OTUD7B knockdown inhibits the proliferation and stemness of breast cancer cells by destabilizing FOXM1

HEBING WANG[1*], SUMEI HAN[2*], JIAN XIAO[1], XINGHANG FU[1], WENFENG CHEN[2] and DEXIANG ZHUO[3]

Departments of [1]Breast Surgery, [2]Dermatology, and [3]Laboratory Medicine,
Sanming First Hospital Affiliated to Fujian Medical University, Sanming, Fujian 365000, P.R. China

Abstract. Breast cancer is a leading cause of cancer-related death in women worldwide; therefore, there is an urgent need to develop novel therapies and drugs that prolong the survival and improve the quality of life of patients with breast cancer. In the present study, the effects and underlying mechanisms of OTU domain-containing 7B (OTUD7B) knockdown on breast cancer were investigated using MDA-MB-468, MDA-MB-453 and MCF7 cell lines. The results of Cell Counting Kit 8, colony formation and tumor sphere formation experiments showed that OTUD7B knockdown caused a significant decrease in the proliferation and sphere formation ability of MDA-MB-468, MDA-MB-453 and MCF7 cells *in vitro*. Moreover, western blotting results showed that CD44, EpCAM, SOX2 and Nanog protein levels were significantly decreased following OTUD7B knockdown. These findings indicated that OTUD7B knockdown reduced the proliferation and stemness of breast cancer cells. Co-immunoprecipitation assays demonstrated that OTUD7B interacted with forkhead box protein M1 (FOXM1) and reduced the polyubiquitylation of FOXM1 in breast cancer cells; accordingly, FOXM1 protein levels were significantly decreased by OTUD7B knockdown. Furthermore, the overexpression of FOXM1 reduced the inhibitory effects of OTUD7B knockdown on breast cancer cells. The findings of the present study provide new insights into the oncogenic role of OTUD7B in breast cancer and indicate that OTUD7B may serve as a therapeutic target for breast cancer.

Introduction

Breast cancer is the most common cancer type and a leading cause of cancer-related death in women worldwide. Breast cancer is clinically classified into four subtypes that are characterized by the expression of hormone receptors: Estrogen receptor (ER)-positive, progesterone receptor (PR)-positive, human epidermal receptor 2 (HER2)-positive, and triple-negative for ER, PR and HER2 (1,2). Breast cancer is treated by various methods, including surgery, chemotherapy, radiotherapy and endocrine therapy (3,4). For early-stage breast cancer, there is a good chance of patients achieving cancer-free status following multimodal therapy (3). By contrast, advanced breast cancer is typically regarded as incurable, with patients surviving for only 2-4 years (5). Thus, there is an urgent need to develop novel therapies and drugs that can prolong survival and improve the quality of life of patients with advanced breast cancer.

Deubiquitinases are components of the proteasome system that remove conjugated ubiquitin molecules from target proteins, and thus regulate their stability and bioactivity (6). Deubiquitinases are involved in several biological processes related to cancer development, including proliferation, cell cycle progression, apoptosis and metastasis (7). Increasing evidence has shown that deubiquitinases contribute to breast cancer growth, metastasis, immunosuppression, chemosensitivity and radiosensitivity (8). OTU domain-containing 7B (OTUD7B), a deubiquitinase is a member of the A20 subgroup of the ovarian tumor protein superfamily, which is often dysregulated in human cancer and may serve as a valuable prognostic predictor (9-14). In ERα-positive breast cancer, OTUD7B deubiquitylates ERα, leading to its stabilization; through this mechanism, OTUD7B promotes the proliferation of breast cancer cells (15). In ERα-negative breast cancer, silencing OTUD7B markedly inhibits the migration and

Correspondence to: Professor Dexiang Zhuo, Department of Laboratory Medicine, Sanming First Hospital Affiliated to Fujian Medical University, 29 Liedong Street, Sanyuan, Sanming, Fujian 365000, P.R. China
E-mail: wubishou@fjmu.edu.cn

Dr Wenfeng Chen, Department of Dermatology, Sanming First Hospital Affiliated to Fujian Medical University, 29 Liedong Street, Sanyuan, Sanming, Fujian 365000, P.R. China
E-mail: wenfengchen@fjmu.edu.cn

*Contributed equally

invasion of cancer cells *in vitro*, and significantly impairs lung metastasis *in vivo* (16). Furthermore, high OTUD7B expression is associated with poor paclitaxel response in patients with triple-negative breast cancer (17).

In the present study, co-immunoprecipitation (co-IP) assay was used to investigate whether OTUD7B interacts with forkhead box protein M1 (FOXM1) and reduces FOXM1 ubiquitination to stabilize the FOXM1 protein. FOXM1 is a proliferation-specific transcription factor that is involved in the initiation, progression, metastasis and drug resistance of breast cancer (18). Furthermore, the present study investigated whether OTUD7B inhibits the proliferation and stemness of breast cancer cells by regulating the protein stability of FOXM1.

Materials and methods

Cell culture and tumor tissues. MCF-10A, MDA-MB-436, MDA-MB-231, MDA-MB-468, MDA-MB-453 and MCF-7 cells were obtained from Xiamen Immocell Biotechnology Co., Ltd., and were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C and 5% CO_2 in an incubator.

A total of 10 pairs of breast cancer tissue samples and adjacent normal tissue samples (1 cm away from the edge of the tumor and free of tumor cells) were obtained from Sanming First Hospital Affiliated to Fujian Medical University (Sanming, China). The age range of the 10 female patients was 43-62 years (mean age, 53±6.5 years). All of the patients did not receive any other treatment, such as chemotherapy, target therapy or other preoperative treatment, except for surgical operation.

Plasmid construction. Plasmid encoding His-ubiquitin (His-Ub) was obtained from Xiamen Anti-HeLa Biological Technology Trade Co., Ltd. The plasmids encoding hemagglutinin (HA)-OTUD7B and Flag-FOXM1 were constructed by subcloning OTUD7B and FOXM1 cDNA into pCDNA3.1-HA and pCDNA3.1-Flag vectors (Xiamen Anti-HeLa Biological Technology Trade Co., Ltd.), respectively. FOXM1 cDNA was also inserted into plv-EF1a-MCS-c-Flag-bsd vector (Xiamen Anti-HeLa Biological Technology Trade Co., Ltd), and the resulting plasmid was named plv-Flag-FOXM1, which was used to produce stable OTUD7B-knockdown and FOXM1-overexpression cells. Plasmids encoding a short hairpin (sh)RNA targeting OTUD7B (shOTUD7B) and a negative control shRNA (shNC) were constructed using the pLKO.1 vector (Xiamen Anti-HeLa Biological Technology Trade Co., Ltd). The sequences for plasmid construction are listed in Table I.

To determine the knockdown or overexpression efficiency of plasmids in different breast cancer cells, MDA-MB-468, MDA-MB-453 and MCF7 cells ($2x10^6$ cells/well) were cultured in six-well plates and 5 µg shNC, shOTUD7B, pCDNA3.1 vector, HA-OTUD7B or Flag-FOXM1 were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 24 h, cells were collected for western blotting. The results of which showed that plasmids encoding Flag-FOXM1

or HA-OTUD7B markedly increased the expression of FOXM1 or OTUD7B in cells, whereas shOTUD7B markedly decreased the expression of OTUD7B in cells. Notably, shOTUD7B-1 had the most significant effect and was thus used in subsequent experiments (Fig. S1).

Construction of stable OTUD7B-knockdown cell lines and OTUD7B-knockdown and FOXM1-overexpressing cell lines. Stable OTUD7B-knockdown MDA-MB-468, MDA-MB-453 and MCF7 cells; and stable OTUD7B-knockdown and FOXM1-overexpressing MDA-MB-468, MDA-MB-453 and MCF7 cells were constructed as follows: 9 µg shNC, shOTUD7B or plv-Flag-FOXM1, 3 µg pMD2G (Xiamen Anti-HeLa Biological Technology Trade Co., Ltd) and 6 µg psPAX2 (Xiamen Anti-HeLa Biological Technology Trade Co., Ltd) were co-transfected into 293T cells (Xiamen Immocell Biotechnology Co., Ltd.) in a 10-cm dish using Lipofectamine 2000. After 48 h cultivation at 37°C, the supernatant containing the lentivirus encoding shOTUD7B or FOXM1 was collected. The lentivirus was enriched and the titer was determined. To construct the stable OTUD7B-knockdown cell lines, the lentivirus encoding shOTUD7B was transduced into MDA-MB-468, MDA-MB-453 and MCF7 cells at a multiplicity of infection of 10 in the presence of 8 µg/ml polybrene. After 48 h, the medium was replaced with fresh medium containing 1 µg/ml puromycin (Gibco; Thermo Fisher Scientific, Inc.). At 10 days post-infection, cells were collected for gene expression analysis. To construct the stable OTUD7B-knockdown and FOXM1-overexpressing cell lines, the lentivirus encoding FOXM1 was transduced into stable OTUD7B-knockdown MDA-MB-468, MDA-MB-453 and MCF7 cells at a multiplicity of infection of 10 in the presence of 8 µg/ml polybrene. After 48 h, the medium was replaced with fresh medium containing 5 µg/ml blasticidin (Gibco; Thermo Fisher Scientific, Inc.). At 10 days post-infection, cells were collected for gene expression analysis.

