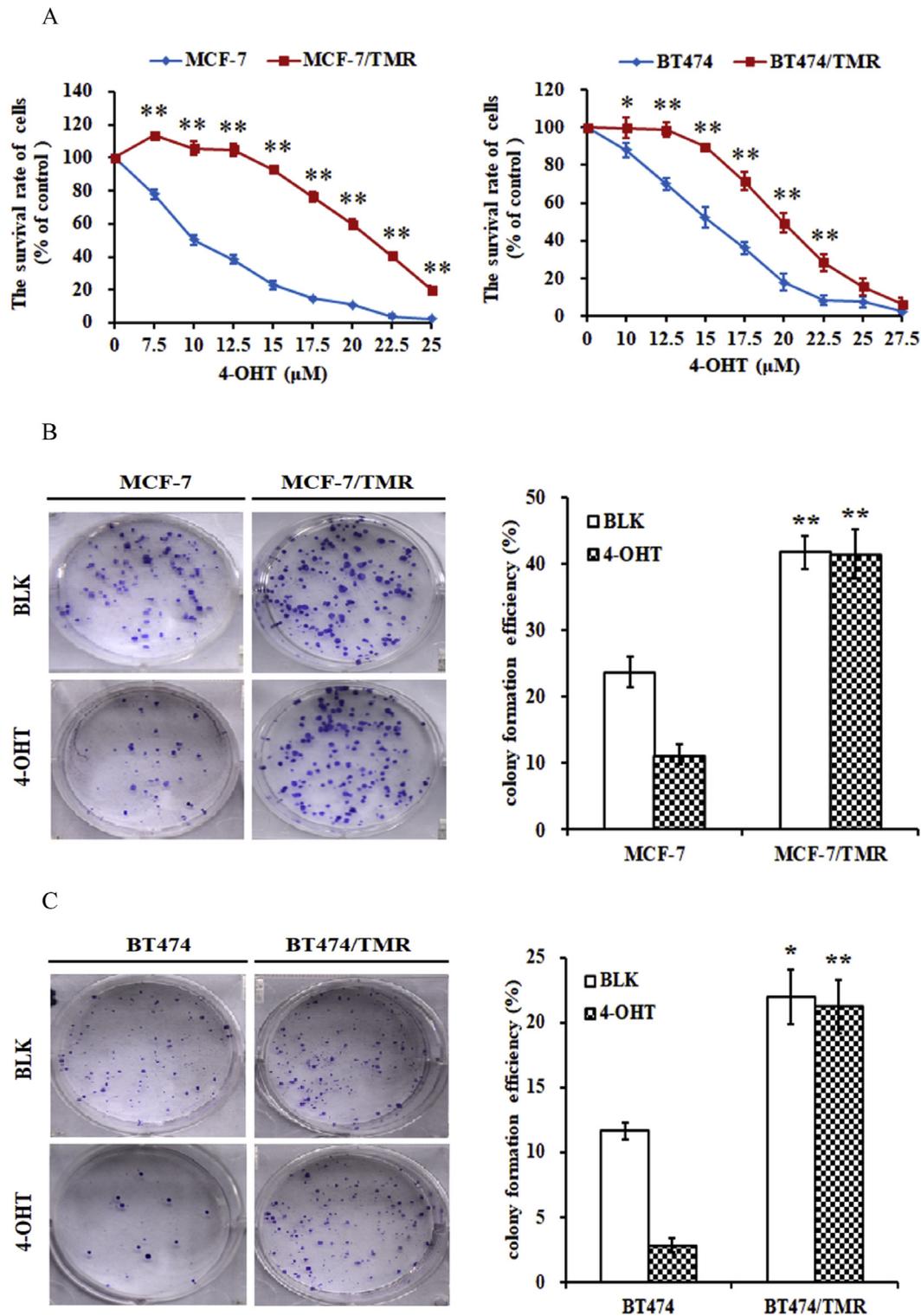
### Microarray analysis

Gene expression microarray analysis was entrusted to Shanghai OE Biotech (Shanghai, China) and executed using a SurePrint G3 Microarray (Array Type: Human Gene Expression v3; Agilent, Foster, CA, USA). Briefly, total RNA was extracted from MCF-7 and MCF-7/TMR cells using an RNeasy Mini kit (Qiagen, Hilden, Germany), labeled using a Quick Amp One-Color Labeling Kit (Agilent, Foster, CA, USA), and hybridized by use of a Gene Expression Hybridization Kit (Agilent, Foster, CA, USA). Finally, the hybridized chips were scanned using an Agilent G2505C Scanner (Agilent, Foster, CA, USA). Raw data were collected using Agilent Feature Extraction Software and normalized with the quantile algorithm.

Then, differentially expressed genes were screened through fold change (FC) screening as well as by *P*-value, as calculated using the Student's *t*-test. The experiments were performed in triplicate as required.

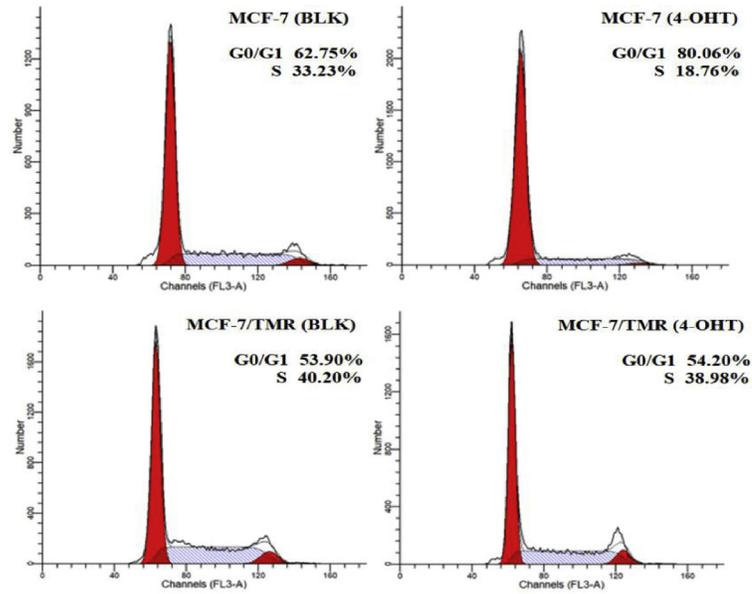
### Plasmid construction and transfection

To overexpress CXXC4 in specified cells, we purchased the lentiviral overexpression vector pGV358, containing a full-length cDNA for CXXC4 (NM\_025212) with control (Genechem, Shanghai, China). Lentiviral constructs were transfected into cells,

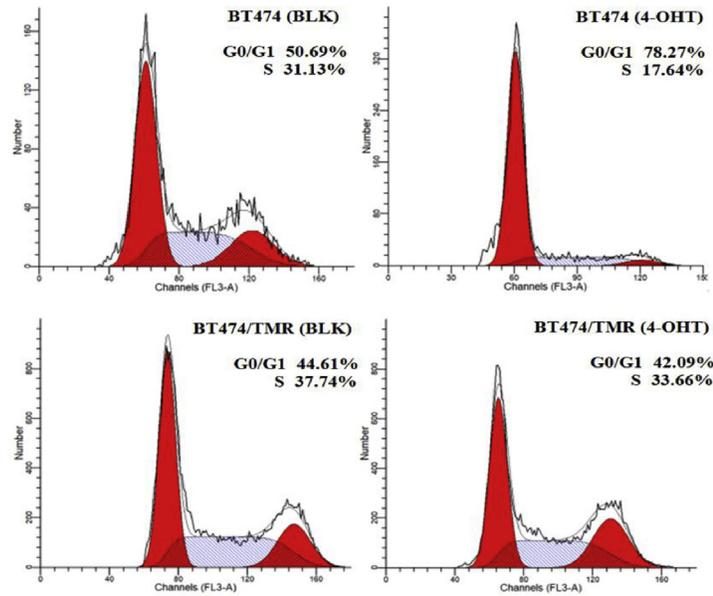


**Figure 1.** Characterization of tamoxifen-resistant cell lines. (A) Cell viability of parental cells (MCF-7, BT474) and tamoxifen-resistant cells (MCF-7/TMR, BT474/TMR) was detected by MTT assays after treated with 4-OHT of the indicated concentrations for three days ( $*P < 0.05$ ,  $**P < 0.01$ ). (B) Plate colony formation in MCF-7 and MCF-7/TMR cells treated with 4-OHT (10  $\mu\text{M}$ ) ( $**P < 0.01$ ). (C) Plate colony formation in BT474 and BT474/TMR cells treated with 4-OHT (15  $\mu\text{M}$ ) ( $*P < 0.05$ ,  $**P < 0.01$ ). (D) MCF-7 and MCF-7/TMR cells and (E) BT474 and BT474/TMR cells were, respectively, treated with 10  $\mu\text{M}$  and 15  $\mu\text{M}$  4-OHT, and cell cycle distribution was detected by flow cytometry analysis after three days. (F) Bar chart showed the percentage of G0/G1 phase in parental cells and tamoxifen-resistant cells (n.s. for no significance,  $**P < 0.01$ ). (G) MCF-7/TMR cells are more tumorigenic than the parental MCF-7 cells ( $**P < 0.01$ ). MTT, methyl thiazolyl tetrazolium; 4-OHT, 4-hydroxytamoxifen.

