Cancer Cell International

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OTUD3 inhibits breast cancer cell metastasis by regulating TGF-β pathway through deubiquitinating SMAD7

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Abstract

Background Breast cancer (BRCA) is the most common malignant tumor in women, and distant metastasis is an important cause of death. Epithelial mesenchymal transition (EMT) is an important factor in tumor cell metastasis, in which TGF-β signaling pathway plays an important role. SMAD7 can inhibit TGF-β pathway. Previously, we found that ovarian tumor domain-containing protein 3(OTUD3) could maintain the stability of multiple molecules through deubiquitination. In this study, multiple experiments were conducted to verify whether OTUD3 can inhibit TGF-β pathway by deubiquitinating SMAD7.

Methods Firstly, bioinformatics was used to search the expression of OTUD3 in breast cancer and its correlation with SMAD7 in the TCGA database. The correlation between the protein and mRNA expression levels of OTUD3 and SMAD7 in multiple BRCA cell lines was verified. Also, the OTUD3 and SMAD7 expression in human BRCA samples and its influence on prognosis were verified by immunohistochemical experiments. Then, the CO-IP experiment was performed by transfecting OTUD3 and SMAD7 in HEK293T cells to confirm whether OTUD3 could maintain SMAD7 protein stability through deubiquitination. Furthermore, luciferase reporting assay, in vitro protein interaction, and transwell assay were used to verify whether OTUD3 could inhibit TGF-β pathway by deubiquitinating SMAD7 and affect cell invasion. Western blot and RT-qPCR were used to detect the correlation between OTUD3 and molecules regulated by the TGF-β pathway. Finally, the effect of OTUD3 on tumor cells was determined by 3D matrigel cell culture.

Results The expression of OTUD3 was low in BRCA and positively correlated with SMAD7. Cytological experiments and immunohistochemistry confirmed that OTUD3 was positively correlated with the expression of SMAD7, and the patients with a low expression of OTUD3 had a short recurrence-free survival (RFS). Cell experiments confirmed that OTUD3 could regulate the TGF-β pathway by deubiquitinating SMAD7, which affected EMT and inhibited cell invasion. OTUD3 was found to inhibit the stemness of tumor cells by 3D matrigel cell culture.

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Conclusions Our findings indicated OTUD3 inhibited BRCA metastasis associated with TGF-β signaling by deubiquitination to stabilize SMAD7 protein levels.

Keywords OTUD3, TGF-β, SMAD7

Background

According to the incidence rate and prevalence data of tumors in 185 countries released by the International Agency for Research on Cancer (IARC) in 2020, breast cancer (BRCA) is the most common malignant tumor in women. With the progress of medicine, the treatment of BRCA is diversified and individualized, including surgery, radiotherapy, chemotherapy, endocrine therapy, and immunotherapy. Nevertheless, because of the high incidence rate, BRCA is still the leading cause of death for women [1], and distant metastasis accounts for the majority of cancer deaths [2], in which the 5-year survival rate of patients with distant metastasis is less than 30% [3]. Therefore, an in-depth exploration of the molecular mechanism of BRCA proliferation and metastasis can not only improve the pathogenesis of BRCA but also provide new ideas for the preparation of targeted drugs for BRCA metastasis.

Epithelial mesenchymal transition (EMT) is a process that involves embryonic development and pathological features of tumors. It refers to the loss of E-cadherin and other epithelial cell junction components in cells, producing mesenchymal cell cytoskeleton, promoting motility and invasiveness [4], thereby facilitating tumor invasion and spread. Studies by multiple scholars have shown that TGF-β is an effective EMT inducer, and abundant TGF-β can be found in cells with EMT at the forefront of human tumor metastasis [5, 6].

In most cells, TGF-β always combines with latency-associated protein (LAP) to form a complex and lacks biological activity [7, 8]. When the signal is activated, TGF-β will separate from the complex and subsequently associate with the receptor TGF-βR1/2 on the cell membrane. The binding could induce a cascade reaction by phosphorylating SMAD2/3 protein to transfer signals from the cell membrane to the nucleus and then regulate the expression of related target genes, such as SNAIL, SLUG, and TWIST, to promote EMT [9, 10]. SMAD7 has been shown to interact with SMAD2/3 and mediate their polyubiquitination and degradation and antagonize the execution of TGF-β signaling events [11, 12].

Recently, we found that ovarian tumor domain-containing protein 3 (OTUD3) was a tumor suppressor by inhibiting metastasis. The laboratory work revealed the regulatory mechanism of OTUD3 was to inhibit SMAD7 ubiquitination leading to the TGF-β signal block.

Methods

Data and software availability

We downloaded STAR-counts data and corresponding clinical information for BRCA specimens and corresponding normal tissues from the TCGA database (https://portal.gdc.cancer.gov). We then extracted data in TPM format and performed normalization using the $\log_2(TPM+1)$ transformation. Tests included T test, the Wilcoxon rank sum test, and Welch one-way ANOVA. The correlation of OTUD3 and SMAD7 mRNA expression was analyzed using the Spearman coefficient. All the study was carried out and visualized by the R software (3.6.3 version) with the package ggplot2(3.3.6), stats (4.2.1), and car (3.1-0). The Kaplan-Meier Plotter database (http://kmplot.com) was used to assess the impact of OTUD3 on patient survival [13]. Patients with BRCA were divided into two groups based on the expression level of OTUD3. Cox regression models were built to estimate hazard ratios (HRs), and survival curves were plotted.

Cell culture

HEK293T, SK-BR-3, MDA-MB-453, ZR-75-1, MCF-7, Hs-578T and MDA-MB-231 cell lines were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100U penicillin/streptomycin. MCF10A cell line and a particular medium (CM-0525) were purchased from Procell Life Science &Technology Co. Ltd., China. All cell lines mentioned above were incubated at +37 °C, 95% humidity, and 5% CO_2. The absence of mycoplasma contamination was verified by the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Patient samples and immunohistochemistry (IHC)

This study was approved by the Qilu Hospital of Shandong University (Qingdao) Research Ethics Committee (Approval no. KYLL-2021028, date of approval: 28 January 2021). Two hundred tumors and adjacent normal tissues with clinical records were collected from Qilu Hospital of Shandong University (Qingdao). All patients underwent surgical resection at Qilu Hospital of Shandong University (Qingdao). All patients gave informed consent.

Paraffin-embedded sections were dewaxed and dehydrated in an ethanol gradient. Heating slides in ethylenediaminetetraacetic acid (EDTA, pH 8.0) for 10 min to perform antigen retrieval, then slides were washed three times with phosphate-buffered saline (PBS). Sections

were incubated in antibodies overnight at 4 °C; after washing with PBS, secondary antibodies were dropped and incubated at room temperature for 1 h. The sections were then stained with diaminobenzidine (DAB) and visualized. The following primary antibodies were used: anti-OTUD3 antibody (HPA028544; Sigma; 1:200), anti-SMAD7 antibody (sc-365846; Santa; 1:100). The score of the IHC signals was blindly judged by two independent pathologists, and the results were determined according to the extent (percent of stained cells) and intensity of staining [14]. Immunohistochemical staining was graded as follows: intensities (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and the percentages of positive cells (0 = 0%, 1 = 1–24%, 2 = 25–49%, 3 = 50–74%, and 4 = 75–100%). The IHC scores were determined by multiplying IHC intensity with a fraction of positive cells.