Cell proliferation assay. MDA-MB-468, MDA-MB-453 and MCF7 cells; stable OTUD7B-knockdown MDA-MB-468, MDA-MB-453 and MCF7 cells; stable OTUD7B-knockdown and FOXM1-overexpressing MDA-MB-468, MDA-MB-453 and MCF7 cells were seeded into 96-well plates ($1x10^4$ cells/well) and cultured overnight. After 0, 24, 48 or 72 h, 10 µl Cell Counting Kit 8 (CCK8) solution (Beyotime Institute of Biotechnology) was added and cells were incubated for 4 h at 37°C. The optical density was determined using an absorbance reader (SpectraMax®; Molecular Devices, LLC) at 450 nm.

Colony formation assay. Clusters of cells containing >50 cells were considered colonies. The same cells used in the cell proliferation assay were seeded in 6-well plates at a density of $1x10^3$ cells/well. After 14 days of culture, cell colonies were fixed with 4% paraformaldehyde overnight at 4°C. The fixed colonies were then stained with 0.1% crystal violet for 10 min at 26°C. A light microscope was used to count the colony numbers.

Sphere formation assay. The same cells used in the cell proliferation assay were seeded in 6-well plates ($1x10^4$ cells/well) containing serum-free DMEM supplemented with 2% B27

Table I. Sequences for plasmid construction used in the present study.

Plasmid name	Sequence, 5'-3'
shOTUD7B-1	F: CCGGCAGATTCTGTGGCTAACAAACCTCGAGGTTTGTTAGCCACAGAATCTGTTTTT
	R: AATTAAAAACAGATTCTGTGGCTAACAAACCTCGAGGTTTGTTAGCCACAGAATCTG
shOTUD7B-2	F: CCGGTTGAAGAGTTTCACGTCTTTGCTCGAGCAAAGACGTGAAACTCTTCAATTTTT
	R: AATTAAAAATTGAAGAGTTTCACGTCTTTGCTCGAGCAAAGACGTGAAACTCTTCAA
shOTUD7B-3	F: CCGGTGGAAATGCTCACGGTTTATACTCGAGTATAAACCGTGAGCATTTCCATTTTT
	R: AATTAAAAATGGAAATGCTCACGGTTTATACTCGAGTATAAACCGTGAGCATTTCCA
shNC	F: CCGGTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTT
	R: AATTAAAAATTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAA
Flag-FOXM1	F: GTACCGAGCTCGGATCCGCCACCATGAAAACTAGCCCCCGTCG
	R: TCTTTGTAGTCCTCGAGCTGTAGCTCAGGAATAAACTG
HA-OTUD7B	F: CTAGAGAATTCGGATCCATGACCCTGGACATGGATGC
	R: GCTTCCATGGCTCGAGTCAGAACCTGTGCACCAGGAG
Plv-Flag-FOXM1	F: CTAGAGAATTCGGATCCATGAAAACTAGCCCCCGTCG
	R: CCATGGCTCGAGCCCGGGCTGTAGCTCAGGAATAAACTG

F, forward; R, reverse; OTUD7B, OTU domain-containing 7B; FOXM1, forkhead box protein M1; sh, short hairpin; NC, negative control.

NeuroMix (Absin Bioscience, Inc.), 20 ng/ml epithelial growth factor (Sigma-Aldrich; Merck KGaA), 20 ng/ml basic fibroblast growth factor (Sigma-Aldrich; Merck KGaA) and 4 μg/ml insulin. After 7 days of culture at 37˚C, the sphere diameters were measured under a light microscope.

Flow cytometric analysis. The same cells used in the cell proliferation assay were plated in 6-well plates ($2x10^6$ cells/well). After 24 h, the cells were harvested and incubated with FITC-conjugated antibodies for CD44 (cat. no. 12211-MM02-F; 1:200; Sino Biological, Inc.), CD24 (cat. no. 655154; 1:200; BD Biosciences) or EpCAM (cat. no. 10694-MM06-F; 1:200; Sino Biological, Inc.) at 26˚C for 30 min. The fluorescence intensity was determined using a NovoCyte flow cytometer (ACEA Biosciences; Agilent Technologies, Inc.) and NovoExpress software (1.4.1; ACEA Biosciences; Agilent Technologies, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from MCF-10A, MDA-MB-436, MDA-MB-231, MDA-MB-468, MDA-MB-453 and MCF-7 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using a reverse transcriptase cDNA synthesis kit (cat. no. RR036Q; Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was conducted on the obtained cDNA with a Roche Lightcycler 480 system (Roche Diagnostics) and TB Green® premix (cat. no. RR820Q; Takara Biotechnology Co., Ltd.). The qPCR program included preheating (95˚C for 30 sec), 40 cycles of heating (95˚C for 5 sec), cooling and extension (60˚C for 30 sec), and dissolution curve procedures (95˚C for 5 sec, 60˚C for 60 sec and 95˚C for 1 sec). The relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (19). The qPCR primers are listed in Table II with 18S used as the reference gene.

Table II. Reverse transcription-quantitative PCR primers used in the present study.

Gene	Sequence, 5'-3'
18S	F: AGGCGCGCAAATTACCCAATCC
	R: GCCCTCCAATTGTTCCTCGTTAAG
OTUD7B	F: AGTTGAGAAGGAAGCGTTGA
	R: TATACCAGCCCTGACTCTTTATTC

F, forward; R, reverse; OTUD7B, OTU domain-containing 7B.