D



E



F

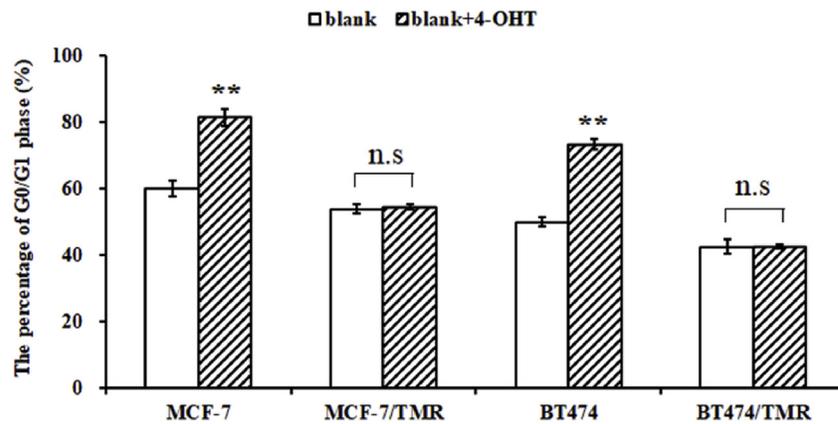


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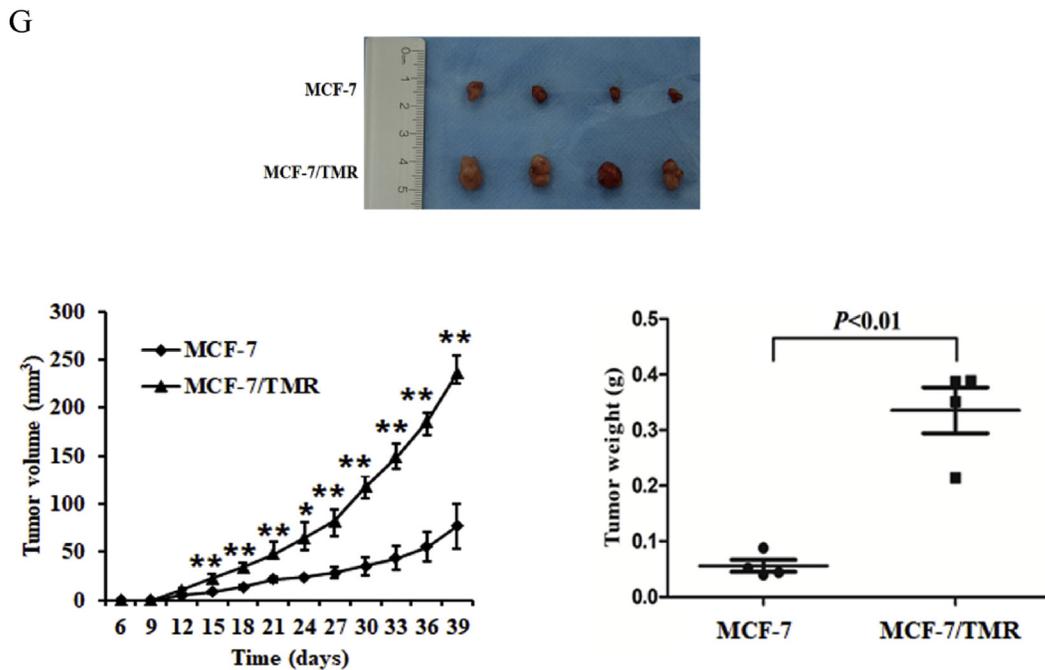


Figure 1. (continued).

and CXXC4 mRNA expression was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The validated lentiviruses were used to generate cells stably expressing CXXC4 by selection with puromycin (0.5–1 μg/mL). To knock down CXXC4 expression in parental cells, CXXC4 shRNA (sh-CXXC4) or negative

control shRNA (sh-NC) was subcloned into the lentivirus vector pGV248 (Genechem, Shanghai, China). The target shRNA with the best knockdown efficiency was used for all subsequent experiments (Figure S4). The aforementioned procedures are detailed in Supplementary Table S3.

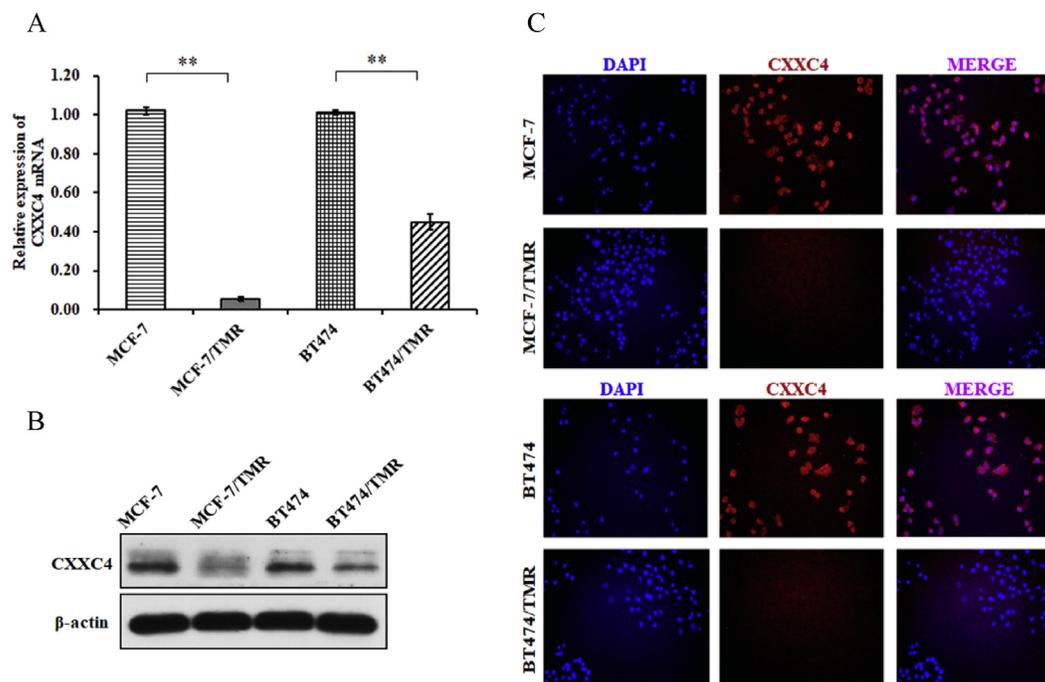
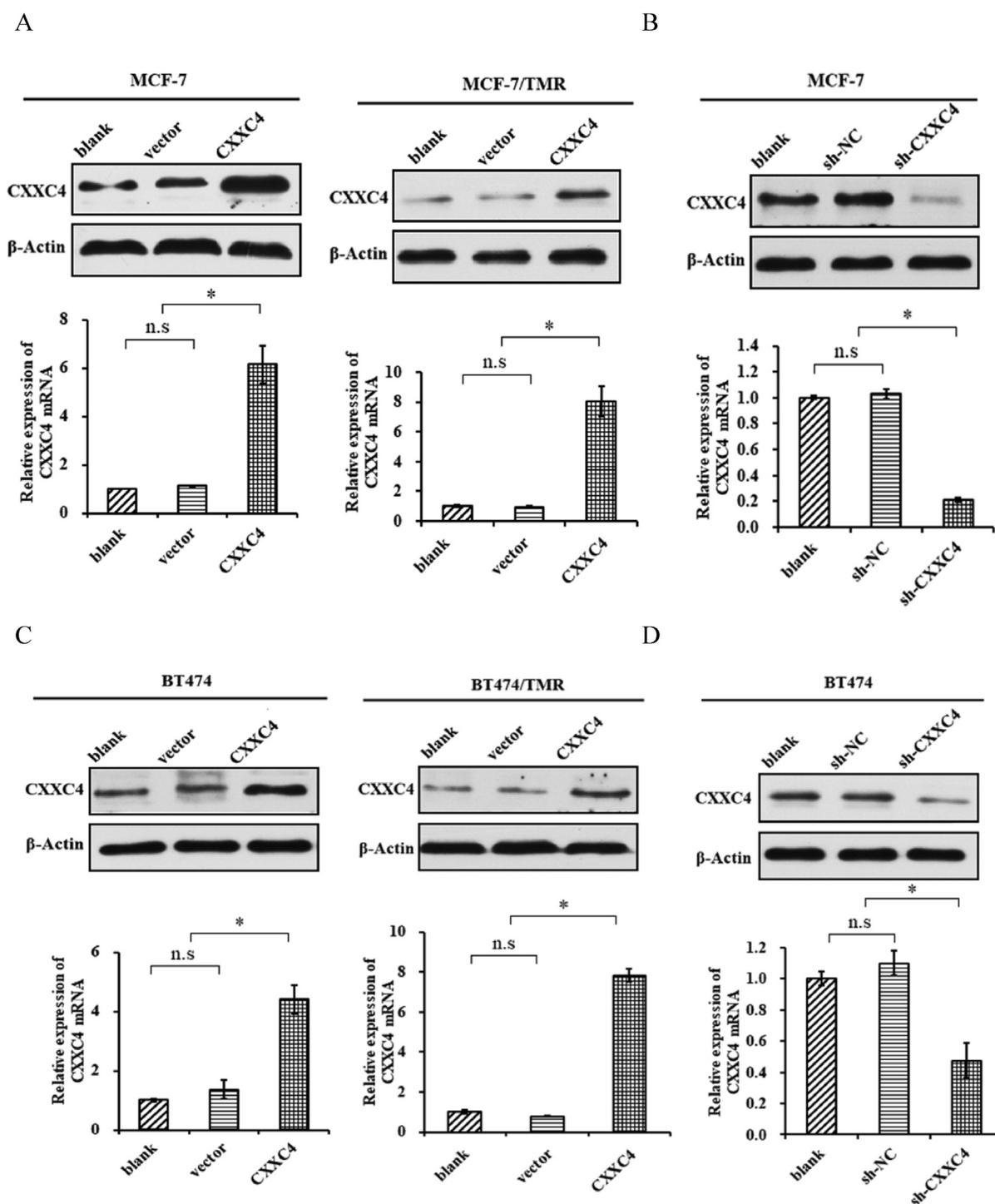


Figure 2. Tamoxifen-resistant cells express lower levels of CXXC4 than parental cells. (A) Transcript levels of CXXC4 in parental cells (MCF-7, BT474) and resistant cells (MCF-7/TMR, BT474/TMR) were measured by qRT-PCR (\*\* $P < 0.01$ ). (B) Protein expression levels of CXXC4 in the cells were detected by Western blot analysis. (C) CXXC4 expression in parental cells and resistant cells was investigated by immunofluorescence analysis ( $\times 100$ ). qRT-PCR, quantitative reverse transcription polymerase chain reaction.



**Figure 3.** Upregulation or knockdown of CXXC4 in breast cancer cell lines. (A) Ectopic expression of CXXC4 in MCF-7 and MCF-7/TMR cells transfected with pGV358-CXXC4 (Western blotting and qRT-PCR) ( $*P < 0.05$ ). (B) Downregulation of CXXC4 in MCF-7 cells infected with pGV248-sh-CXXC4 ( $*P < 0.05$ ). (C) Ectopic expression of CXXC4 in BT474 and BT474/TMR cells ( $*P < 0.05$ ). (D) Knockdown of CXXC4 in BT474 cells ( $*P < 0.05$ ). qRT-PCR, quantitative reverse transcription polymerase chain reaction.