Transcriptional regulation reporter assay

The HEK293T cells were seeded in 24-well plates in an antibiotic-free medium 24 h before transfection. On the day of transfection, the medium was changed prior to adding the indicated plasmids and calcium phosphate. The luciferase activity was measured using a VICTOR Light luminometer (PerkinElmer). Results were normalized to renilla transfection controls. Each experiment was repeated three times, and the data represent the mean ± SD of three independent experiments.

Immunoblot (IB) and Immunoprecipitation (IP) analyses

Cultured cells were washed with PBS and harvested by scraping into a lysis buffer (Pierce) supplemented with protease inhibitors and phosphatase inhibitors (Sigma). The Lysates were centrifuged at $1.2 \times 10^3 g$ for 15 min, and the supernatant was transferred to a new tube. Protein concentrations were quantified using Bradford assay (Bio-Rad), and equal amounts of lysate were used for immunoprecipitation. The lysates were incubated on a rotator with the anti-FLAG M2 magnetic beads for 1.5 h at 4 °C. Beads were washed three times with IP buffer containing protease inhibitors for 10 min each wash, and then the recovered immuno-complexes were washed five times with NETN buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris, and 1 mM EDTA). The immunoprecipitated proteins were processed for SDS-PAGE following standard procedures.

Plasmids and antibodies

The human deubiquitylase library was purchased from OriGene. Myc-RNF12, Myc-OTUD3-wt/CA, HA-ubiquitin and Flag-SMAD7 were cloned into the pCMV-Myc or pFlag-CMV-2 vectors as indicated. His-SMAD7 was cloned into the pET28a vector, and GST-RNF12 was cloned into the pGEX-4T-2 vector.

Primary antibodies used for IB in this study: anti-OTUD3 antibody (HPA028544; Sigma; 1:500), anti-SMAD7 antibody (sc-365846; Santa;1:800), anti-SMAD4 antibody (46535 S; Cell Signaling Technology; 1:200), anti-SMAD2/3 antibody (5678 S; Cell Signaling Technology; 1:50), anti-Phospho-SMAD2 antibody (18338 S; Cell Signaling Technology; 1:100), anti-Myc-Tag antibody (5678 S; Cell Signaling Technology; 1:250), anti-Flag-Tag antibody (14793 S; Cell Signaling Technology; 1:50), anti-Actin antibody (ab179467; Abcam; 1:40), anti-N-cadherin antibody (13116 S; Cell Signaling Technology; 1:50), anti-Vimentin antibody (ab137321; Abcam; 1:100), anti-Fibronectin antibody (26836 S; Cell Signaling Technology; 1:100). For the detection of proteins with similar or the same molecular weight, after the first chemiluminescence, the original antibody was stripped and the membrane was incubated with other antibodies.

Lentiviral infection and generation of stable cell lines

Lentiviral vectors (GV112) encoding short-hairpin RNA (shRNA) to OTUD3 along with a non-silencing control vector were designed and synthesized by Shanghai GeneChem Co., Ltd (Shanghai, China). Lentiviral infections of HEK293T, SK-BR-3, MDA-MB-231 and MCF10A cell lines were performed following the manufacturer's instructions. The successfully infected cells were screened by puromycin. Clones were picked, expanded, and screened for knockdown by western blot. The shRNA sequences were described as follows:

OTUD3 no. 1: 5'-TGGAAATCAGGGCTTAAAT-3';
no. 2, 5'-GAGTTACACATCGCATATC-3';
the non-targeting control:5'-TTCTCCGAACGTGTCACGT-3'.

Single guide RNAs (sgRNAs) design and OTUD3 disruption by CRISPR/Cas9

The guide RNA (gRNA) target sites were designed with an online tool, ZIFIT Targeter (http://zifit.partners.org/ZiFiT/CSquare9Nuclease.aspx). The OTUD3 sgRNA oligos and SMAD7 sgRNA oligos were purchased from Sangon and ligased into the lentiCRISPRv2 plasmid. To generate the virus, the plasmid carrying the sgRNA oligos was co-transfected into HEK293T cells with packaging plasmid and helper plasmid. The HEK293T, MDA-MB-231 and MCF 10 A cells were transiently transfected with two CRISPR/Cas9 constructs targeting OTUD3. The next day, cells were selected with puromycin for two days and subcloned to form single colonies. The obtained clones were validated by qPCR and immunoblot using an antibody against OTUD3.

The sgRNA sequences were described as follows:

OTUD3 sg1: 5'-ATTCCGCCGCTCCTTGGCCA-3';
sg2: 5'-CAAGGAGCGGCGGAATCGGC-3';
SMAD7 sg: 5'- CGCGCGGCCCGCGCCCTGCG-3'.

RT-qPCR

Total RNA was extracted with Trizol (Invitrogen). Total RNA (1 μg) was reverse transcribed into cDNA using HiScript II Q RT SuperMix (Vazyme Biotech, Nanjing, China, P212-03). A 20 μl volume reaction consisted of 1 μl reverse transcription product and 250 nM of each primer.

For qPCR, technical triplicates of each of at least three biological replicates were run for 40 cycles as 20 μl reactions using Applied Biosystems StepOne 96-well System. Gene expression was calculated relative to GAPDH using the $2^{-\Delta\Delta CT}$ formula, and fold change in gene expression was calculated relative to the average gene expression of control groups. The primers used for the indicated gene products are described in Supplementary Table 1.

Ubiquitylation and deubiquitylation assay

For ubiquitylation reaction, the substrates including ubiquitin-activating enzyme E1, E2 (Ube1 40 nM), Ubiquitin (8 μM), UbcH6 (0.7 μM), 5 μM His-SMAD7, 5 μM GST-RNF12, and 5 μM OTUD1-wt/CA proteins were incubated at 30 °C in buffer containing 25 mM Tris HCl, pH 7.4, 2mM ATP, 5mM $MgCl_2$, 5mM $MnCl_2$ and 0.1mM DTT for 1 h. For the deubiquitylation reaction, the reactant containing purified OTUD3 protein and purified poly-HA-ubiquitinated Flag-SMAD7 was performed in deubiquitylation buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1mM EDTA, 10 mM DTT, 5% glycerol) overnight at 16 °C. Reaction mixtures were stopped by adding SDS-PAGE loading buffer followed by heat denaturation at 95 °C for the indicated time. Reaction products were detected by immunoblotting using specific antibodies.

Protein purification and pulldown assays

After transfection with HA-ubiquitin, His-SMAD7, and GST-RNF12, the cells were used to obtain the corresponding protein. The HEK293T cells were transfected with OTUD1-wt/CA plasmids and immunoprecipitated with α-Flag-M2 resin (Sigma) overnight. Then Reaction mixtures were eluted by Flag peptide (Sigma, 1 mg/ml in 50mM HEPES (pH 7.5), 100 mM NaCl, 0.1% NP40, 5% glycerol).

The purification of His-SMAD7 protein was under nickel bead pulldown assay. The eluted cell samples were resuspended in lysis buffer (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, and 10mM imidazole). Protein was extracted by vigorous sonication and freeze/thaw cycles. Cell debris was removed by centrifugation. The supernatant was used and incubated with Talon beads (BD Biosciences) in the presence of 20 mM imidazole. Bead washing was performed and washed three times with a lysis buffer lacking imidazole. Then, proteins were eluted with lysis buffer supplemented with 200 mM imidazole.

Protein–protein interactions

In vitro protein–protein interactions were assayed using IB for coprecipitating proteins.