Western blotting. MCF-10A, MDA-MB-436, MDA-MB-231, MDA-MB-468, MDA-MB-453 and MCF-7 cells; stable OTUD7B-knockdown MDA-MB-468, MDA-MB-453 and MCF7 cells; stable OTUD7B-knockdown and FOXM1-overexpressing MDA-MB-468, MDA-MB-453 and MCF7 cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Beyotime Institute of Biotechnology). Protein quantification was performed using the bicinchoninic acid method. A total of 20 μg protein/lane was separated by SDS-PAGE on 10% gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). After blocking with 5% skimmed milk at 25˚C for 1 h, the membranes were incubated with primary antibodies against OTUD7B (cat. no. 16605-1-AP; 1:1,000), Nanog (cat. no. 14295-1-AP; 1:1,000), SOX2 (cat. no. 11064-1-AP; 1:1,000), Flag (cat. no. 20543-1-AP; 1:20,000), HA (cat. no. 51064-2-AP; 1:5,000), His (cat. no. 10001-0-AP; 1:1,000), β-actin (cat. no. 20536-1-AP; 1:1,000), β-tubulin (cat. no. 10094-1-AP; 1:2,000), FOXM1 (cat. no. 13147-1-AP; 1:2,000) or GAPDH (cat. no. 10494-1-AP; 1:5,000) (all from Proteintech Group, Inc.) at 25˚C for 2 h, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit
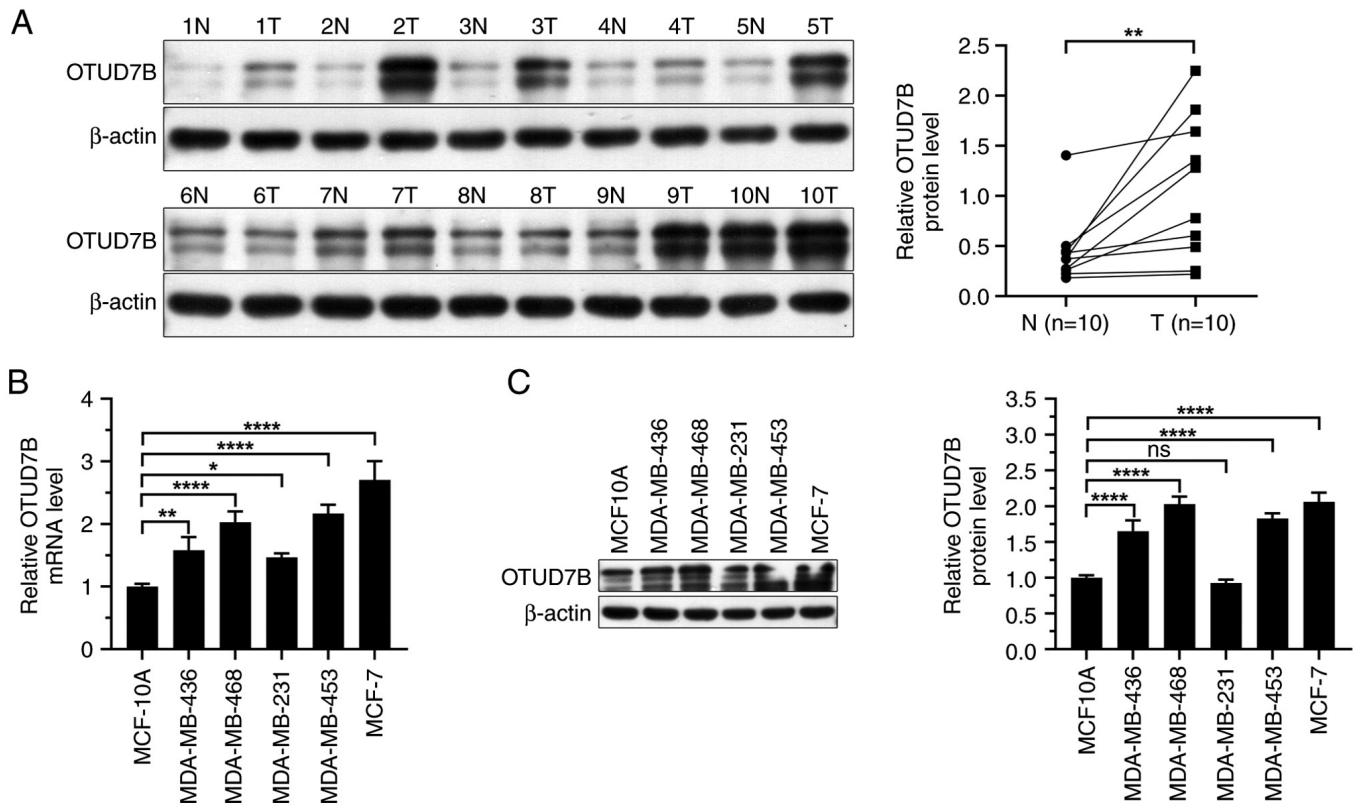
Figure 1. OTUD7B is upregulated in breast cancer tissues and cells. (A) Western blotting of OTUD7B protein in breast cancer tissues and adjacent normal tissues, and statistical analysis of the relative protein expression levels of OTUD7B. (B) Reverse transcription-quantitative PCR analysis of OTUD7B expression in breast cancer cell lines. (C) Western blotting of OTUD7B expression in breast cancer cell lines. *P<0.05, **P<0.01 and ****P<0.0001. OTUD7B, OTU domain-containing 7B; N, normal tissue; T, tumor tissue; ns, not significant.

IgG (cat. no. SA00001-2; 1:2,000; Proteintech Group, Inc.) at 25˚C for 1 h. An enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) was used to visualize the membranes. ImageJ v1.48 (National Institutes of Health) was used for densitometry. β-actin, β-tubulin and GAPDH were used as internal reference proteins.

Co-IP. MDA-MB-468 cells were seeded in 6-well plates (2x10⁶ cells/well) overnight at 37˚C and then transfected with 1.6 μg pCDNA3.1 vector, pCDNA3.1-HA-OTUD7B, pCDNA3.1-Flag-FOXM1 or His-Ub per well with Lipofectamine 2000 following the manufacturer's instructions for 24 h at 37˚C. Subsequently, cells were harvested and lysed in immunoprecipitation buffer (150 mmol/l NaCl; 50 mmol/l Tris-HCl, Ph 7.4; 40 mmol/l β-glycerophosphate; 1 mmol/l Na_4OV_3; 10 mmol/l NaF; 2 mmol/l EDTA) supplemented with 1 mmol/l PMSF and protease inhibitor on ice for 30 min. The cells in immunoprecipitation buffer were centrifuged at 4˚C at 14,000 x g for 15 min, and the supernatant was immediately transferred to a new centrifuge tube to obtain the cell lysate. protein A/G agarose beads (50 μl; Beyotime Institute of Biotechnology) were incubated with anti-HA (cat. no. 66006-2-Ig; 1 μg) or anti-Flag (cat. no. 66008-4-Ig; 1 μg) (both from Proteintech Group, Inc.) at 4˚C overnight, followed by incubation with 500 μl cell lysates at 4˚C for 6 h. The mixture was centrifuged at 14,000 x g at 4˚C for 15 min and the supernatant was discarded. The agarose bead-antigen-antibody complex was then cleaned three times

with immunoprecipitation buffer. After the supernatant was discarded, the precipitation was added to 60 μl 2X loading buffer, heated at 100˚C for 5 min, and then subjected to western blotting.

Detection of the effect of OTUD7B on FOXM1 degradation. MDA-MB-468 cells were seeded in 6-well plates (2x10⁶ cells/well) overnight at 37˚C and then transfected with 1.6 μg pCDNA3.1 vector, pCDNA3.1-HA-OTUD7B or pCDNA3.1-Flag-FOXM1 with Lipofectamine 2000 for 24 h at 37˚C, according to the manufacturer's instructions. Subsequently, the cells were cultured in medium containing 50 μg/ml cyclohexane (MilliporeSigma) for 0, 2, 4, 8 and 12 h at 37˚C. Subsequently, the cells were collected for western blotting. Subsequently, ImageJ v1.48 was used to calculate FOXM1 protein levels at different time points.

Statistical analysis. The experiments were independently repeated three times. Data are presented as the mean ± standard deviation. Unpaired Student's t-test was used for comparisons between unpaired groups, paired Student's t-test was used for comparisons between paired groups, and one-way ANOVA followed by Tukey's post-hoc test was used for comparisons among multiple groups. Prior to statistical analysis, the normality of data was verified by the Shapiro-Wilk test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism v5.0 (Dotmatics).
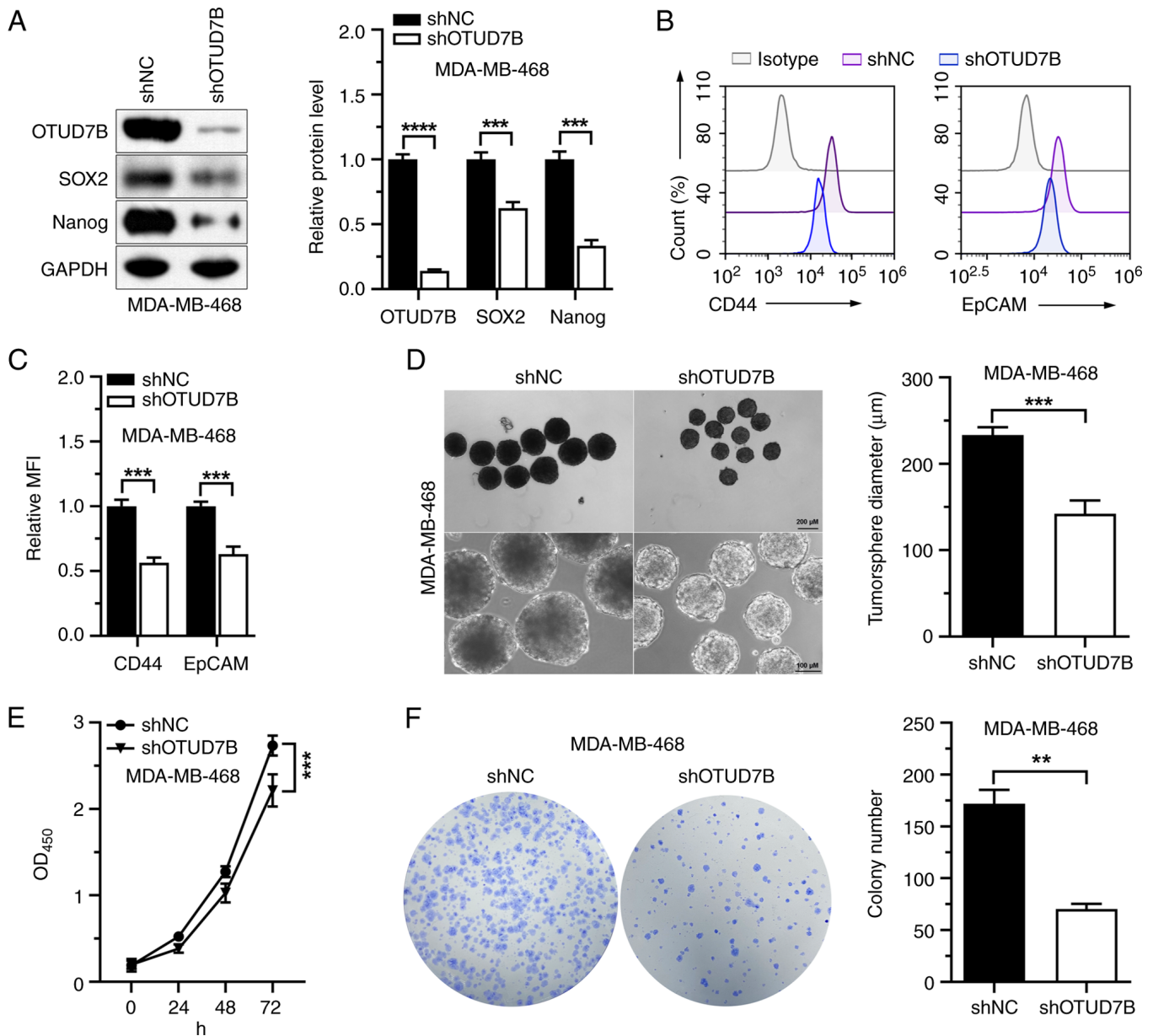
Figure 2. OTUD7B knockdown reduces the stemness and proliferation of MDA-MB-468 cells. (A) Western blotting of SOX2 and Nanog proteins in OTUD7B-knockdown MDA-MB-468 cells. (B) Flow cytometric analysis of CD44 and EpCAM proteins in OTUD7B-knockdown MDA-MB-468 cells. (C) Statistical analysis of mean fluorescence intensity. (D) Tumorsphere diameter analysis of OTUD7B-knockdown MDA-MB-468 cells. (E) Cell Counting Kit 8 analysis of cell proliferation in OTUD7B-knockdown MDA-MB-468 cells. (F) Colony formation capacity analysis of OTUD7B-knockdown MDA-MB-468 cells. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$. OTUD7B, OTU domain-containing 7B; NC, negative control; sh, short hairpin; MFI, mean fluorescence intensity.