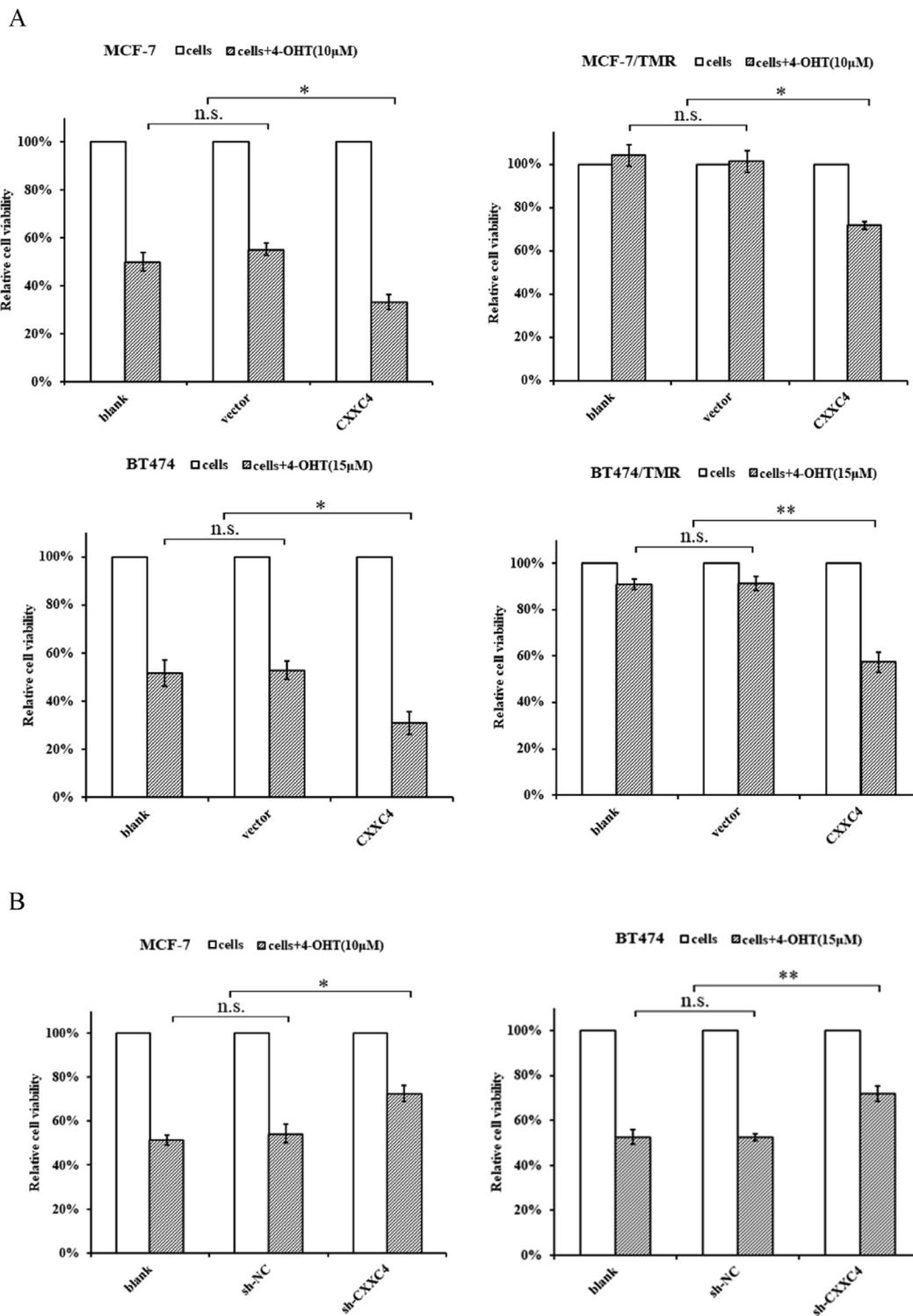
#### Cell viability assay

Cells were seeded at the indicated density (4000 cells per well) in 96-well plates (Costar, Cambridge, MA, USA) overnight. After 4-OHT treatment for three days, cell viability was assessed by methyl thiazolyl tetrazolium (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). Relative absorbance at 490 nm was detected using a microplate reader (Bio-Rad Laboratories, Inc.). The survival rates of cells were

calculated using the measured absorbance values. All reactions were carried out with three replicates.

#### Colony formation assay

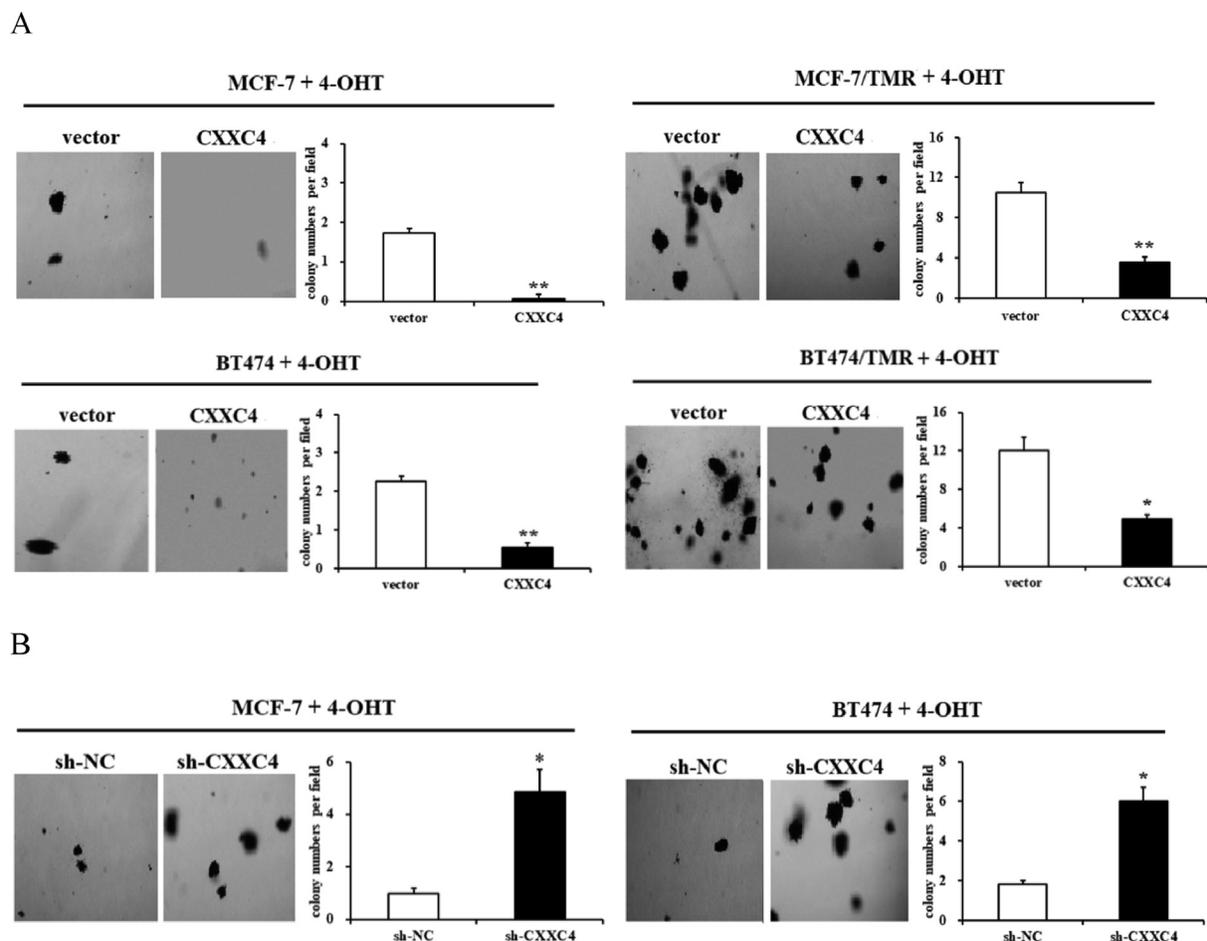
Plate colony formation assays were performed as follows: Cells were plated at the indicated density (300 cells per well) in six-well plates and incubated for at least 24 h. After 24-h tamoxifen treatment,



**Figure 4.** Decreased CXXC4 expression renders tamoxifen resistance. (A) CXXC4 overexpression increases cell sensitivity to 4-OHT (\* $P < 0.05$ , \*\* $P < 0.01$ ). (B) Knockdown of CXXC4 reduces tamoxifen sensitivity (\* $P < 0.05$ , \*\* $P < 0.01$ ). 4-OHT, 4-hydroxytamoxifen.

nonattached cells were removed and attached cells were cultured in fresh medium for 10–14 days. Finally, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then stained with Giemsa for 20–30 min. Soft agar colony formation assay was executed by the following procedure: Cells were planted in 500 µL of 0.33% (*w/v*) agar (Sigma–Aldrich, St. Louis, MO, USA) containing a

certain concentration of 4-OHT overlaid on 1 mL of 0.5% (*w/v*) solidified agar bottom layer in 12-well plates. The top media containing 4-OHT was changed every two to three days. The plates were incubated at 37 °C until colonies were stained by MTT reagents, counted, and photographed. All the experiments were performed in triplicate.



**Figure 5.** Decreased CXXC4 expression promotes soft agar colony formation. (A) Elevated CXXC4 expression suppresses soft agar colony formation in parental cells and resistant cells treated with 4-OHT (\* $P < 0.05$ , \*\* $P < 0.01$ ). (B) Reduced CXXC4 expression promoted soft agar colony formation in MCF-7 and BT474 cells treated with 4-OHT (\* $P < 0.05$ ). 4-OHT, 4-hydroxytamoxifen.

### Cell cycle analysis

Cell cycle assays were performed using a cell cycle analysis kit (KeyGEN Biotech, Nanjing, China) in accordance with the manufacturer's instructions. Cells were seeded at  $10^5$  cells per well into six-well plates. After 72-h treatment with 4-OHT, cells were harvested and rinsed with ice-cold phosphate buffered saline (PBS) and fixed in 70% ethanol at 4 °C overnight. After PBS washes, the cells were incubated with RNase A for 30 min at 37 °C, stained with propidium iodide (PI), and analyzed by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA). The cell cycle distribution was assessed using Modifit software.