Using binding buffer (25 mM HEPES [pH 7.5], 100 mM KCl, 2 mM $MgCl_2$, 0.1% NP40, and 5% glycerol) for the dilution of target proteins. Immunoprecipitation between the target protein and anti-OTUD3 or anti-SMAD7 antibody was performed at 4℃ for 3 h. Then, protein A-Sepharose beads were added and incubated for 1 h. The mix was washed three times with the same buffer before IB.

Organoids were grown in 3D matrigel cultures

Cells were cultured in Growth Factor Reduced Basement Membrane Matrix (Corning) in reference to the methods described in a previous study [15]. The cell morphology was observed with an inverted microscope, spheres were counted, and pictures were taken.

Transwell invasion assay

The invasion assay was performed using the invasion chamber (Corning). The normal-growth MDA-MB-231 cells were trypsinized, washed in PBS, and resuspended in serum-free medium. About 5×10^4 cells were seeded into the upper chamber of Matrigel Invasion Chambers and Control inserts (Corming, #354480 and #3354578, respectively) containing 8 μm pores in serum-free medium. The lower chamber contained a 10% FBS culture medium. 24 h post-seeding, the cells that had invaded into the wells. Cleaning the inner surface of the upper chambers without disturbing the outer side. After air drying, the invasive cells were stained with crystal violet solution (0.1% crystal violet, 20% methanol in water) for visualization. Images were taken with an inverted microscope.

Statistical analyses

Each experiment was performed at least three times. Data are represented as mean ± SD unless otherwise specified. Data are presented as the means of experimental replicates with their respective standard deviations (SD). Student's two-tailed unpaired *t*-test or specified analytic method was used for analyses. p values < 0.05 were considered significant (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Baseline characteristics of BRCA patients in the TCGA-BRCA database

We collected the patient's clinicopathological data, including age, race, menopause status, T stage, N stage, M stage, pathologic stage, histological type, estrogen receptor (ER) status, progesterone receptor (PR) status, HER2 status and PAM50 subtype, anatomic neoplasm subdivisions. Then, we performed logistic analysis on

the mRNA expression level of OTUD3 and these clinical characteristics to detect the correlation. As shown in Table 1, OTUD3 expression level was correlated with histological type, ER status, PR status, and PAM50 subtype and had statistical differences ($p < 0.05$).

The expression level of OTUD3 was down-regulated in BRCA tissues

In the TCGA-BRCA database, the mRNA expression level of OTUD3 was down-regulated in tumor tissues than adjacent normal tissues ($p < 0.001$) (Fig. 1A). In addition, we obtained a similar result in paired sample analysis ($p < 0.001$) (Fig. 1B). It is lower in tumor tissues than that in normal tissues. However, there was no significant difference in the expression level across the pathological stage (Fig. 1C). In analyzing the expression correlation between OTUD3 and SMAD7, we found that the expression of these two molecules was positively correlated significantly ($p < 0.001$) (Fig. 1D).

OTUD3 expression level correlated with SMAD7 positively and is associated with poor prognosis

The above bioinformatic results suggested that OTUD3 showed low expression in BRCA and was positively correlated with SMAD7 expression. To verify the above results, we extracted RNA and protein from normal mammary epithelial cells, MCF10A, and multiple BRCA cell lines (SK-BR-3, MDA-MB-453, ZR-75-1, MCF-7, Hs-578T, and MDA-MB-231). The mRNA and protein expression levels of OTUD3 and SMAD7 were detected, respectively. As shown in Fig. 2A, OTUD3 and SMAD7 protein expression levels were generally lower in BRCA cell lines. At the same time, the expression levels of OTUD3 and SMAD7 mRNA were also lower in BRCA cell lines, and the expression trends of the two molecules were similar (Fig. 2B-C). Then, we collected 200 breast cancer samples from patients, all of which were surgically removed and pathologically confirmed as invasive breast cancer, and we used them for tissue microarray analysis. Immunohistochemical (IHC) analysis of OTUD3 and SMAD7 levels showed a statistically significant positive correlation (Fig. 2D-F). In the Kaplan-Meier Plotter database, we found that patients with high OTUD3 tend toward a good recurrence-free survival (RFS) (Fig. 2G).

Based on the above results, we found that OTUD3 was related to SMAD7 expression. To further confirm the correlation between the two molecules, SK-BR-3 and MDA-MB-231 BRCA cell lines, both relatively highly expressed, were selected to knock down OTUD3. The results showed that the expression level of SMAD7 was decreased simultaneously. As shown, OTUD3 was positively correlated with SMAD7 (Fig. 2H).

A series of previous studies suggested that OTUD3 could inhibit the proliferation and metastasis of tumor cells. As a member of the OTU DUB family, we found the functional properties of OTUD3 in cancer mainly unexplored. Our previous research reported that OTUD3 could deubiquitinate and stabilize p53 to suppress tumor progression [16]. Based on these findings, we hypothesized that OTUD3 prevents breast cancer metastasis by de-ubiquitinate SMAD7.

OTUD3 maintains the stability of SMAD7 through deubiquitylation

In cells whether were treated with the proteasome inhibitor MG132 or not, we found that overexpression wild-type OTUD3 (OTUD3-wt), instead of OTUD3 deubiquitinase-defective mutant (OTUD3-CA), could deubiquitinate SMAD7, including conjugation with Lysine 48 (Lys48)-linked polyubiquitin chains (as shown

Table 1 Logistic analysis of the correlation between OTUD3 mRNA expression and clinical characteristics in breast cancer

Characteristics	Total (*n*)	OR (95% CI)	*p* value
Age (> 60 vs. <= 60)	1087	0.967 (0.762–1.229)	0.786
Race (Black or African American&White vs. Asian)	997	1.558 (0.912–2.663)	0.105
Menopause status (Pre&Peri vs. Post)	976	0.944 (0.713–1.250)	0.690
Pathologic T stage (T2&T3&T4 vs. T1)	1084	0.762 (0.580–1.002)	0.052
Pathologic N stage (N1&N2&N3 vs. N0)	1068	0.848 (0.667–1.078)	0.178
Pathologic M stage (M1 vs. M0)	925	0.802 (0.329–1.954)	0.627
Pathologic stage (Stage III&Stage IV vs. Stage I&Stage II)	1063	0.857 (0.648–1.133)	0.278
Histological type (Infiltrating Ductal Carcinoma vs. Infiltrating Lobular Carcinoma&Infiltrating Carcinoma NOS&Medullary Carcinoma&Mixed Histology &Mucinous Carcinoma)	1034	0.733 (0.552–0.973)	0.031*
ER status (Positive vs. Negative&Indeterminate)	1039	1.494 (1.117–1.998)	0.007*
PR status (Positive vs. Negative&Indeterminate)	1038	1.360 (1.049–1.763)	0.020*
HER2 status (Positive vs. Negative&Indeterminate)	729	0.549 (0.383–0.788)	0.001*
PAM50 (Normal&LumA&LumB&Her2 vs. Basal)	1087	1.272 (0.932–1.736)	0.130
Anatomic neoplasm subdivisions (Left vs. Right)	1087	1.105 (0.871–1.401)	0.413