Results

OTUD7B is upregulated in breast cancer tissues and cells. To clarify the role of OTUD7B in breast carcinogenesis, first the expression levels of OTUD7B in breast cancer tissues and cells were examined. Western blotting showed that the expression levels of OTUD7B were higher in breast cancer tissues than those in adjacent normal tissues (Fig. 1A). Next, the mRNA and protein expression levels of OTUD7B were analyzed in several breast cancer cell lines. OTUD7B mRNA and protein expression levels were increased in breast cancer cell lines compared with those in MCF-10A cells (Fig. 1B and C). Among the breast cancer cell lines, MDA-MB-468, MDA-MB-453 and MCF-7 cells showed relatively higher OTUD7B expression

levels; therefore, these three cell lines were selected for further analysis.

OTUD7B knockdown reduces the stemness and proliferation of MDA-MB-468 cells. A previous study has shown that OTUD7B maintains the stemness of neural progenitor cells by removing polyubiquitin conjugates from SOX2 protein, increasing its stability (20). Thus, whether OTUD7B affects the stemness of MDA-MB-468 cells was investigated. Western blotting showed that shOTUD7B was successfully transfected into MDA-MB-468 cells and reduced the protein expression levels of OTUD7B in the cells (Fig. 2A). The protein expression levels of SOX2 and Nanog, two stemness-associated proteins, were markedly suppressed by OTUD7B knockdown
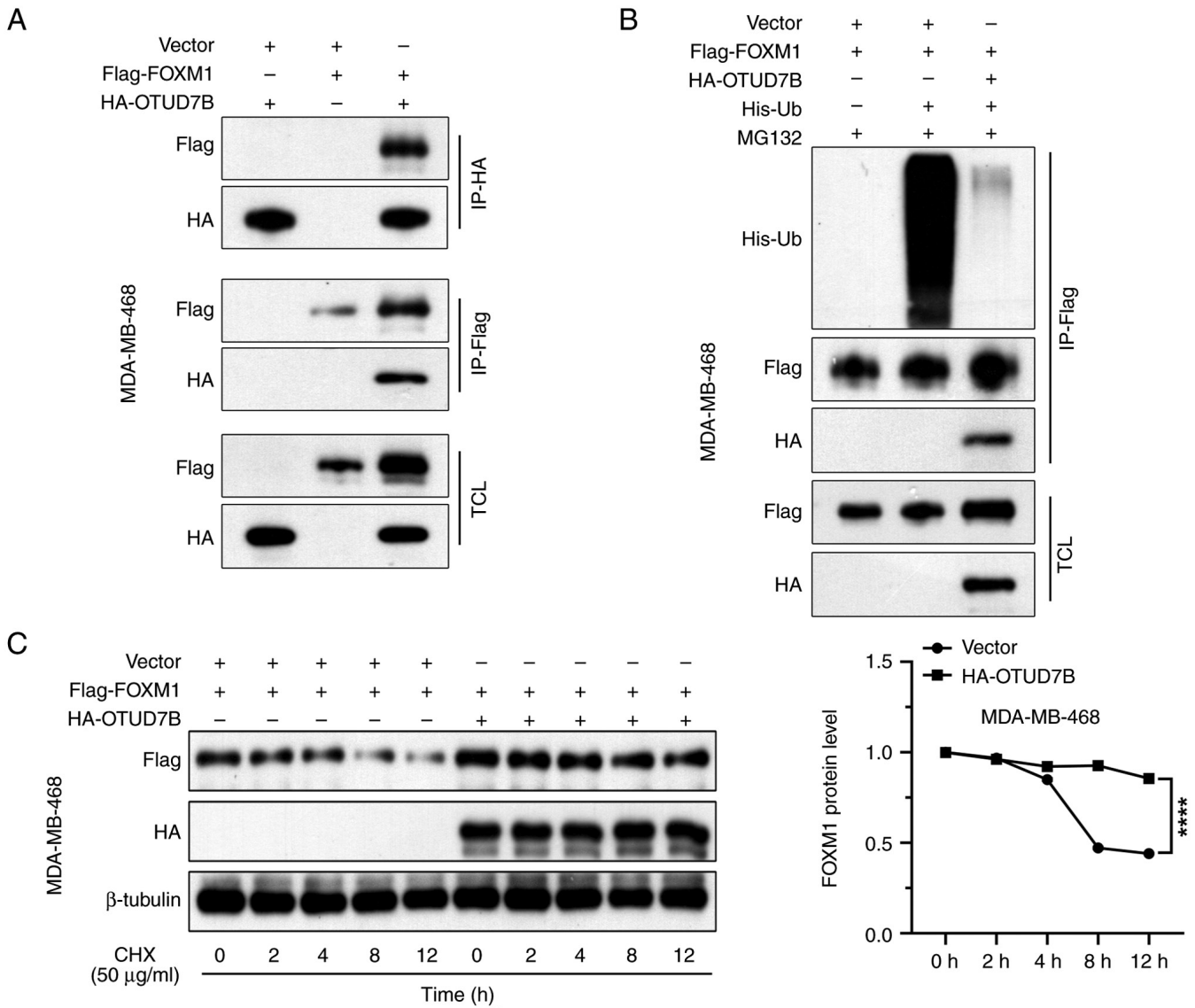
Figure 3. OTUD7B interacts with and stabilizes FOXM1. (A) Interaction between OTUD7B and FOXM1 analyzed using co-IP. (B) Effect of OTUD7B on FOXM1 ubiquitination analyzed using co-IP. (C) Effect of OTUD7B on FOXM1 degradation detected by western blotting. ****$P<0.0001$. OTUD7B, OTU domain-containing 7B; CHX, cycloheximide; FOXM1, forkhead box protein M1; HA, hemagglutinin; co-IP, co-immunoprecipitation; His-Ub, His-ubiquitin; TCL, total cell lysate.

(Fig. 2A). Furthermore, the mean fluorescence intensity of CD44 and EpCAM were also significantly decreased in MDA-MB-468 cells with stable knockdown of OTUD7B (Fig. 2B and C). Sphere formation assays showed that the tumorsphere diameters of MDA-MB-468 cells were significantly reduced by OTUD7B knockdown (Fig. 2D). The impact of OTUD7B on cell proliferation was investigated and it was found that OTUD7B knockdown inhibited the proliferation and colony formation abilities of MDA-MB-468 cells (Fig. 2E and F). These data indicated that OTUD7B knockdown may suppress the stemness and proliferation of MDA-MB-468 cells.