### Quantitative reverse transcription polymerase chain reaction

Total RNAs were isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After RNA concentration and purity were measured using a NanoDrop 1000 (Nanodrop Technologies, Wilmington, DE, USA), cDNA was synthesized using an iScript™ gDNA Clear cDNA synthesis kit (Bio-Rad Laboratories, Inc.). Real-time qRT-PCR analysis was performed using SsoFast™ Eva Green Supermix (Bio-Rad Laboratories, Inc.) in accordance with the manufacturer's instructions. All gene expressions were normalized to GAPDH. The relative fold expressions were calculated using the  $2^{-\Delta\Delta CT}$  method. RNA was extracted from at least three independent

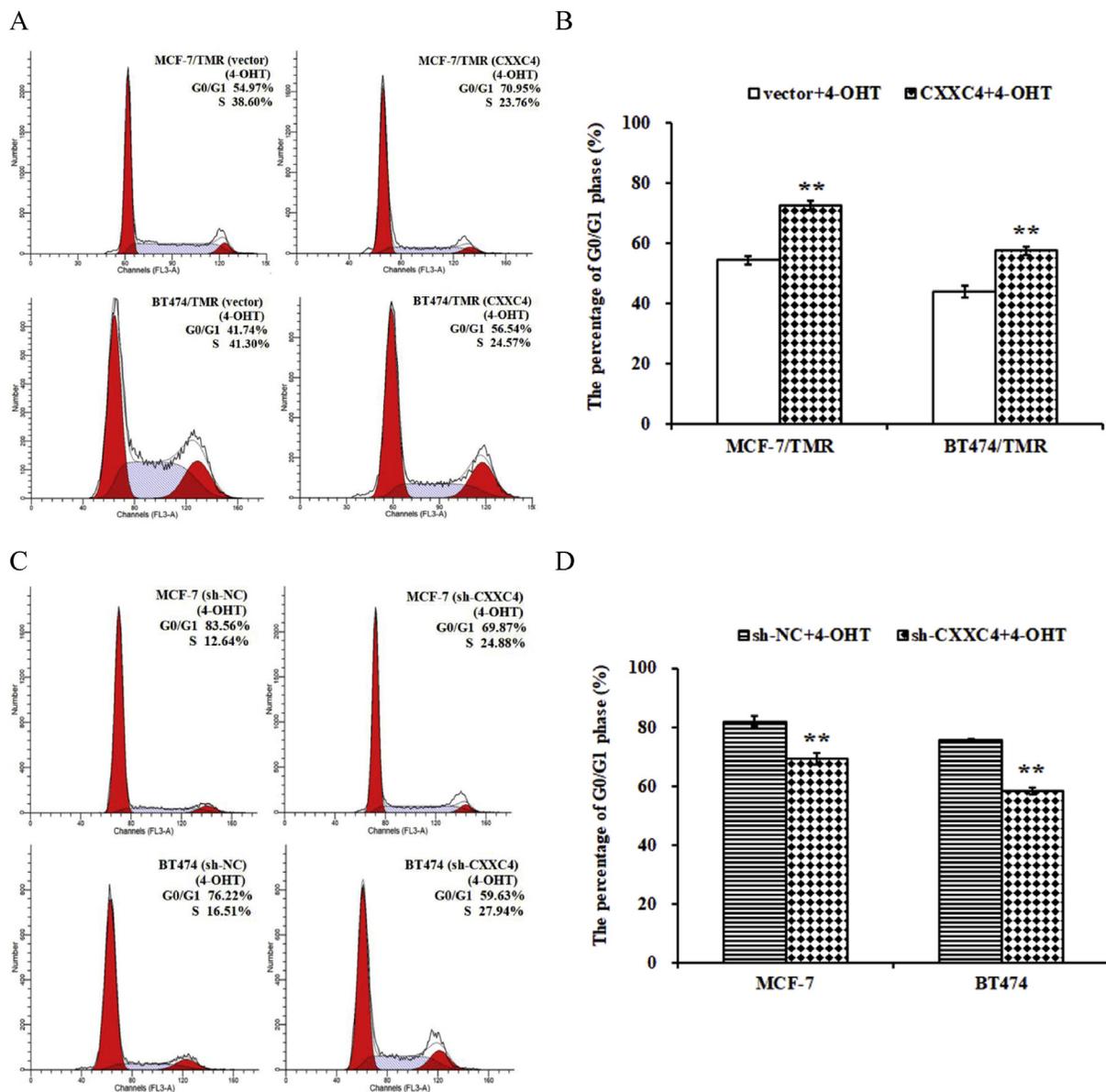
sets of cells, and all reactions were performed in triplicate. The primer sequences are listed in [Supplementary Table S4](#).

### Western blot analysis and antibodies

Protein purification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrical transfer, and immunoblotting were implemented as per the instructions. Primary antibodies for CXXC4 (1:1000; cat. no. ab105400; Abcam, Cambridge, MA, USA), p-GSK3 $\beta$  (Ser9) (1:1000; cat. no. 310010; ZenBio, Inc., Chengdu, China),  $\beta$ -catenin (1:1000; cat. no. 250110; ZenBio, Inc., Chengdu, China), cyclinD1 (1:1000; cat. no. 380999; ZenBio, Inc., Chengdu, China), and c-myc (1:1000; cat. no. 380784; ZenBio, Inc., Chengdu, China) were used to bind the target proteins. All immunoreactive protein complexes were incubated with horseradish peroxidase-conjugated secondary antibodies until they were detected.

### Immunocytofluorescence

Parental cells and resistant cells were seeded on cover slips, fixed in 4% paraformaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Unspecific antigens were blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. Cells were incubated with a primary antibody for CXXC4 (1:50; cat. no. ab105400; Abcam, Cambridge, MA, USA) overnight at 4 °C and then incubated with secondary



**Figure 6.** CXXC4 overexpression reinforces the antitumor effect of 4-OHT through cell cycle retardation. (A) CXXC4-overexpressing MCF-7/TMR and BT474/TMR cells were separately treated with 10  $\mu$ M, 15  $\mu$ M 4-OHT, then detected by flow cytometry analysis after three days. (B) Bar chart presented the proportion of G0/G1 phase in resistant cells transfected with vector (control) or pGV358-CXXC4 plasmid after treated with 4-OHT for three days (\*\* $P < 0.01$ ). (C) CXXC4-knockdown MCF-7 and BT474 cells treated as described previously, and cell cycle was evaluated using flow cytometry. (D) Bar chart presented the proportion of G0/G1 phase in breast cancer cells (MCF-7, BT474) transfected with lentiviral sh-NC (control) or sh-CXXC4 plasmid after treated with 4-OHT for three days (\*\* $P < 0.01$ ). 4-OHT, 4-hydroxytamoxifen.

antibody (anti-rabbit Alexa Fluor® 568; cat. no. ab175471; Abcam, Cambridge, MA, USA) for 1 h at 37 °C. Cell nuclei were stained using 1  $\mu$ g/mL DAPI (Vector Laboratories, Burlingame, CA, USA) for 10 min. Slides were observed under a fluorescence microscope (Olympus, Japan).

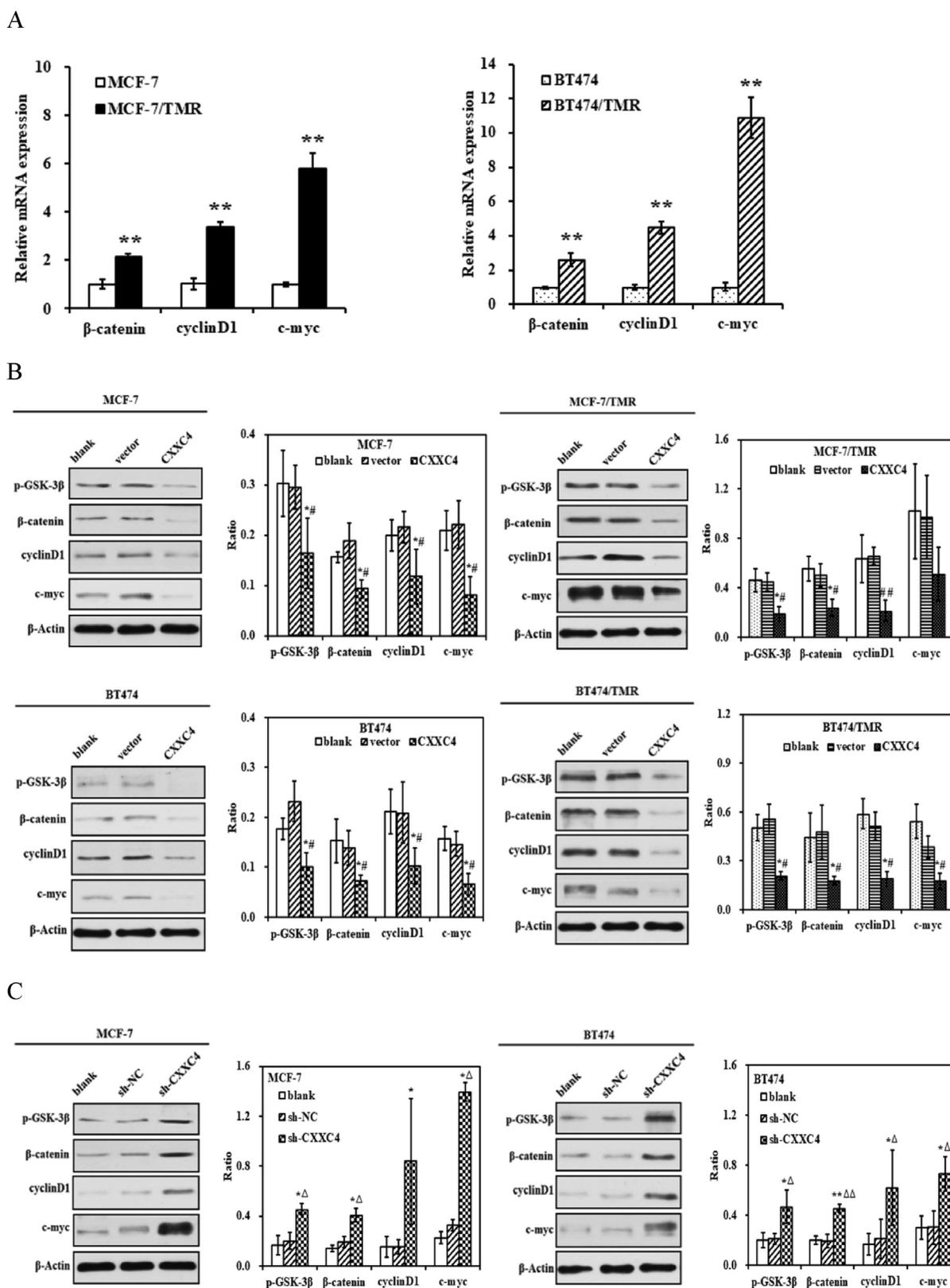
**In vivo tumorigenicity study**

Five-week-old female nude mice with BALB/c backgrounds (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were used for tumorigenicity assays. Animal studies were carried out in accordance with the regulations of the Institutional Animal Care and Use Committee. MCF-7 cells, MCF-7-sh-NC cells,

MCF-7-sh-CXXC4 cells, MCF-7/TMR cells, MCF-7/TMR-vector cells, and MCF-7/TMR-pGV358-CXXC4 cells were injected subcutaneously in the right lower flanks of the nude mice. Implanted tumors were measured, and tumor volumes were obtained by the formula: volume = 0.5  $\times$  length  $\times$  width<sup>2</sup>. The mice were sacrificed 39 days later, the weights of the xenograft tumors were measured, and the tumor samples were collected for further research.