*$p < 0.05$ was considered significant

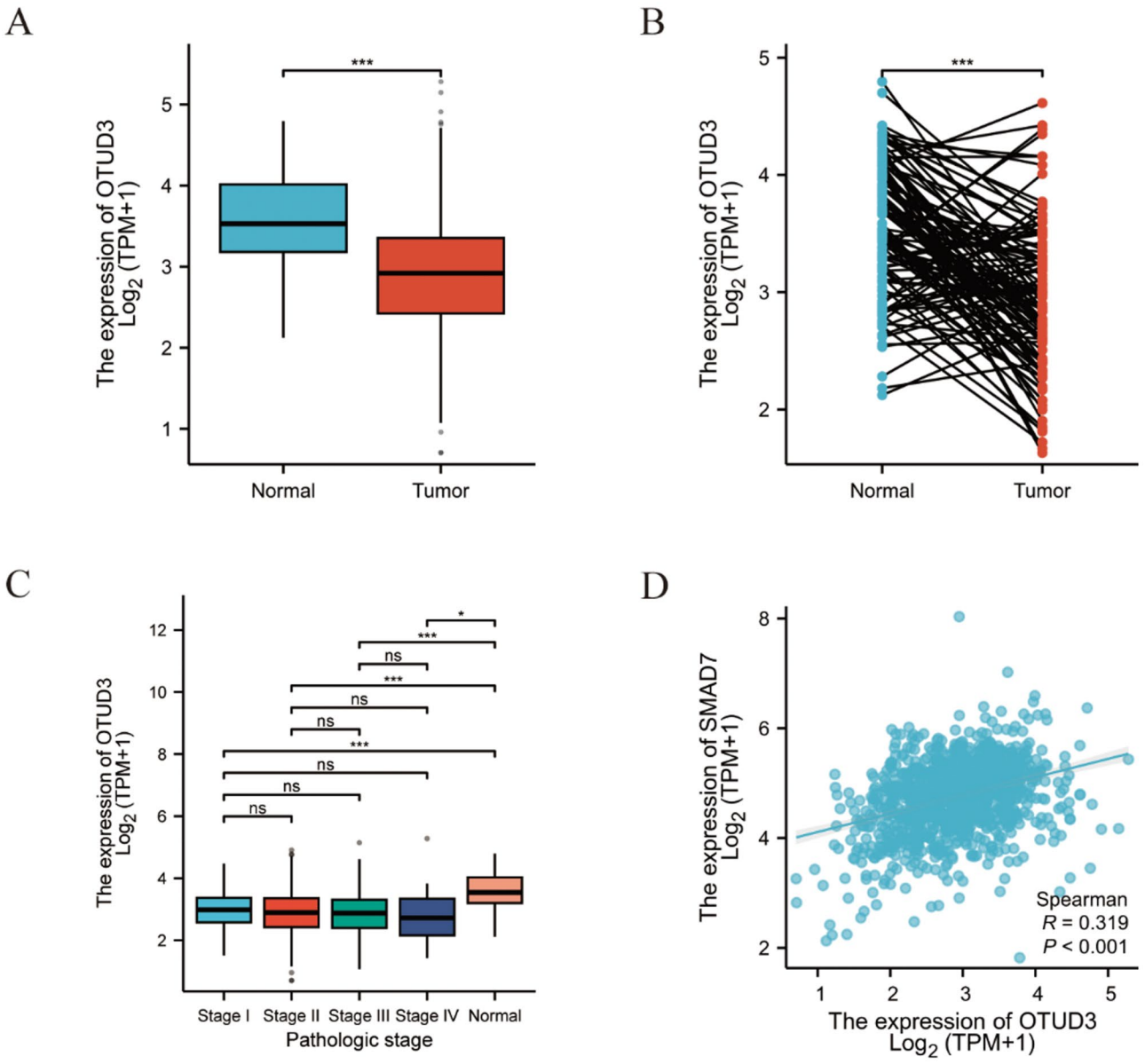

Fig. 1 Bioinformatics analysis of OTUD3 in the TCGA-BRCA database. **A-B** The results of unpaired (**A**) and paired analysis (**B**) both show that the expression level of OTUD3 mRNA in cancer tissues is lower than that in adjacent tissues. **C** There is no significant difference in the expression level of OTUD3 mRNA in different tumor stages, but it is lower than that in normal tissues. **D** The mRNA expression levels of OTUD3 and SMAD7 are positively correlated with statistical difference ($p < 0.001$)

by K48-linked specific antibodies (Fig. 3A). As RNF12 could promotes the polyubiquitination and degradation of SMAD7 [17], we observed this phenomenon in control cells, OTUD3-CA-expressing cells and OTUD3-wt-expressing cells separately. As revealed in Fig. 3B, RNF12 promoted SMAD7 polyubiquitination in both control and OTUD3-CA-expressing cells, but not in OTUD3-wt-expressing cells. In addition, at both the basal and RNF12-induced levels, the deficiency of endogenous OTUD3 promoted polyubiquitination of SMAD7 (Fig. 3C).

We created conditional knockout HEK293T cell lines for OTUD3 by CRISPR-Cas9. Endogenous SMAD7 was precipitated by anti-SMAD7 antibodies, and co-precipitated OTUD3 was examined in OTUD3$^{-/-}$ and OTUD3$^{+/+}$ cells. Compared to OTUD3$^{+/+}$cells, polyubiquitination of SMAD7 showed a significant increase in the OTUD3$^{-/-}$ cells, while the effect was reversed when we restored the expression of OTUD3 (Fig. 3D). To assess whether the circulation of SMAD7 was affected by OTUD3, we detected the turnover rate of SMAD7 protein. Such an experiment was shown in Fig. 3E; in cells overexpressing OTUD3-wt but not OTUD3-CA, the