OTUD7B interacts with and stabilizes FOXM1 via deubiquitination. FOXM1 is a transcriptional activator that is involved in the stemness of several types of human cancer cells (21), and its degradation rates are accelerated in OTUD7b-depleted cells (22). Whether FOXM1 is involved in the effects of

OTUD7B on the stemness of breast cancer cells was explored in the present study, as was whether OTUD7B interacts with FOXM1 in breast cancer cells. HA-OTUD7B and Flag-FOXM1 plasmids were co-transfected into breast cancer cells and the interaction between OTUD7B and FOXM1 was detected by co-IP experiments. FLAG-tagged FOXM1 was detected in the HA-OTUD7B immunoprecipitates and vice versa (Fig. 3A).

OTUD7B is a deubiquitinase; therefore, whether OTUD7B affects FOXM1 ubiquitination and stability was explored. As shown in Fig. 3B, in the immunoprecipitate pulled down by the Flag antibody, the His-Ub level in the group with overexpression of HA-OTUD7B was markedly lower than that in the group without overexpression of HA-OTUD7B, indicating that OTUD7B reduced the ubiquitination level of Flag-FOXM1. Protein biosynthesis was pharmacologically inhibited using cycloheximide in MDA-MB-468 cells and FOXM1 protein levels over 12 h with or without OTUD7B overexpression
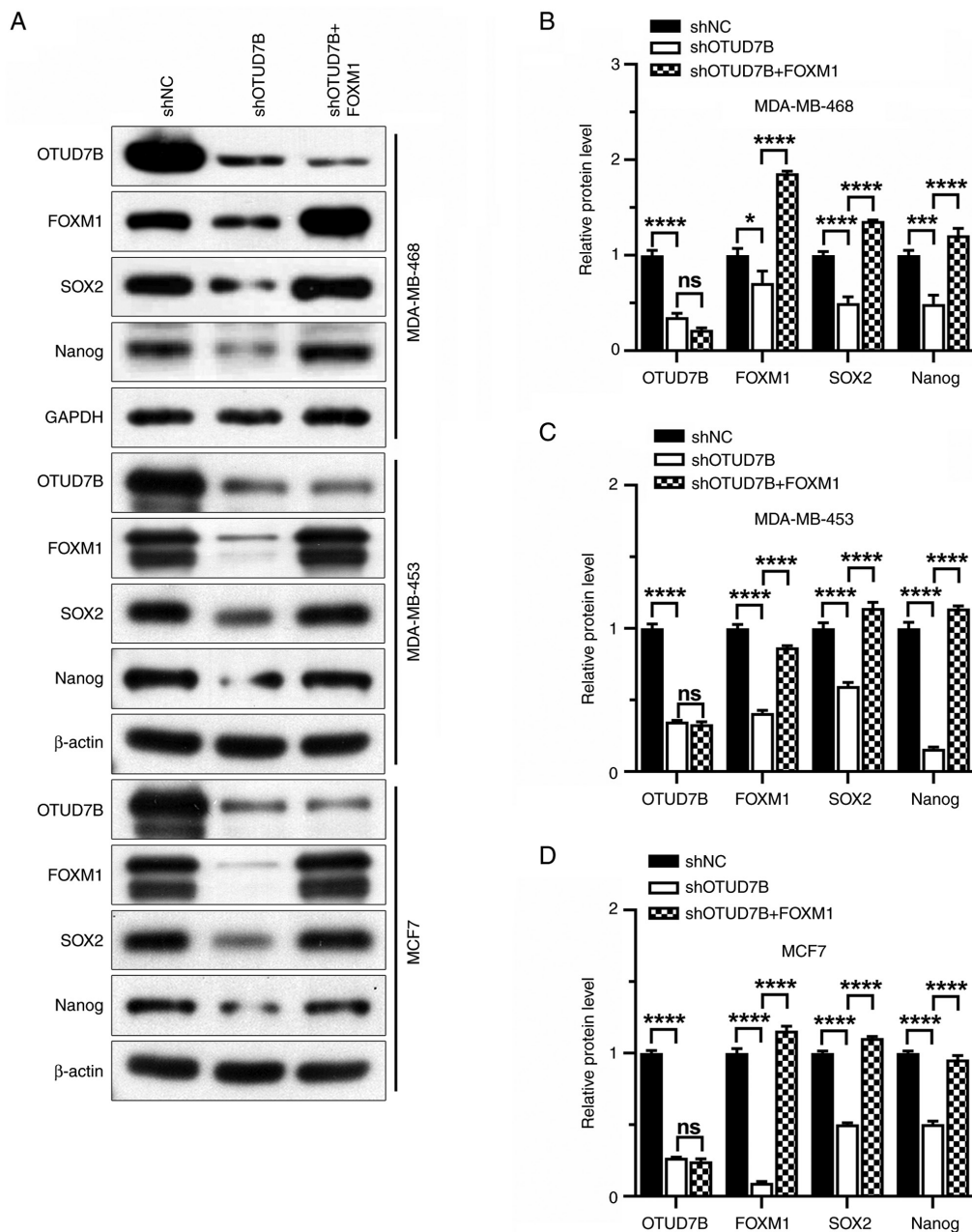
Figure 4. Overexpression of FOXM1 increases the protein expression levels of SOX2 and Nanog in OTUD7B-knockdown breast cancer cells. (A) Western blotting of OTUD7B, FOXM1, SOX2 and Nanog proteins in MDA-MB-468, MDA-MB-453 and MCF7 cells. Statistical analysis of relative protein expression levels of OTUD7B, FOXM1, SOX2 and Nanog in (B) MDA-MB-468, (C) MDA-MB-453 and (D) MCF7 cells. *P<0.05, ***P<0.001 and ****P<0.0001. OTUD7B, OTU domain-containing 7B; FOXM1, forkhead box protein M1; NC, negative control; sh, short hairpin ns, not significant.

were evaluated. It was found that Flag-FOXM1 protein levels consistently decreased from 0 to 12 h after cycloheximide treatment and this decrease was significantly alleviated by OTUD7B overexpression in MDA-MB-468 cells (Fig. 3C). These findings suggested that OTUD7B stabilizes FOXM1 protein by reducing its polyubiquitination.

FOXM1 overexpression rescues the OTUD7B knock-down-induced inhibition of stemness and proliferation in breast cancer cells. To examine the role of FOXM1 in the OTUD7B-mediated effects on the proliferation and stemness of breast cancer cells, the effects of FOXM1 overexpression

on the expression of stemness markers were analyzed. It was revealed that the protein expression levels of SOX2, Nanog, CD44, CD24 and EpCAM were increased by FOXM1 overexpression in OTUD7B-knockdown breast cancer cells (Figs. 4 and 5). The reduced tumorsphere diameter of OTUD7B-knockdown breast cancer cells was also reversed by FOXM1 overexpression (Fig. 6).