**Immunohistochemistry**

Immunohistochemistry (IHC) was conducted as follows. Paraffin-embedded tissue sections (4  $\mu$ m) were dewaxed and rehydrated using graded ethanol. Antigen retrieval was performed by



**Figure 7.** CXXC4 knockdown activates the Wnt/ $\beta$ -catenin signaling. (A) The mRNA expression levels of  $\beta$ -catenin, cyclinD1, and c-myc in parental cells and resistant cells were quantified using qRT-PCR (\*\* $P < 0.01$ ). (B) Phosphorylation of GSK-3 $\beta$  (ser9) and protein expression of  $\beta$ -catenin, cyclinD1, and c-myc in CXXC4-overexpressing breast cancer cells were examined by Western blot analysis after treated with 4-OHT for three days ( $*P < 0.05$  compared with the blank group;  $\#P < 0.05$ ,  $\#\#P < 0.01$  compared with the vector group). (C) Phosphorylation of GSK-3 $\beta$  (ser9) and protein expression of  $\beta$ -catenin, cyclinD1, and c-myc in CXXC4-knockdown MCF-7 and BT474 cells were detected using Western blot assay ( $*P < 0.05$ ,  $**P < 0.01$  compared with the blank group;  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  compared with the sh-NC group). (D) The protein expressions of  $\beta$ -catenin, cyclinD1, and c-myc

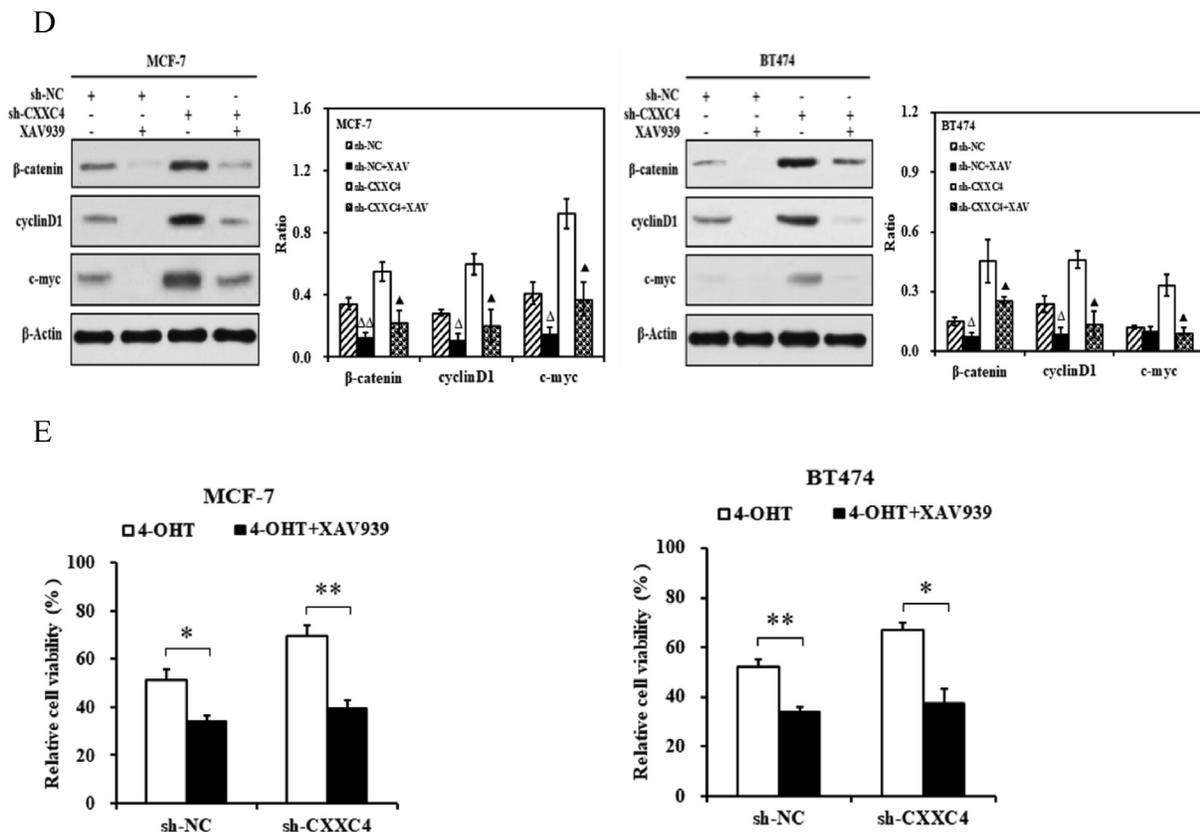


Figure 7. (continued).

heat-induced epitope retrieval (microwaving) for 15 min in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase was blocked by 3% hydrogen peroxide. Slides were then incubated with normal serum at room temperature for 30 min. The sections were incubated overnight in a 4 °C humid chamber with primary antibodies against CXXC4 (1:100, cat. no. ab105400; Abcam, Cambridge, MA, USA),  $\beta$ -catenin (1:100; cat. no. BM3905; Boster, Inc., Wuhan, China), cyclinD1 (1:100; cat. no. 380999; ZenBio, Inc., Chengdu, China), and c-myc (1:100; cat. No 0.380784; ZenBio, Inc., Chengdu, China) followed by incubation with secondary peroxidase-labeled antibody (1:300; cat. no. K400311-2; EnVision/HRP, Dako, Denmark). Professional pathologists evaluated the IHC results.

### Statistical analysis

Statistical analysis was performed using SPSS, version 20.0, (SPSS, Inc.). Group comparisons were performed using a two-tailed Student t-test for comparison of two groups and one-way analysis of variance (ANOVA) for comparison of three groups. A  $P$  value < 0.05 was considered as statistically significant. Other  $P$  values were regarded as nonsignificant (n.s.).

## Results

### Characterization of tamoxifen-resistant cell lines and comparison of antitumor effects of 4-OHT on parental and resistant cells

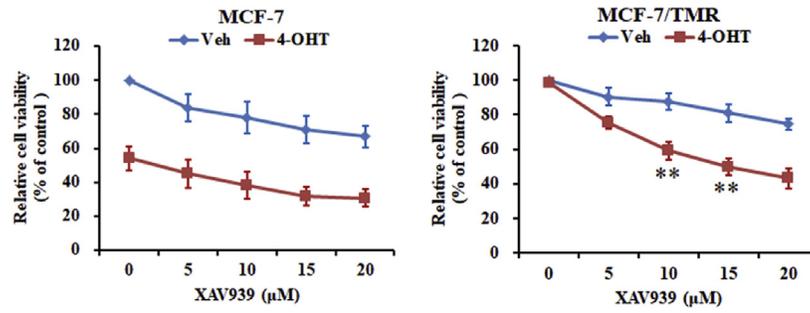
The ER + breast cancer cell lines MCF-7 and BT474 were subjected to high-concentration short-term shock and progressive concentration induction, eventually adapting to the tamoxifen-containing culture environment and thus generating the tamoxifen-resistant sublines MCF-7/TMR and BT474/TMR.

To examine cell sensitivity to 4-OHT, we treated these cell lines with different doses of the drug for three days. As shown in Figure 1A, the relative cell viabilities of the resistant cell lines are obviously higher than those of the parental cell lines ( $*P < 0.05$ ,  $**P < 0.01$ ). The half-maximal inhibitory concentrations ( $IC_{50}$ ) of 4-OHT for MCF-7/TMR and BT474/TMR cells are 1.94-fold and 1.30-fold higher, respectively, than those of their corresponding parental cells ( $P < 0.01$ ).

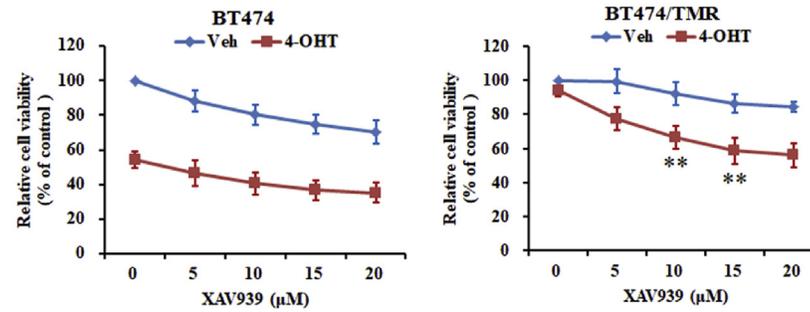
In terms of colony formation capacity, the resistant cells show stronger proliferation capacities than the parental cells, especially in

before and after CXXC4 knockdown in the presence or absence of 10  $\mu$ M of XAV939 were analyzed by Western blot ( $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  compared with the sh-NC group;  $\blacktriangle P < 0.05$  compared with the sh-CXXC4 group). (E) The effects of CXXC4 knockdown in the presence of 4-OHT (10  $\mu$ M for MCF-7, 15  $\mu$ M for BT474) and in the presence or absence of 10  $\mu$ M of XAV939 on the growth of MCF-7 and BT474 cells were measured by MTT analysis ( $*P < 0.05$ ,  $**P < 0.01$ ). 4-OHT, 4-hydroxytamoxifen; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