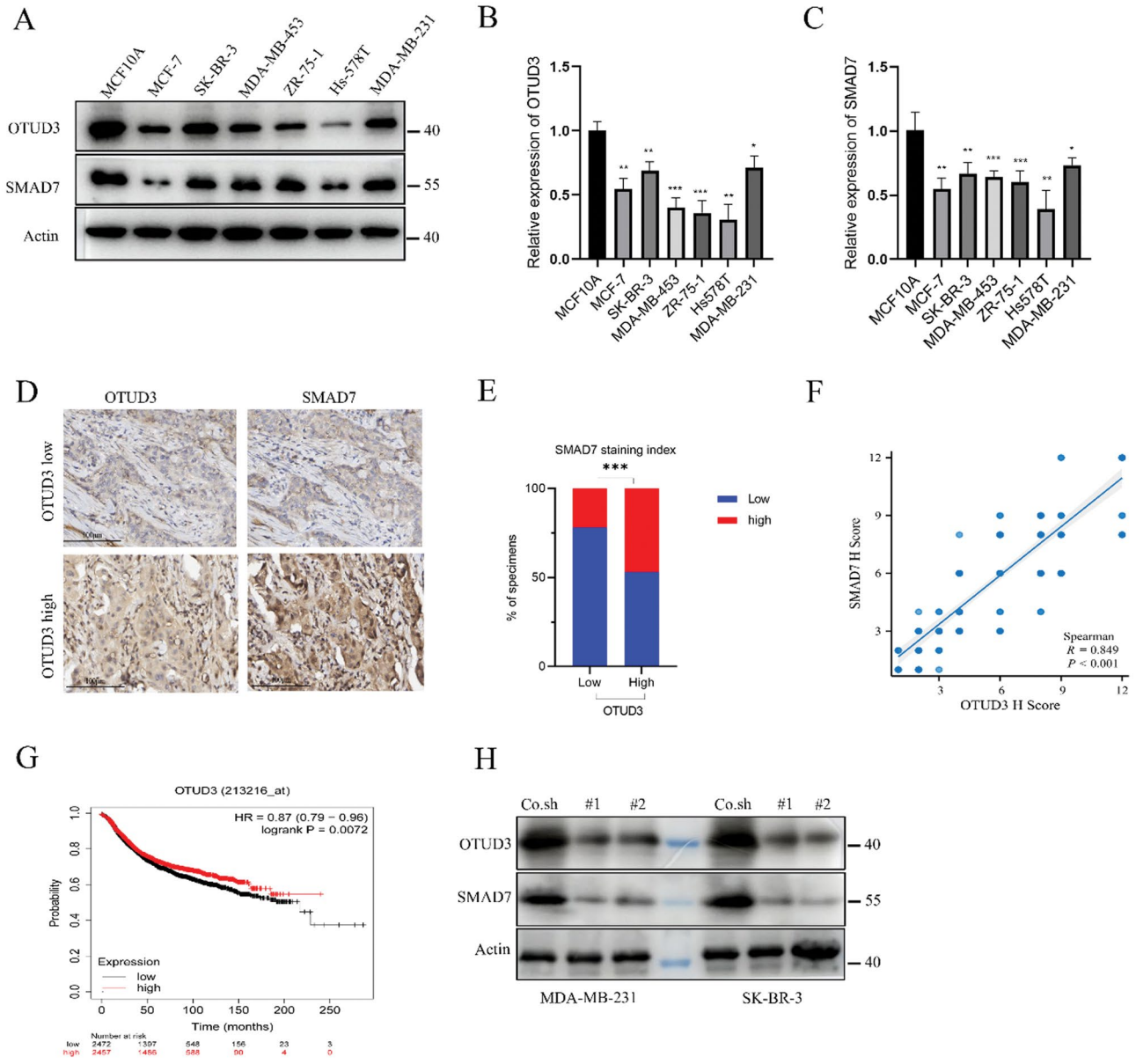

Fig. 2 Expression levels of OTUD3 and SMAD7 and the correlation of both in cells and tissues. **A** The protein levels of OTUD3 and SMAD7 in MCF10A and BRCA cells. **B-C** The expression levels of mRNA of OTUD3 (**B**) and SMAD7 (**C**) in MCF10A and BRCA cells. **D-F** OTUD3 and SMAD7 antibodies were successively used to stain the breast cancer tissue microarray with immunohistochemical staining and correlation analysis. **D** Representative pictures. **E** Percentage of high or low SMAD7 expression in tissues expressing high or low OTUD3. **F** The scatterplot showed that the expression levels of OTUD3 and SMAD7 were positively correlated ($p < 0.001$). **G** In Kaplan-Meier Plotter database, the recurrence-free survival (RFS) was positively correlated with the expression of OTUD3 ($p = 0.0072$). **H** Knockdown (#1,#2) of OTUD3 in MDA-MB-231 and SK-BR-3 cells. Western blotting was used to detect the protien levels of OTUD3 and SMAD7. Co.sh, non-targeting shRNA

protein stability of SMAD7 increased which was measured by cycloheximide (CHX) treatment. We, therefore, considered that endogenous OTUD3 deubiquitinated SMAD7 to maintain the stability of SMAD7.

OTUD3 inhibits TGF-β signal transduction pathway and EMT by deubiquitinating SMAD7 in vitro

Next, we investigated the molecular mechanism of the inhibition of TGF-β signals by OTUD3. As OTUD3 is a number of deubiquitinating enzymes (DUBs), the most likely mode of action was targeting to repressive factor of TGF-β signals, especially SMAD7. Previous studies pointed out that activation of TGF-β signal pathway via SMAD2/3 could be measured with ARE-Luc and CAGA-Luc transcriptional reporters [18, 19]. HEK293T were co-transfected with a CAGA-luciferase reporter and the OTUD3 overexpression vector. After stimulated by TGF-β, we found that the overexpression of

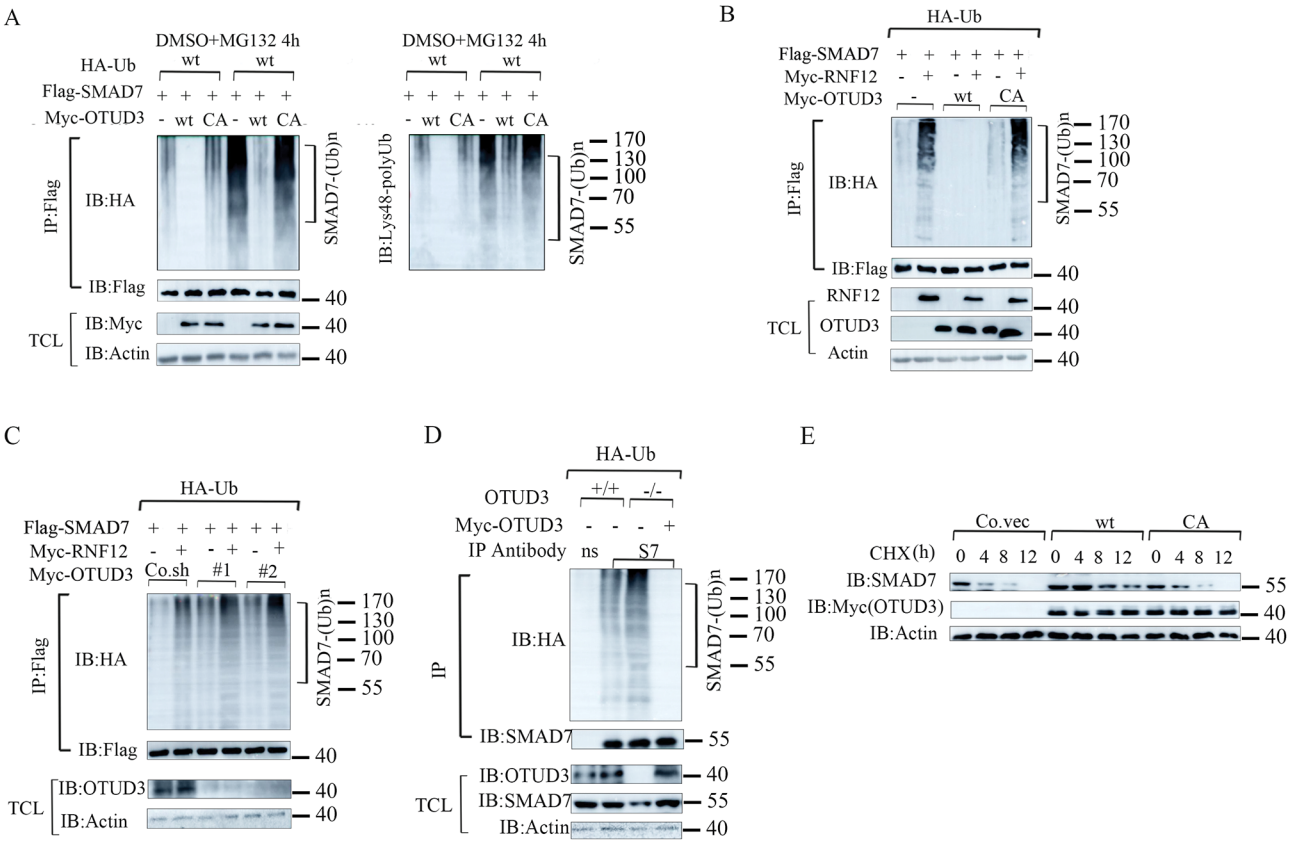

Fig. 3 OTUD3 maintains SMAD7 stability through deubiquitylation. **A** HEK293T cells were transfected with HA-Ub, Flag-SMAD7, Myc-OTUD3-wt/CA, and treated with MG132 (5 μM) for 4 h, while the control group was treated with DMSO. The total cell lysate (TCL) and immunoprecipitates were extracted for IB. HA-Ub labeled HEK293T cells were transfected with Flag-SMAD7, Myc-RNF12, and Myc-OTUD3-wt/CA (**B**) or OTUD3 shRNA (#1 and #2) (**C**), respectively. IB of TCL and immunoprecipitates were peformed. **D** Transfecting Myc-OTUD3 plasmid into HA-Ub labeled OTUD3-/- and OTUD3+/+ HEK293T cells respectively, then TCL and immunoprecipitates of transfected and control cells were extracted for IB, and endogenous polyubiquitination SMAD7 was immunoprecipitated with anti-SMAD7 antibody. **E** Cells expressing empty vector (Co.vec), Myc-OTUD3-wt or Myc-OTUD3-CA were treated by cycloheximide (CHX, 20 μg/ml) for different time, and lysates were collected for IB to detect the turnover rate of SMAD7 protein