To determine the role of FOXM1 in OTUD7B knock-down-mediated cell proliferation inhibition, FOXM1 was overexpressed in OTUD7B-knockdown breast cancer cells and the proliferation of the cells was analyzed by CCK8 assay. As shown in Fig. 7A-C, OTUD7B knockdown inhibited the
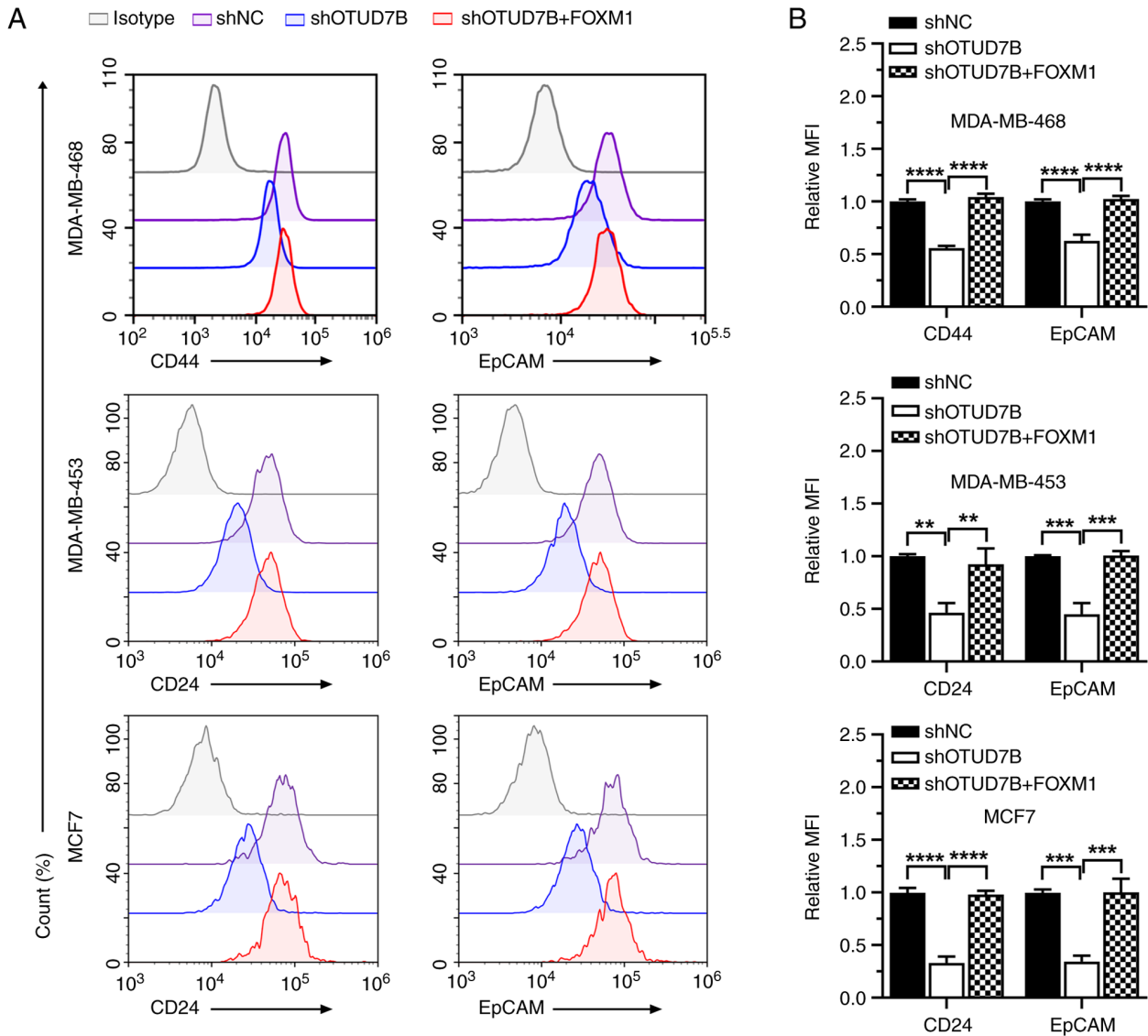
Figure 5. Overexpression of FOXM1 increases the expression levels of stemness markers in OTUD7B-knockdown breast cancer cells. (A) Flow cytometric analysis of the expression levels of CD44, CD24 and EpCAM in MDA-MB-468, MDA-MB-453 and MCF7 cells. (B) Statistical analysis of the MFI of CD44, CD24 and EpCAM in MDA-MB-468, MDA-MB-453 and MCF7 cells. $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$. OTUD7B, OTU domain-containing 7B; FOXM1, forkhead box protein M1; NC, negative control; sh, short hairpin; MFI, mean fluorescence intensity.

proliferation of MDA-MB-468, MDA-MB-453 and MCF-7 cells, whereas the overexpression of FOXM1 blocked the inhibitory effect induced by OTUD7B knockdown. Similar results were observed in colony formation assays, which showed that overexpression of FOXM1 alleviated the inhibitory effect of OTUD7B silencing on the colony formation ability of MDA-MB-468, MDA-MB-453 and MCF-7 cells (Fig. 7D).

Discussion

In the present study, it was revealed that OTUD7B interacts with and deubiquitinates FOXM1 in breast cancer cells, leading to the stabilization of FOXM1. Furthermore, knocking down OTUD7B inhibited the proliferation and stemness of breast cancer cells by enhancing FOXM1 degradation.

OTUD7B was first identified as a negative regulator of NF-κB that inhibits the polyubiquitination of

receptor-interacting protein 1 signal adapter proteins (23-25). OTUD7B dysregulation has been found to participate in the development of numerous types of cancer, but studies on the role of OTUD7B in oncogenesis have yielded conflicting results. In breast, pancreatic and endometrial cancer, OTUD7B may play an oncogenic role by facilitating the proliferation, progression and metastasis of tumor cells (9,13,26). However, in hepatocellular carcinoma (HCC), OTUD7B may function as a tumor suppressor, which is evidenced by the fact that OTUD7B knockdown accelerates the migration and invasion of HCC cells *in vitro* and promotes metastasis *in vivo*. Furthermore, reduced OTUD7B expression is associated with poor prognosis in patients with HCC (10,12). The opposite results have been shown in lung cancer. Zhang *et al* (11) reported that OTUD7B expression was negatively correlated with tumor size, lymph node metastasis and tumor-node-metastasis stage in patients with non-small cell lung cancer. However, patients with high OTUD7B expression had favorable prognoses. Additionally,
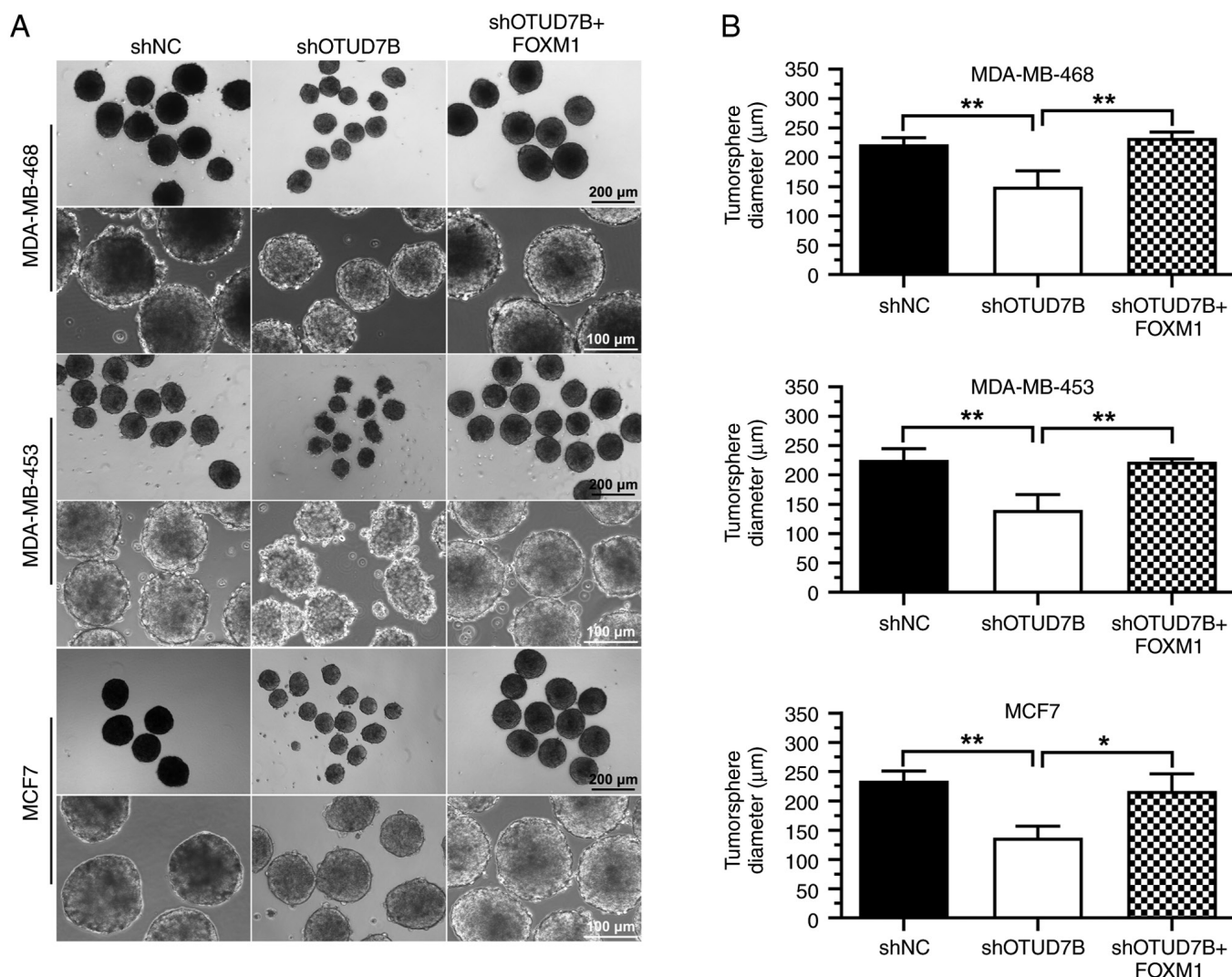
Figure 6. Overexpression of FOXM1 alleviates the reduced tumorsphere diameter of OTUD7B-knockdown breast cancer cells. (A) Tumorsphere diameter analysis of MDA-MB-468, MDA-MB-453 and MCF7 cells. (B) Statistical analysis of tumorsphere diameter of MDA-MB-468, MDA-MB-453 and MCF7 cells. *P<0.05 and **P<0.01. OTUD7B, OTU domain-containing 7B; FOXM1, forkhead box protein M1; NC, negative control; sh, short hairpin.