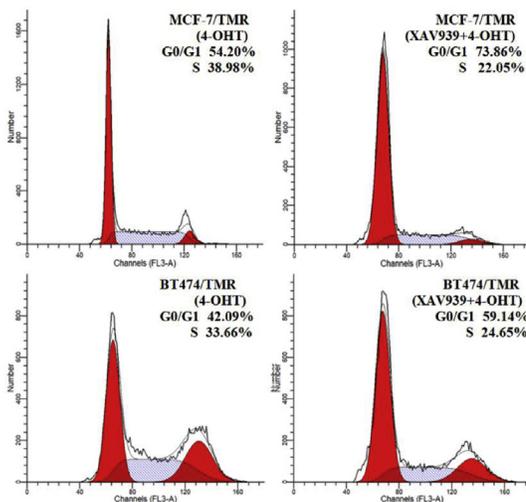
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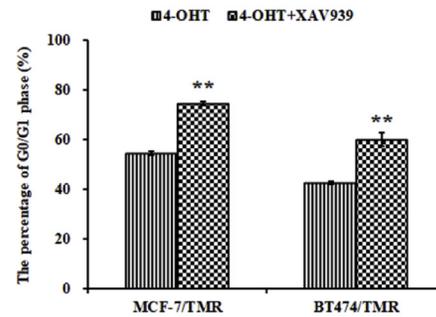
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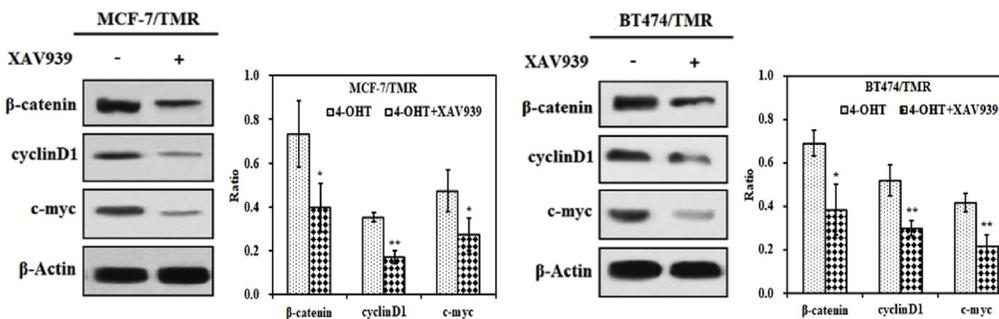
**C**



**D**



**E**



the presence of 4-OHT (Figure 1B and C) ( $*P < 0.05$ ,  $**P < 0.01$ ). Thus, the MCF-7/TMR and BT474/TMR cells were successfully imbued with resistance to tamoxifen.

Previous studies have indicated that tamoxifen can suppress cell proliferation through cell cycle arrest. In this study, flow cytometry was used for cell cycle analysis. We found that the percentage of cells in the G0/G1 phase increases markedly in MCF-7 and BT474 cells after treatment with 4-OHT, while there is no significant change in the resistant cells (Figure 1D and E).

Furthermore, subcutaneous inoculation of MCF-7/TMR cells to nude mice leads to larger and faster growing tumors, suggesting that MCF-7/TMR cells are more tumorigenic than the parental MCF-7 cells (Figure 1F).

### *Reduced expression of CXXC4 in tamoxifen-resistant cells*

To screen the differential expression genes (DEGs) between MCF-7 cells and MCF-7/TMR cells, we subjected both cell lines to mRNA microarray analysis. Hierarchical clustering analysis demonstrated that there are indeed numerous transcriptomal differences between the parental cells and the resistant cells (Figure S1). We defined  $FC \geq 2$  with  $P < 0.05$  ( $t$ -test) as a meaningful differential expression. We then selected the genes with high FC values, such as FGF13, PTPRR, AKAP12, CXXC4, EEF1A2, GPRC5B, EMP3, and IFI16 as candidates and preliminarily validated them by qRT-PCR (Tables S1 and S2, Figures S2A and S2B). As a result, CXXC4, a negative regulator of Wnt/ $\beta$ -catenin signaling, became our focus.

The expression level of CXXC4 in MCF-7/TMR cells is 10.74 times that in MCF-7 cells under routine culture conditions ( $P = 0.003$ ) and 14.35 times that in tamoxifen-containing culture conditions ( $P = 0.001$ ). qRT-PCR analysis verified that the expression of CXXC4 is 20-fold lower in MCF-7/TMR cells than that in the control cells ( $P < 0.01$ ) and 2.2-fold lower in BT474/TMR cells than that in the control cells ( $P < 0.01$ ) (Figure 2A).

Western blot analysis showed that CXXC4 expression is distinctly decreased both in MCF-7/TMR cells and BT474/TMR cells (Figure 2B), corroborating the PCR data. Furthermore, immunofluorescence tests showed that CXXC4 expression is much weaker in MCF-7/TMR and BT474/TMR cells than that in the parental cells (Figure 2C). Thus, CXXC4 expression in tamoxifen-resistant cells is significantly lower than that in the corresponding parental breast cancer cells.

### *Alteration of CXXC4 expression affects cell proliferation and tamoxifen sensitivity*

To investigate whether CXXC4 is involved in the acquisition of resistance to tamoxifen, we depleted CXXC4 expression with lentiviral CXXC4 shRNA cells in parental cells and overexpressed CXXC4 with recombinant lentivirus in both parental cells and

resistant cells (Figures S3A–S3C, Figure 3A–D). After a three-day exposure to 4-OHT, the survival rates of the cells were obtained by MTT assay.

In comparison with blank/vector control, upregulation of CXXC4 expression in cells makes them more sensitive to 4-OHT (Figure 4A;  $*P < 0.05$ ,  $**P < 0.01$ ), while depletion of CXXC4 expression in parental cells increases survival rate to some extent (Figure 4B;  $*P < 0.05$ ,  $**P < 0.01$ ).

Furthermore, the effects of CXXC4 on sensitivity to tamoxifen treatment were assessed by colony formation assay. Ectopic CXXC4 expression increases the inhibitory effect of 4-OHT on cell proliferation, whereas CXXC4 knockdown weakens the effect of 4-OHT (Figure 5A and B;  $*P < 0.05$ ,  $**P < 0.01$ ). These data indicate that CXXC4 is associated with the development of tamoxifen resistance.

### *CXXC4 is involved in the acquisition of tamoxifen resistance by regulating the cell cycle*

As shown previously, 4-OHT blocks cell cycle progression in parental cells but not in tamoxifen-resistant cells. Accordingly, we investigated whether CXXC4 influences the antitumor effects of 4-OHT through regulation of cell cycle process.

Compared with that in the negative control cells, the percentage of G0/G1 phase cells is increased in CXXC4-overexpressing resistant cells after treatment with 4-OHT (Figure 6A and B). However, the number of cells arrested in the G0/G1 phase is decreased to some degree upon eliminating CXXC4 expression in MCF-7 cells or BT474 cells (Figure 6C and D;  $*P < 0.05$ ,  $**P < 0.01$ ). Thus, we inferred that CXXC4 overexpression can partially restore cell sensitivity to tamoxifen through cell cycle arrest, both in MCF-7/TMR and BT474/TMR cells, but decreasing CXXC4 expression in MCF-7 and BT474 cells attenuates the cell cycle blocking effects of 4-OHT. Accordingly, we concluded that CXXC4 is involved in the acquisition of tamoxifen resistance by modulating the cell cycle.

### *Downregulation of CXXC4 expression activates the canonical Wnt signaling pathway*

To the best of our knowledge, CXXC4 does not regulate the cell cycle directly. It has been reported that CXXC4 acts as a negative regulator of the Wnt/ $\beta$ -catenin signaling pathway in RCC and GC. Thus, we reasoned that CXXC4 might affect cell cycle progression through regulation of the downstream target of the Wnt signaling pathway.

CyclinD1 and c-myc proteins are important for cell cycle transition. Besides the mammalian target of rapamycin and nuclear factor- $\kappa$ B pathways, the Wnt/ $\beta$ -catenin signaling pathway is also an upstream regulator of these two proteins. The reduction of CXXC4 expression in tamoxifen-resistant cells is demonstrated in the preceding sections.

**Figure 8.** Inhibition of Wnt/ $\beta$ -catenin signaling partly reverses tamoxifen resistance. (A) The survival rates of MCF-7 and MCF-7/TMR cells treated with the increasing concentrations of XAV939 (from 5  $\mu$ M to 20  $\mu$ M) and 10  $\mu$ M 4-OHT were analyzed by MTT assay ( $**P < 0.01$ ). (B) The survival rates of BT474 and BT474/TMR cells treated with the increasing concentrations of XAV939 (from 5  $\mu$ M to 20  $\mu$ M) and 15  $\mu$ M 4-OHT were analyzed by MTT assay ( $**P < 0.01$ ). (C) MCF-7/TMR and BT474/TMR cells were respectively growing in the presence of 10  $\mu$ M, 15  $\mu$ M 4-OHT and in the presence or absence of 10  $\mu$ M XAV939, and cell cycle was then detected by flow cytometry analysis after three days. (D) Bar chart showed the proportion of G0/G1 phase in resistant cells in the presence or absence of XAV939 ( $**P < 0.01$ ). (E) The protein expressions of  $\beta$ -catenin, cyclinD1, and c-myc of the tamoxifen-resistant cells in the presence or absence of 10  $\mu$ M of XAV939 were analyzed by Western blot ( $*P < 0.05$ ,  $**P < 0.01$ ). 4-OHT, 4-hydroxytamoxifen.

$\beta$ -Catenin is the core of the canonical Wnt signaling pathway. The results of qRT-PCR analysis showed that mRNA expression of  $\beta$ -catenin as well as cyclinD1 and c-myc increase in both tamoxifen-resistant cell lines (Figure 7A; \* $P < 0.05$ , \*\* $P < 0.01$ ). This phenomenon highlights the possible involvement of CXXC4 to the Wnt signaling pathway in breast cancer. Accordingly, we felt it necessary to confirm CXXC4 regulation of canonical Wnt signaling in breast cancer.