OTUD3 could inhibit TGF-β signals in control cells, while almost no effect in SMAD7 deficient cells. It indicated that SMAD7 may be the main target of OTUD3 in TGF-β signal pathway (Fig. 4A). We further confirmed these findings in human BRCA cells. We constructed MDA-MB-231 OTUD3-KO and SMAD7-KO cell lines with CRISPR-Cas9 technology, respectively. In MDA-MB-231 OTUD3-KO cells, we found that overexpression of SMAD7 could inhibit cell invasion compared to control cells (Fig. 4B). While in subsequent MDA-MB-231 SMAD7-KO cells, we found that OTUD3 overexpression suppressed the invasion in control cells. At the same time, no significant inhibitory effect was observed in SMAD7 knockout cells (Fig. 4C). This phenomenon suggested the anti-metastatic activity of OTUD3 was mainly by the regulation of SMAD7. Consistent with this finding, the purified SMAD7 protein was found to interact with OTUD3 in vitro directly (Fig. 4D). Myc-OTUD3 and Flag-SMAD1-7 expressing vectors were co-transfected into HEK293T cells, and the cell lysate was subjected to co-immunoprecipitation. As shown in Fig. 4E, there was a strong binding between OTUD3 and SMAD7. Meanwhile, endogenous OTUD3 interacted with SMAD7 in breast cancer cells (Fig. 4F).

The expression of SMAD7 protein is regulated by post-translational modifications, including ubiquitination [20]. Firstly, we co-transfected Flag-tagged SMAD7 and HA-tagged ubiquitin vector into HEK293T cells and extracted affinity-purified SMAD7. Through HA antibody co-immunoprecipitation assay, we found that polyubiquitination was the primary modification of SMAD7. To test whether OTUD3 could directly deubiquitinate SMAD7, we conducted deubiquitylation experiments in vitro. It was found that purified OTUD3-wt could remove the poly-ubiquitin chains from SMAD7, while purified OTUD3-CA could not, and more than 50% of poly-ubiquitinated SMAD7 poly-ubiquitin chains could be cleaved within 20 min (Fig. 4G). Then we purified poly-ubiquitinated SMAD7 and observed the deubiquitylation ability of both OTUD3-wt and OTUD3-CA on