Lin *et al* (27) showed that OTUD7B augmented EGF-induced Akt signal transduction, promoting lung cancer cell proliferation and migration. These results indicated that the functions of OTUD7B in cancer may be cancer dependent.

In ERα-positive breast cancer, OTUD7B directly interacts with and stabilizes ERα and knocking down OTUD7B inhibits cell proliferation by promoting ERα degradation (15). In ERα-negative breast cancer, silencing OTUD7B inhibits cell migration and invasion *in vitro* and impairs lung metastasis of cancer cells *in vivo* by increasing lysine-specific demethylase 1 (LSD1)-Ub and targeting LSD1 for p62-mediated proteolysis (16). In the present study, it was demonstrated that knocking down OTUD7B inhibited the proliferation and stemness of breast cancer cells by enhancing FOXM1 degradation, which indicates that OTUD7B may function as an oncoprotein in breast cancer.

Cancer stem cells are characterized by increased self-renewal and differentiation capacity, leading to metastasis, drug resistance and cancer relapse, which ultimately result in treatment failure (28,29). As a proliferation-specific transcription factor, FOXM1 has been implicated in the initiation, progression, metastasis and drug resistance of breast cancer (22). In agreement with the findings of the present study, several recent studies have also reported that FOXM1 facilitates the stemness of both ER-positive and ER-negative breast cancer cells (30-32).

Due to the important roles played by deubiquitinases in each stage of breast cancer progression, much effort has been put into developing inhibitors for breast cancer treatment, which has led to promising results. A number of small molecule inhibitors of different deubiquitinases including ZRANB1, CSN5, UCHL3, USP14, USP1 and USP2 have been developed, and are beginning to be used to interfere with proliferation, apoptosis, metastasis and DNA repair, and to enhance sensitivity to immunotherapy and chemotherapy in breast cancer (33-38). In the present study, the oncogenic role played by OTUD7B in breast cancer was demonstrated and the results suggested that OTUD7B is a possible target for drug design in this malignancy. MDA-MB-468 and MDA-MB-453 cells are triple-negative breast cancer cells (39,40) and MCF-7 cells are double-positive breast cancer cells (41). The results of the present study showed that OTUD7B and FOXM1 may
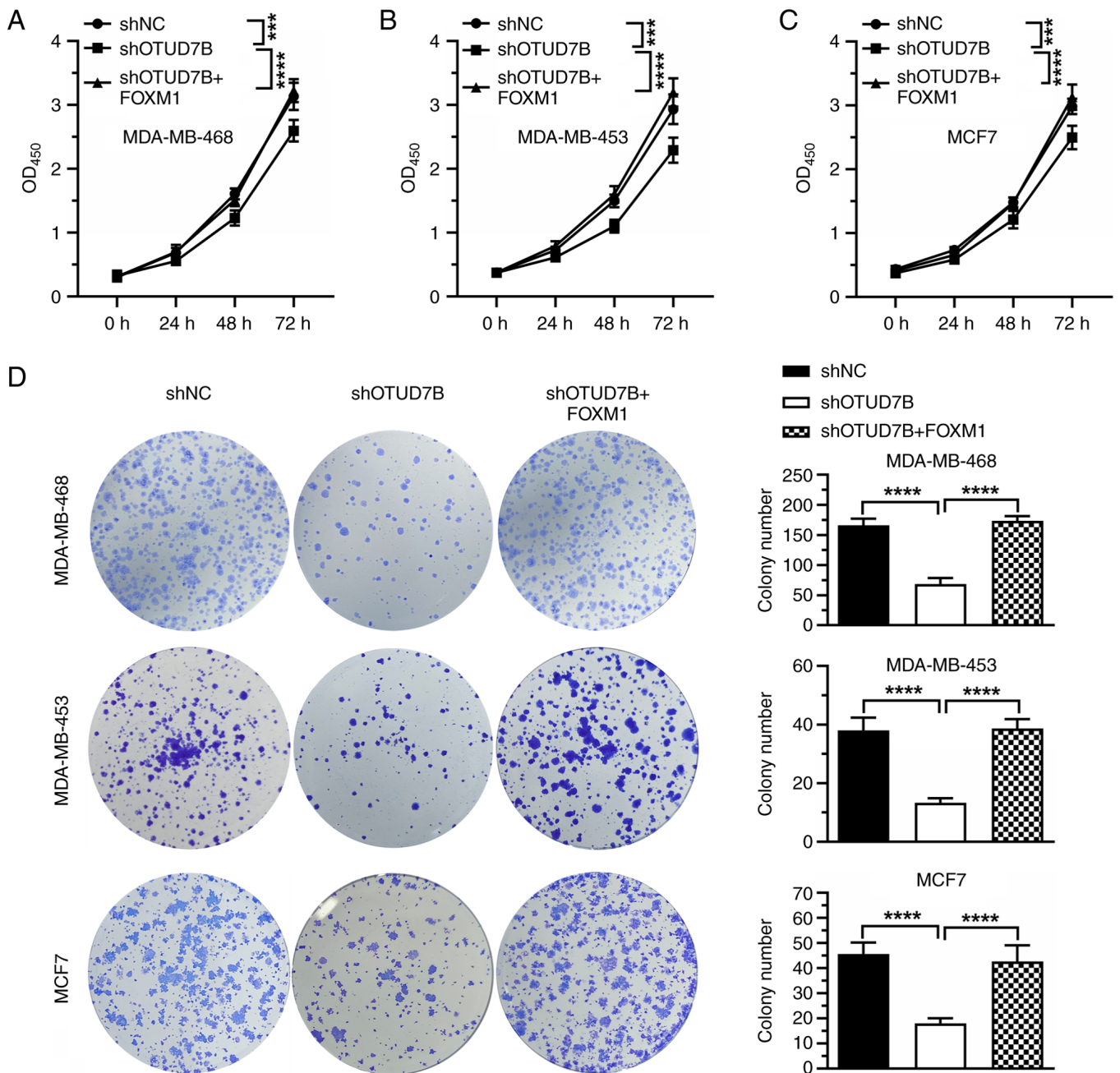
Figure 7. Overexpression of FOXM1 reverses the decreased proliferation of OTUD7B-knockdown breast cancer cells. Cell Counting Kit 8 analysis of cell proliferation in (A) MDA-MB-468, (B) MDA-MB-453 and (C) MCF7 cells. (D) Colony formation assay of MDA-MB-468, MDA-MB-453 and MCF7 cells. ***P<0.001 and ****P<0.0001. OTUD7B, OTU domain-containing 7B; FOXM1, forkhead box protein M1; NC, negative control; sh, short hairpin.

regulate the proliferation and stemness of these three cell lines and suggested that drugs targeting OTUD7B or FOXM1 may have the potential to treat triple-negative breast cancer and double-positive breast cancer.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DZ and WC were involved in conceptualization of the present study. XF performed the statistical analysis. HW and SH

performed the experiments. JX obtained the informed consent from the patients and collected the cancer tissues. DZ wrote the original draft. HW, SH, XF, WC and JX revised the manuscript. DZ supervised the study and was involved in project administration. HW and DZ acquired the funding. HW, SH and DZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript and have agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