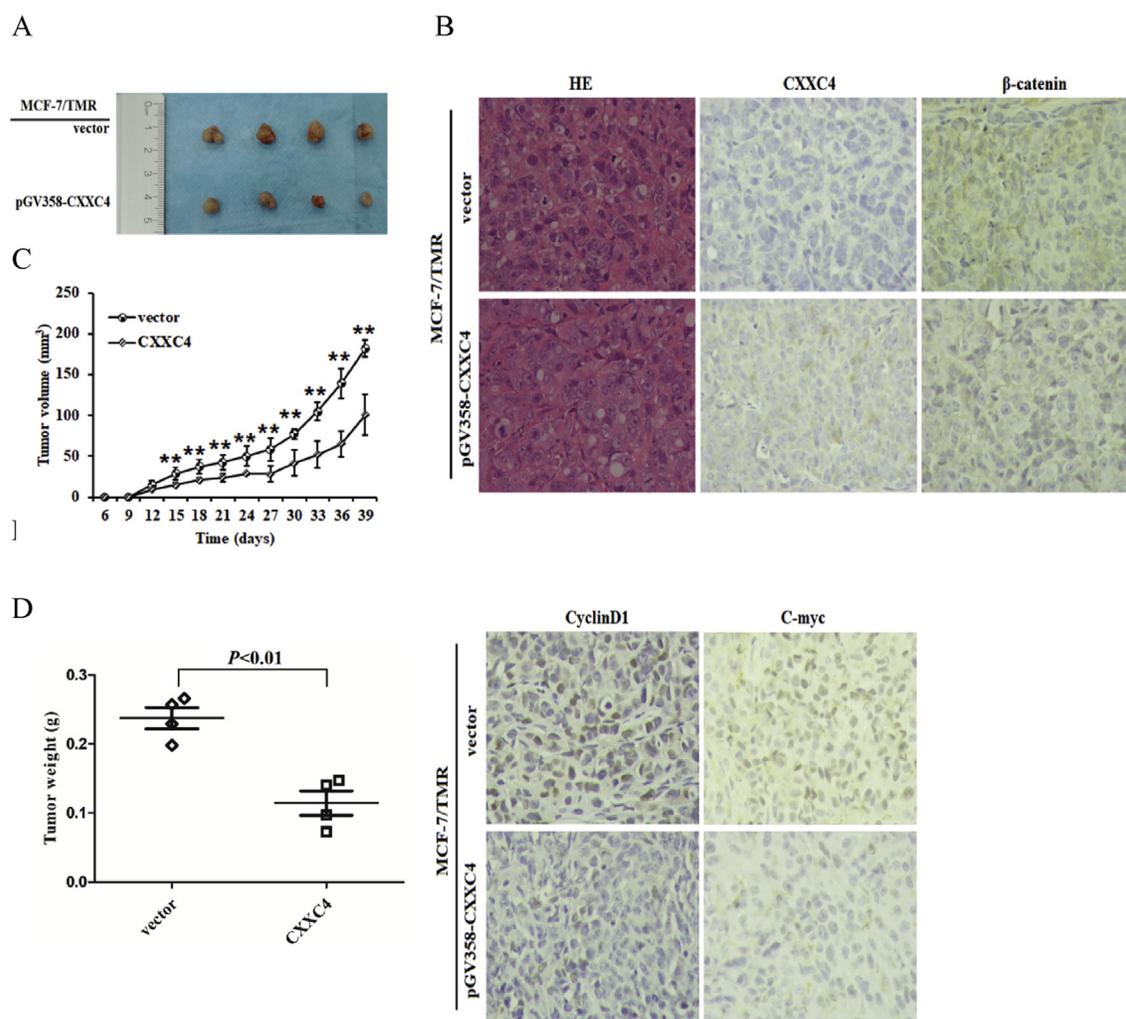
After 4-OHT treatment, we measured the protein levels of  $\beta$ -catenin, cyclinD1, and c-myc in all cells. In MCF-7 and BT474 cells with depleted CXXC4, the protein expressions of  $\beta$ -catenin, cyclinD1, and c-myc are increased (Figure 7C; \* $P < 0.05$ , \*\* $P < 0.01$ ,  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ ). Conversely, elevated CXXC4 expression in both parental and resistant cells inhibits the expressions of these proteins (Figure 7B; \* $P < 0.05$ , # $P < 0.05$ , ## $P < 0.01$ ).

To ascertain whether CXXC4 affects breast cancer cells acquiring a tamoxifen-resistant phenotype through regulation of canonical Wnt signaling, we used the small-molecule inhibitor XAV-939,

which degrades  $\beta$ -catenin by stabilizing the degradation complex containing Axin and can selectively inhibit  $\beta$ -catenin-mediated transcription.

Upon addition of XAV939, CXXC4-knockdown breast cancer cells do not exhibit higher expression of  $\beta$ -catenin, cyclinD1, or c-myc (Figure 7D;  $\blacktriangle P < 0.05$ ,  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ ) and do not gain resistance to tamoxifen (Figure 7E; \* $P < 0.05$ , \*\* $P < 0.01$ ). This implies that decreased expression of CXXC4 promotes breast cancer cell resistance to tamoxifen by activating Wnt/ $\beta$ -catenin signaling and that XAV939 can counteract the impact of CXXC4 knockdown on canonical Wnt signaling to some extent.

GSK-3 $\beta$  is an important kinase in the  $\beta$ -catenin degradation complex. There is a negative correlation between the activity of GSK-3 $\beta$  and the phosphorylation of its ser9 site. Ectopic CXXC4 expression suppresses the phosphorylation of GSK-3 $\beta$  and reduces the expression of  $\beta$ -catenin. Conversely, lowering CXXC4 expression increases the phosphorylation of GSK-3 $\beta$  and the amount of  $\beta$ -catenin (Figure 7B and C). Thus, we concluded that CXXC4



**Figure 9.** Growth of subcutaneously implanted tumor in nude mice: MCF-7/TMR vector group and MCF-7/TMR-CXXC4 group. (A) Implanted tumors in MCF-7/TMR-CXXC4 group are smaller than that in MCF-7/TMR vector group (\*\* $P < 0.01$ ). (B) Tumor sections were stained using hematoxylin and eosin (HE). The expressions of CXXC4,  $\beta$ -catenin, cyclinD1, and c-myc in tumor xenografts were analyzed by IHC staining. (C) Tumor growth curve in MCF-7/TMR vector group and MCF-7/TMR-CXXC4 group. Tumor volume in MCF-7/TMR-CXXC4 group is less than that in MCF-7/TMR vector group. (D) The final tumor weight in MCF-7/TMR-CXXC4 group is lower than that in MCF-7/TMR vector group ( $P < 0.01$ ). IHC, immunohistochemistry.

may affect the integrity of the  $\beta$ -catenin degradation complex by modulating the phosphorylation of GSK-3 $\beta$ , resulting in inhibition of Wnt signaling.

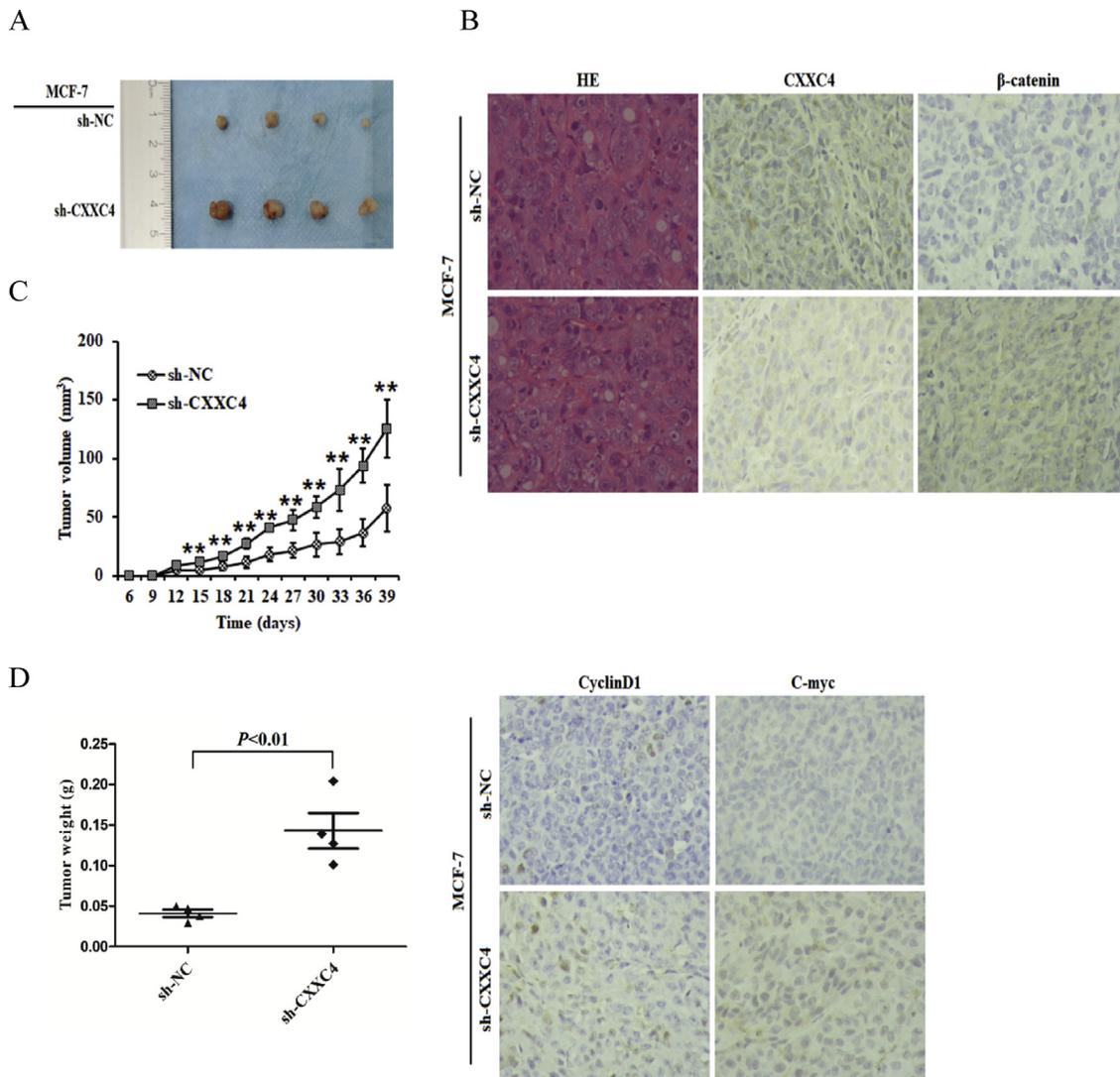
**Inhibiting canonical Wnt signaling may partially reverse tamoxifen resistance**