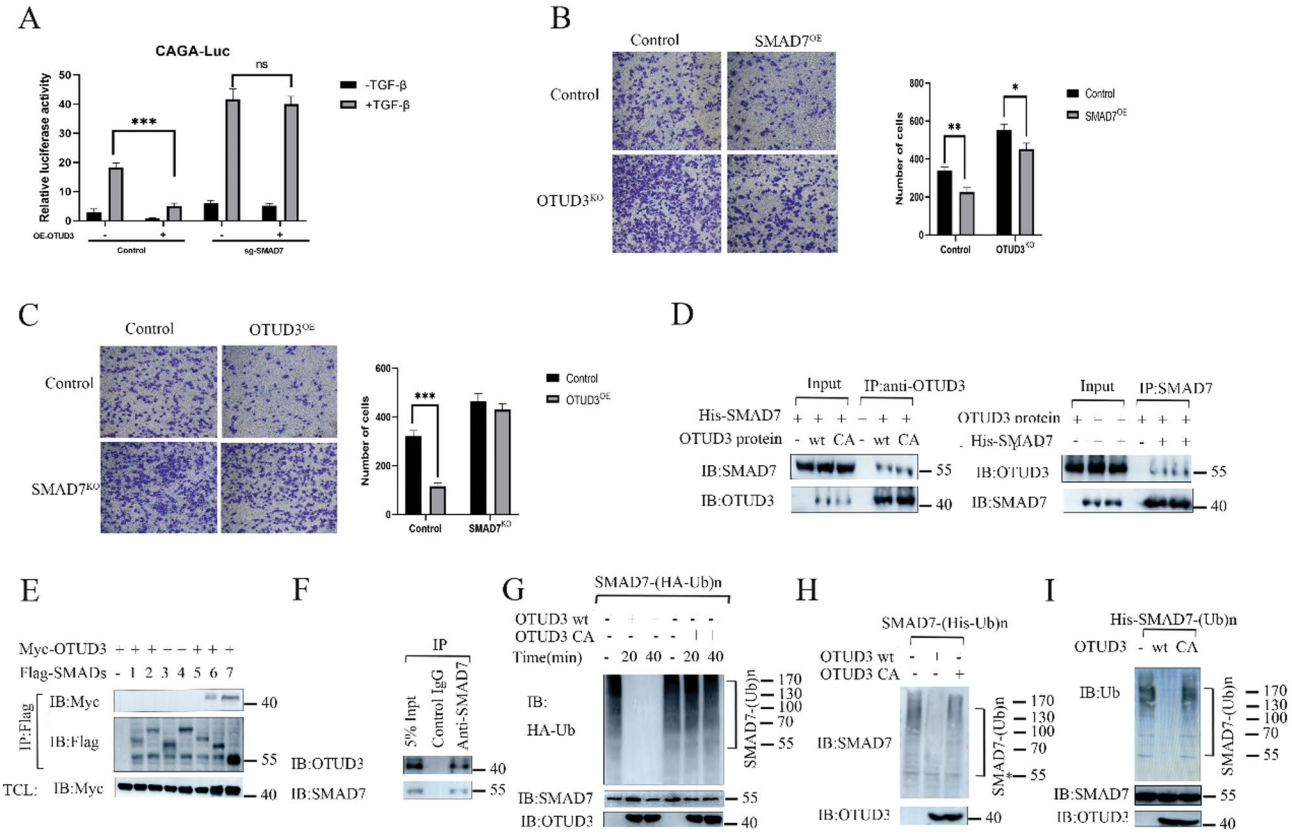

Fig. 4 OTUD3 inhibits TGF-β signaling and EMT by deubiquitinating SMAD7. **A** The effect of OTUD3 overexpression on CAGA-Luc transcriptional response in SMAD7-deficient HEK293T and control cells treated with TGF-β (2.5 ng/ml) for 16 h. **B**-**C** The effect of SMAD7 or OTUD3 overexpression on cell invasion in OTUD3-deficient (**B**) or SMAD7-deficient (**C**) MDA-MB-231 and control cells was detected by transwell assay. Left is a cell picture, enlarged images were taken using a 10× magnification lens; right is a statistical analysis. **D** Purified SMAD7 interacts with OTUD3-wt/CA in vitro. The left image shows the immunoprecipitation of purified SMAD7 protein with OTUD3 antibody, and the right image shows the immunoprecipitation of purified OTUD3 protein with SMAD7 antibody. The results were shown by immunoblotting. **E** HEK293T cells were transfected with Myc-OTUD3 and Flag-SMAD1-7 vectors, respectively, and TCL and immunoprecipitants were extracted for IB analysis. **F** To detect the correlation between OTUD3 and SMAD7, the anti-SMAD7 immunoprecipitate of HEK293T cells was subjected to IB, and 5% TCL was used as input. **G-I** OTUD3 deubiquitinats polyubiquitination of SMAD7 in vitro. **G** FLAG-SMAD7 and HA-Ub were transfected into HEK293T cells, respectively. The polyubiquitinized SMAD7 was immunoprecipitated with anti-FLAG M2 magnetic beads and incubated with purified OTUD3-wt/CA protein for different times. The cleavage products were analyzed by IB with antibodies HA-Ub, SMAD7 and OTUD3. **H** FLAG-SMAD7 and His-Ub were introduced into HEK293T cells, respectively. Subsequently, polyubiquitinated SMAD7 was extracted from the cell lysate, pulled off with nickel beads and incubated with purified OTUD3-wt/CA for 60 min. The cell lysate was analyzed by IB with SMAD7 and OTUD3 antibodies, respectively. The asterisk indicates that free SMAD7. **I** At 37℃ in vitro, His-SMAD7 was first incubated with E1,E2(UbcH6), GST-RNF12 and ubiquitin for 3 h, and then immunoprecipitated with SMAD7 antibody. Then it was incubated with purified OTUD3-wt /CA for 60 min, and the mixture was analyzed with Ub, SMAD7 and OTUD3 antibodies, respectively. (*$p < 0.05$;**$p < 0.01$;*** $p < 0.001$)

poly-ubiquitin chains. The results showed that OTUD3-wt could undergo deubiquitylation and lead to the accumulation of free SMAD7 (Fig. 4H). After incubating His-SMAD7 with RNF12 in vitro, the practical cutting ability of OTUD3 wt/CA on the poly-ubiquitin chain of SMAD7 was detected, and only OTUD3-wt could do this (Fig. 4I). Hence, we have confirmed that OTUD3 can deubiquitinate SMAD7 in vitro.

OTUD3 represses the TGF-β signal pathway and EMT

The stem cell properties of breast cancer are related to EMT, which needs the interaction of multiple tumor-promoting signaling pathways, including TGF-β signals. Zhang ZK and his colleagues found that TGF-β could vigorously promote the formation of tumor organoids and tumor spheres in breast cancer, while the TβRI kinase inhibitor SB431542 could suppress the effect, and this showed that TGF-β signals played an active role in backing up stem cell-like properties [21]. Again, we measured the activation of the TGF-β signal pathway via CAGA-Luc transcriptional reporters following the addition of TGF-β. First, we transfected the HEK293T cells with OTUD3-wt and OTUD3-CA separately, and the result is shown in Fig. 5A. As OTUD3-CA did not inhibit TGF-β signals, we indicated that DUB activity was an indispensable factor. Secondly, we knocked down OTUD3 and found that the induced transcriptional response by TGF-β needed the absence of OTUD3

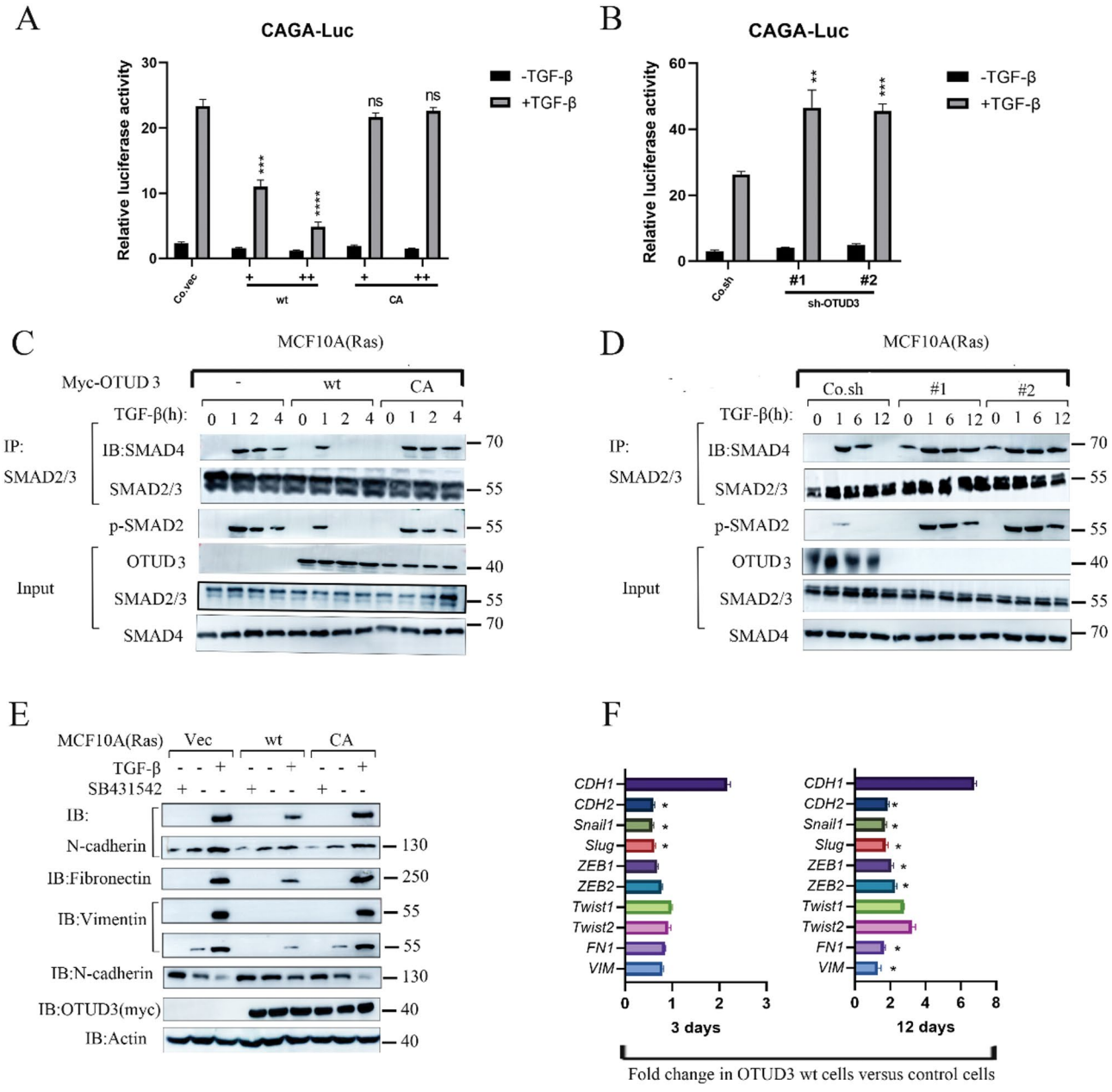

Fig. 5 OTUD3 inhibited TGF-β signal pathway and EMT. In HEK293T cells, the effects of OTUD3 wt and OTUD3CA (**A**) or OTUD3 knockout (#1,#2) (**B**) on TGF-β induced CAGA-luciferase activity were detected respectively. + or ++ represented various concentrations of plasmid pCMV-Myc. Co.vec, empty vector; Co.sh, non-targeting shRNA. **C-D** Myc-OTUD3 wt/CA transfected MCF10A-RAS cells(**C**), or knocked out OTUD3 (#1, #2), and corresponding control cells(**D**) were treated with transformed growth factor-β (5 ng/ml). IB of total cell lysates and SMAD2/3 immunoprecipitates was analyzed for SMAD2/3 and SMAD4 levels. 5% total cell lysate as input. **E** MCF10A-RAS cells were transfected with Myc-OTUD3wt/CA and empty vector, respectively, and treated with transformed growth factor β (2.5 ng/ml) and SB431542(10μM) for 72 h. The cell lysates were extracted for IB. **F** Lentiviral vector OTUD3 wt and control empty vector were used to infect MDA-MB-231 cells, and qRT-PCR was performed at 3 days (left picture) and 12 days (right picture), respectively, to analyze the expression levels of EMT-related genes. (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