The study procedures were approved by The Ethics Committees of Sanming First Hospital Affiliated to Fujian Medical University [Sanming, China, approval no. 2020(12)] and written informed consent was obtained from all of the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M, et al: Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26: 1275-1281, 2008.
2. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y and Pietenpol JA: Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 121: 2750-2767, 2011.
3. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J and Cardoso F: Breast cancer. Nat Rev Dis Primers 5: 66, 2019.
4. Waks AG and Winer EP: Breast cancer treatment: A review. JAMA 321: 288-300, 2019.
5. Barzaman K, Karami J, Zarei Z, Hosseinzadeh A, Kazemi MH, Moradi-Kalbolandi S, Safari E and Farahmand L: Breast cancer: Biology, biomarkers, and treatments. Int Immunopharmacol 84: 106535, 2020.
6. Mevissen TET and Komander D: Mechanisms of deubiquitinase specificity and regulation. Annu Rev Biochem 86: 159-192, 2017.
7. Lai KP, Chen J and Tse WKF: Role of deubiquitinases in human cancers: Potential targeted therapy. Int J Mol Sci 21: 2548, 2020.
8. Li S, Zhang H and Wei X: Roles and Mechanisms of Deubiquitinases (DUBs) in breast cancer progression and targeted drug discovery. Life (Basel) 11: 965, 2021.
9. Pareja F, Ferraro DA, Rubin C, Cohen-Dvashi H, Zhang F, Aulmann S, Ben-Chetrit N, Pines G, Navon R, Crosetto N, et al: Deubiquitination of EGFR by Cezanne-1 contributes to cancer progression. Oncogene 31: 4599-4608, 2012.
10. Wang JH, Wei W, Guo ZX, Shi M and Guo RP: Decreased Cezanne expression is associated with the progression and poor prognosis in hepatocellular carcinoma. J Transl Med 13: 41, 2015.
11. Zhang B, Wang H, Yang L, Zhang Y, Wang P, Huang G, Zheng J, Ren H and Qin S: OTUD7B and NIK expression in non-small cell lung cancer: Association with clinicopathological features and prognostic implications. Pathol Res Pract 212: 893-898, 2016.
12. Wang JH, Zhong XP, Zhang YF, Wu XL, Li SH, Jian PE, Ling YH, Shi M, Chen MS, Wei W, et al: Cezanne predicts progression and adjuvant TACE response in hepatocellular carcinoma. Cell Death Dis 8: e3043, 2017.
13. Lei S, He Z, Chen T, Guo X, Zeng Z, Shen Y and Jiang J: Long noncoding RNA 00976 promotes pancreatic cancer progression through OTUD7B by sponging miR-137 involving EGFR/MAPK pathway. J Exp Clin Cancer Res 38: 470, 2019.
14. Qiu S, Liu Y, Gui A, Xia Z, Liu W, Gu JJ, Zuo J, Yang L and Zhang Q: Deubiquitinase OTUD7B is a potential prognostic biomarker in diffuse large B-cell lymphoma. J Cancer 13: 998-1004, 2022.
15. Tang J, Wu Z, Tian Z, Chen W and Wu G: OTUD7B stabilizes estrogen receptor α and promotes breast cancer cell proliferation. Cell Death Dis 12: 534, 2021.
16. Gong Z, Li A, Ding J, Li Q, Zhang L, Li Y, Meng Z, Chen F, Huang J, Zhou D, et al: OTUD7B Deubiquitinates LSD1 to Govern Its Binding Partner Specificity, Homeostasis, and Breast Cancer Metastasis. Adv Sci (Weinh) 8: e2004504, 2021.
17. Chiu HW, Lin HY, Tseng IJ and Lin YF: OTUD7B upregulation predicts a poor response to paclitaxel in patients with triple-negative breast cancer. Oncotarget 9: 553-565, 2017.
18. Chen H, Yu Y, Yang M, Huang H, Ma S, Hu J, Xi Z, Guo H, Yao G, Yang L, et al: YTHDF1 promotes breast cancer progression by facilitating FOXM1 translation in an m6A-dependent manner. Cell Biosci 12: 19, 2022.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
20. Cui CP, Zhang Y, Wang C, Yuan F, Li H, Yao Y, Chen Y, Li C, Wei W, Liu CH, et al: Dynamic ubiquitylation of Sox2 regulates proteostasis and governs neural progenitor cell differentiation. Nat Commun 9: 4648, 2018.
21. Sher G, Masoodi T, Patil K, Akhtar S, Kuttikrishnan S, Ahmad A and Uddin S: Dysregulated FOXM1 signaling in the regulation of cancer stem cells. Semin Cancer Biol 86: 107-121, 2022.
22. Bonacci T, Suzuki A, Grant GD, Stanley N, Cook JG, Brown NG and Emanuele MJ: Cezanne/OTUD7B is a cell cycle-regulated deubiquitinase that antagonizes the degradation of APC/C substrates. EMBO J 37: e98701, 2018.
23. Evans PC, Taylor ER, Coadwell J, Heyninck K, Beyaert R and Kilshaw PJ: Isolation and characterization of two novel A20-like proteins. Biochem J 357: 617-623, 2001.
24. Evans PC, Smith TS, Lai MJ, Williams MG, Burke DF, Heyninck K, Kreike MM, Beyaert R, Blundell TL and Kilshaw PJ: A novel type of deubiquitinating enzyme. J Biol Chem 278: 23180-23186, 2003.
25. Enesa K, Zakkar M, Chaudhury H, Luong le A, Rawlinson L, Mason JC, Haskard DO, Dean JL and Evans PC: NF-kappaB suppression by the deubiquitinating enzyme Cezanne: A novel negative feedback loop in pro-inflammatory signaling. J Biol Chem 283: 7036-7045, 2008.
26. Zhu QZ, Liu HY, Zhao XY, Qiu LJ, Zhou TT, Wang XY, Chen HP and Xiao ZQ: DJ-1 activates the noncanonical NF-κB pathway via interaction with Cezanne to inhibit the apoptosis and promote the proliferation of Ishikawa cells. Mol Biol Rep 48: 6075-6083, 2021.
27. Lin DD, Shen Y, Qiao S, Liu WW, Zheng L, Wang YN, Cui N, Wang YF, Zhao S and Shi JH: Upregulation of OTUD7B (Cezanne) promotes tumor progression via AKT/VEGF pathway in lung squamous carcinoma and adenocarcinoma. Front Oncol 9: 862, 2019.
28. Prasad S, Ramachandran S, Gupta N, Kaushik I and Srivastava SK: Cancer cells stemness: A doorstep to targeted therapy. Biochim Biophys Acta Mol Basis Dis 1866: 165424, 2020.
29. Ibragimova M, Tsyganov M and Litviakov N: Tumour stem cells in breast cancer. Int J Mol Sci 23: 5058, 2022.
30. Sun HL, Men JR, Liu HY, Liu MY and Zhang HS: FOXM1 facilitates breast cancer cell stemness and migration in YAP1-dependent manner. Arch Biochem Biophys 685: 108349, 2020.
31. Modi A, Purohit P, Roy D, Vishnoi JR, Pareek P, Elhence P, Singh P, Sharma S, Sharma P and Misra S: FOXM1 mediates GDF-15 dependent stemness and intrinsic drug resistance in breast cancer. Mol Biol Rep 49: 2877-2888, 2022.
32. Tsao AN, Chuang YS, Lin YC, Su Y and Chao TC: Dinaciclib inhibits the stemness of two subtypes of human breast cancer cells by targeting the FoxM1 and Hedgehog signaling pathway. Oncol Rep 47: 105, 2022.
33. Zhang P, Xiao Z, Wang S, Zhang M, Wei Y, Hang Q, Kim J, Yao F, Rodriguez-Aguayo C, Ton BN, et al: ZRANB1 Is an EZH2 deubiquitinase and a potential therapeutic target in breast cancer. Cell Rep 23: 823-837, 2018.
34. Xiao H, Claret FX and Shen Q: The novel Jab1 inhibitor CSN5i-3 suppresses cell proliferation and induces apoptosis in human breast cancer cells. Neoplasma 66: 481-486, 2019.

35. Song Z, Tu X, Zhou Q, Huang J, Chen Y, Liu J, Lee S, Kim W, Nowsheen S, Luo K, *et al*: A novel UCHL3 inhibitor, perifosine, enhances PARP inhibitor cytotoxicity through inhibition of homologous recombination-mediated DNA double strand break repair. Cell Death Dis 10: 398, 2019.
36. Liao Y, Xia X, Liu N, Cai J, Guo Z, Li Y, Jiang L, Dou QP, Tang D, Huang H, *et al*: Growth arrest and apoptosis induction in androgen receptor-positive human breast cancer cells by inhibition of USP14-mediated androgen receptor deubiquitination. Oncogene 37: 1896-1910, 2018.
37. Ma A, Tang M, Zhang L, Wang B, Yang Z, Liu Y, Xu G, Wu L, Jing T, Xu X, *et al*: USP1 inhibition destabilizes KPNA2 and suppresses breast cancer metastasis. Oncogene 38: 2405-2419, 2019.
38. He J, Lee HJ, Saha S, Ruan D, Guo H and Chan CH: Inhibition of USP2 eliminates cancer stem cells and enhances TNBC responsiveness to chemotherapy. Cell Death Dis 10: 285, 2019.
39. Ahram M, Abdullah MS, Mustafa SA, Alsafadi DB and Battah AH: Androgen downregulates desmocollin-2 in association with induction of mesenchymal transition of breast MDA-MB-453 cancer cells. Cytoskeleton (Hoboken NJ) 78: 391-399, 2021.
40. Liubomirski Y, Lerrer S, Meshel T, Rubinstein-Achiasaf L, Morein D, Wiemann S, Körner C and Ben-Baruch A: Tumor-stroma-inflammation networks promote pro-metastatic chemokines and aggressiveness characteristics in triple-negative breast cancer. Front Immunol 10: 757, 2019.
41. Pruteanu LL, Braicu C, Módos D, Jurj MA, Raduly LZ, Zǎnoagǎ O, Magdo L, Cojocneanu R, Paşca S, Moldovan C *et al*: Targeting cell death mechanism specifically in triple negative breast cancer cell lines. Int J Mol Sci 23: 4784, 2022.