XAV939 partially restores the sensitivities of MCF-7/TMR and BT474/TMR cells to tamoxifen (Fig. 8A and B; \* $P < 0.05$ , \*\* $P < 0.01$ ). Cell cycle analysis showed that there are more resistant cells arrested in the G0/G1 phase when treated with 4-OHT combined with XAV939 (Fig. 8C and D; \* $P < 0.05$ , \*\* $P < 0.01$ ). Thus, XAV939 enhances the inhibitory effect of 4-OHT on the proliferation of tamoxifen-resistant cells. We observed that XAV939 lowers the expressions of  $\beta$ -catenin and  $\beta$ -catenin-mediated downstream target proteins such as cyclinD1 and c-myc (Figure 8E; \* $P < 0.05$ , \*\* $P < 0.01$ ). These data indicate that XAV939 partially

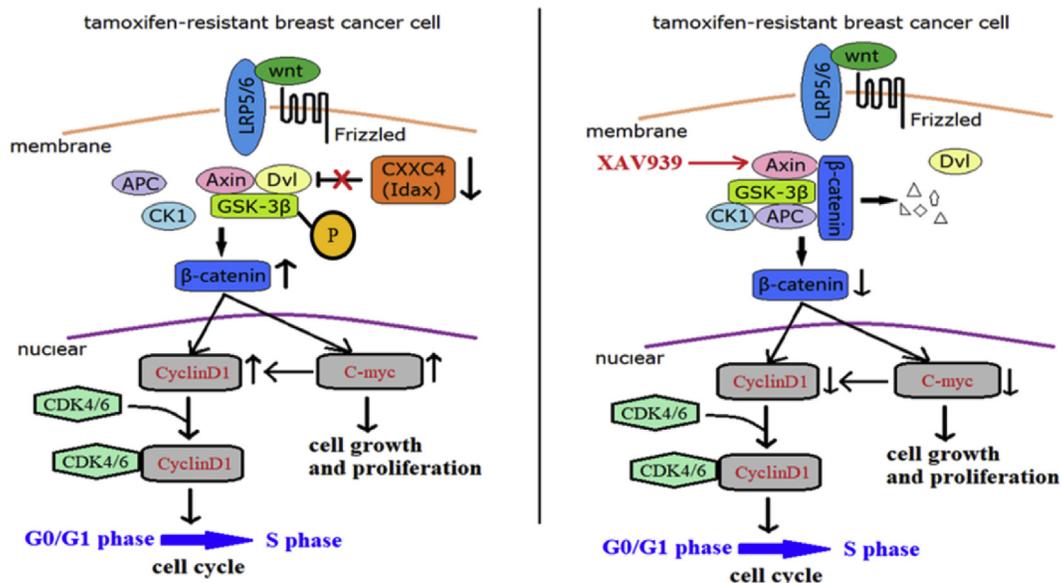
reverses tamoxifen resistance by affecting the cell cycle through inhibition of canonical Wnt signaling.

**Downregulating CXXC4 expression increases the tumorigenicity of breast cancer cells**

To evaluate the influence of CXXC4 on the tumorigenicity of breast cancer cells, MCF-7 cells lacking CXXC4 expression and MCF-7/TMR cells overexpressing CXXC4 were injected subcutaneously near the number five mammary in the right-lower flank of nude mice. Compared with the MCF-7/TMR vector group, the volume and weight of the tumors are decreased in the MCF-7/TMR-CXXC4 group (Figure 9A, C, and 9D; \* $P < 0.05$ , \*\* $P < 0.01$ ). In comparison with the negative control (sh-NC group), CXXC4 depletion in MCF-7 cells increases the growth and weight of tumors (Figure 10A, C, and 10D; \* $P < 0.05$ , \*\* $P < 0.01$ ). This indicates that the tumorigenicity of MCF-7 cells increases as CXXC4 expression



**Figure 10.** Growth of subcutaneously implanted tumor in nude mice: MCF-7-sh-NC group and MCF-7-sh-CXXC4 group. (A) Implanted tumors in MCF-7-sh-CXXC4 group are larger than that in MCF-7-sh-NC group (\*\* $P < 0.01$ ). (B) Tumor sections were stained using hematoxylin and eosin (HE). The expressions of CXXC4,  $\beta$ -catenin, cyclinD1, and c-myc in tumor xenografts were analyzed by IHC staining. (C) Tumor growth curve in MCF-7-sh-NC group and MCF-7-sh-CXXC4 group. Tumor volume in MCF-7-sh-CXXC4 group is more than that in MCF-7-sh-NC group. (D) The final tumor weight in MCF-7-sh-CXXC4 group is higher than that in MCF-7-sh-NC group ( $P < 0.01$ ). IHC, immunohistochemistry.



**Figure 11.** Diagrammatic representation of the possible molecular mechanism of CXXC4-mediated tamoxifen resistance. In resistant cells, downregulation of CXXC4 elevates the expression levels of cyclinD1 and c-myc by activating the Wnt/ $\beta$ -catenin signaling, accelerates cell cycle transition, and finally leads to tamoxifen resistance. XAV939 partially restores the sensitivity of resistant cells to tamoxifen through inhibition of the Wnt/ $\beta$ -catenin signaling.

decreases. Conversely, CXXC4 overexpression reduces the tumorigenic capacity of MCF-7/TMR cells.

As shown by IHC analysis, compared with those in the corresponding control groups, the expression levels of  $\beta$ -catenin, cyclinD1, and c-myc increase in the CXXC4-knockdown group (Figure 10B) but decrease in the CXXC4-overexpression group (Figure 9B). The IHC study data reveal a negative correlation between the expression of CXXC4 and the expressions of  $\beta$ -catenin, cyclinD1, and c-myc, suggesting that CXXC4 inhibits breast tumorigenesis through negative regulation of Wnt/ $\beta$ -catenin signaling.

## Discussion

In accordance with reports in the literature, CXXC4 functions as a tumor suppressor in RCC and GC [32–34]. In the present study, we used ER + breast cancer cell lines (MCF-7 and BT474) to construct tamoxifen-resistant cells and screened for tamoxifen resistance-related genes. A reduction in CXXC4 expression was observed in both resistant cell lines. We found that CXXC4 has an inhibitory effect on cell proliferation and cell cycle progression. More importantly, we demonstrated that CXXC4 knockdown promotes tamoxifen resistance in breast cancer cells, while CXXC4 overexpression partially reverses tamoxifen resistance. We have investigated the mechanisms of the aforementioned phenomenon in depth, revealing valuable knowledge regarding the development of tamoxifen resistance in ER + breast cancer.

In our study, we observed that CXXC4 overexpression inhibits the expression of cyclinD1 and c-myc protein, while CXXC4 silencing enhances their expression. As a crucial transcription factor, c-myc deregulation induces chromosomal instability and triggers uncontrolled activation of a series of downstream genes involved in metabolism, proliferation, survival, and differentiation [35]. In the absence of ER, c-myc can even mimic the proliferation-promoting action of estrogen, indicating that c-myc can partially replace ER and promote tumor cell growth independently of ER signaling [36,37].

c-myc also promotes the formation of the cyclinD-CDK complex [38]. The cyclinD-CDK4/CDK6 axis has a significant effect on tumor cell growth and survival by modulating the cell cycle, and cyclinD1 is the core of the cyclinD-CDK4/CDK6 axis. High expression of cyclinD1 causes the failure of the G1/S transition checkpoint, resulting in acceleration of cell cycle progression [39–41]. We observed high expression levels of cyclinD1 and c-myc in the tamoxifen-resistant cell lines (MCF-7/TMR and BT474/TMR). The resistant cells gain a survival advantage that does not depend on an external mitotic signal. Consequently, 4-OHT alone has no effect on MCF-7/TMR and BT474/TMR cell lines. However, elevated CXXC4 expression in resistant cells could enhance the antitumor effect of 4-OHT by cell cycle arrest. Alteration of CXXC4 expression affects cell cycle progress and cell proliferation through regulating the expression of cyclinD1 and c-myc.

We further investigated the mechanism of CXXC4 regulation of cyclinD1 and c-myc, revealing that CXXC4 might modulate upstream regulators of cyclinD1 and c-myc, such as Wnt/ $\beta$ -catenin [42]. To our best knowledge, CXXC4 is regarded as a negative regulator for the Wnt/ $\beta$ -catenin pathway. It has been demonstrated that CXXC4 interacts with Dvl1 protein and prevents it from destroying the  $\beta$ -catenin degradation complex in GC. In ER + breast cancer, an elevated level of  $\beta$ -catenin, cyclinD1, and c-myc is observed when CXXC4 expression is downregulated, while a reduction in  $\beta$ -catenin, cyclinD1, and c-myc expression is endowed by CXXC4 overexpression. Furthermore, we found that CXXC4 can modulate the phosphorylation of GSK-3 $\beta$ , which affects the integrity of the  $\beta$ -catenin degradation complex and transcription mediated by  $\beta$ -catenin. The small-molecule inhibitor XAV939 stabilizes the  $\beta$ -catenin degradation complex containing Axin and inhibits  $\beta$ -catenin-mediated transcription [43,44].

Our study also revealed that tamoxifen resistance and the activation of Wnt/ $\beta$ -catenin pathway due to CXXC4 knockdown can be suppressed by XAV939. This indicates that downregulation of

CXXC4 leads to tamoxifen resistance in breast cancer cells by accelerating cell cycle progression through activation of the Wnt/ $\beta$ -catenin pathway (Figure 11). Furthermore, XAV939 restores the sensitivity of MCF-7/TMR and BT474/TMR cell lines to tamoxifen to some extent. Thus, these observations highlight the relevance of the Wnt/ $\beta$ -catenin signaling pathway and resistance to tamoxifen in ER + breast cancer.

Undoubtedly, our study reveals merely the tip of the iceberg; CXXC4 and Wnt signaling may be just two of many factors involved in the development of endocrine resistance. Nevertheless, we have provided first-hand information regarding the role of CXXC4 in the acquisition of tamoxifen resistance as well as demonstrating the involvement of canonical Wnt signaling in breast cancer endocrine resistance. Thus, we propose CXXC4 as a possible new biomarker for monitoring endocrine resistance and that inhibitors of the Wnt signaling pathway have potential as agents to reverse tamoxifen resistance.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Funding

This work was supported by the National Key Development Plan for Precision Medicine Research (grant number 2017YFC0910004) and the National Natural Science Foundation of China (grant number 81202099).

### Acknowledgments

The authors are deeply grateful to Hui Li, Jiayuan Li, Kang Liu, Guiqin Song, and Chungmei Wang for their technical support. The authors thank their colleagues in the Laboratory of Molecular Diagnosis of Cancer for their assistance in the development of the project.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2019.12.005>.

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