(Fig. 5B). In Ras-activated MCF10A (MCF10A-RAS) cells, overexpression of OTUD3-wt instead of OTUD3-CA reduces the degree and duration of SMAD2 phosphorylation induced by TGF-β and SMAD2-SMAD4 complex formation (Fig. 5C). The opposite results were obtained in the cells with OTUD3 knockdown (Fig. 5D). We then considered that OTUD3 was an effective antagonist of the TGF-β/SMAD pathway.

The classic markers of EMT include N-cadherin, fibronectin, smooth muscle actin, vimentin, and E-cadherin, and the markers would be up-regulated or down-regulated accordingly in stem cells [22]. In MCF10A-RAS

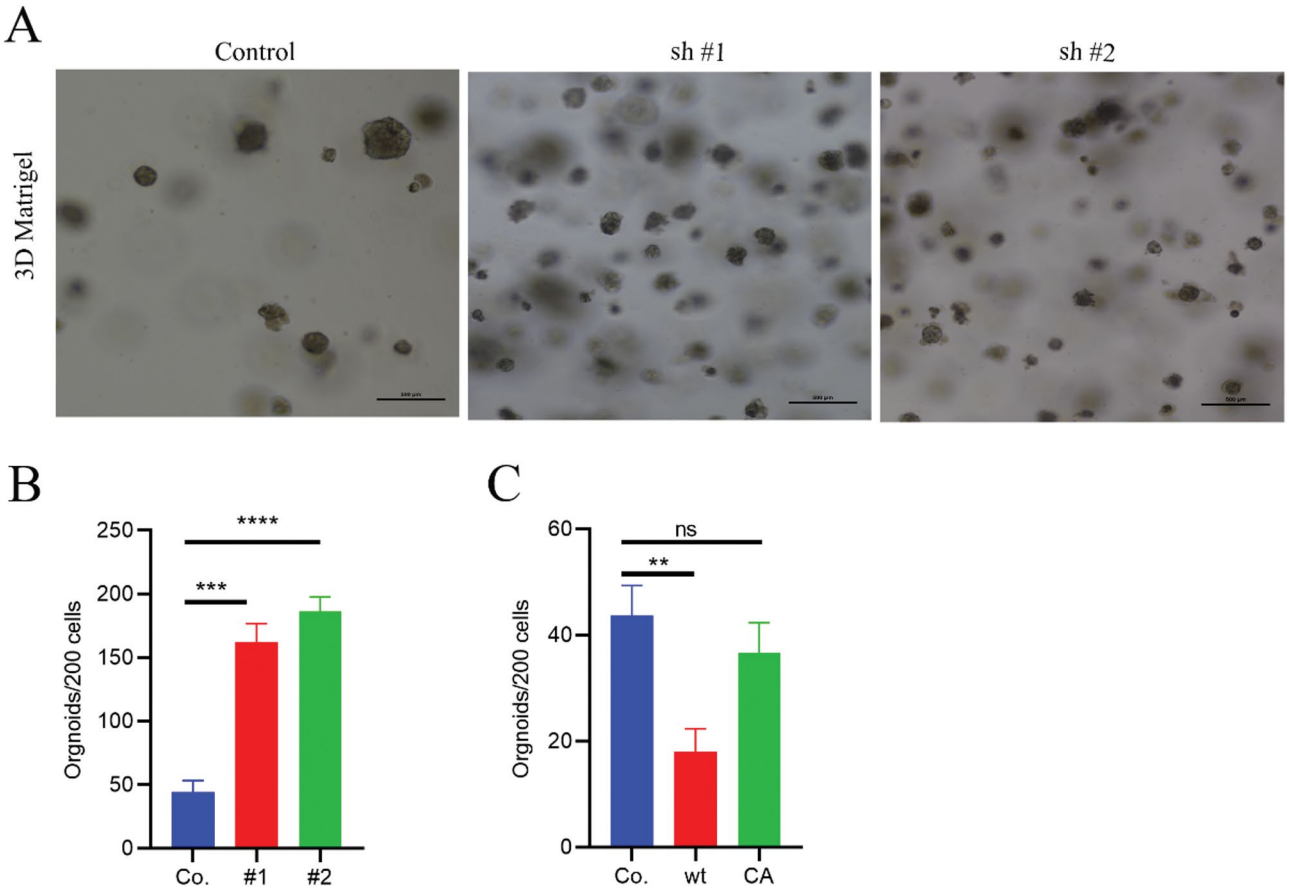

Fig. 6 OTUD3 inhibits cancer stem cell-like properties. **A-B** MCF10A-RAS cells knocked out OTUD3 (#1, #2) and control cells were cultured in 3D Matrigel. Representative cell pictures (**A**). The experiment was repeated three times, where the mean number of organoids per 200 cells (± SD) was statistically analyzed(**B**). **C** OTUD3wt/CA overexpression and empty vector MCF10A-RAS cells were cultured in 3D Matrigel, where the mean number of organoids per 200 cells (± SD) was statistically analyzed (repeated three times)

cells, EMT markers show significant changes when silencing OTUD3. At the same time, the changes in EMT markers were inhibited by overexpression of OTUD3-wt instead of OTUD3-CA (Fig. 5E). Subsequently, we constructed a lentiviral vector expressing OTUD3 and transfected it to the MDA-MB-231 cells. We analyzed the EMT-related genes by qPCR to assess the impact of overexpression of OTUD3 on EMT after three days (immediate response) and 12 days (delayed response) separately (Fig. 5F). Compared with control cells, the epithelial cell marker CDHI increased by the overexpression of OTUD3, and the mesenchymal marker decreased s, including CDH2, FNI, and VIM (Fig. 5F). After 12 days of overexpression of OTUD3, several EMT-related transcription factors such as including SNAIL, SLUG, ZEB1, and ZEB2, were observed to decrease (Fig. 5F), indicating the interstitial cell phenotype of MDA-MB 231 cells reversed. Summing up, we believe that OTUD3 is a key and selective inhibitor of TGF-β signal pathway and EMT.

OTUD3 inhibits cancer stem cell-like properties of human breast cancer cells

We studied the role of OTUD3 on the cytological behavior in breast cancer cells. By analyzing the tumor organoids in 3D Matrigel, the survival and proliferation of MCF10A-RAS cells were enhanced by the absence of endogenous OTUD3 (Fig. 6A- B). In cells with ectopic expression of OTUD3-wt, the number of tumor-like colonies significantly reduced compared with cells with ectopic expression of OTUD3-CA mutant (Fig. 6C). These results indicate that OTUD3 inhibits the stem cell-like properties of BRCA cells.

Discussion

Current cancer research believes that tumorigenesis and development is a gradual process, including the activation of oncogenes and the inhibition of tumor suppressor gene [23], as well as the process of promoting tumor progression [24]. Most cancer-related deaths are caused by metastatic disease. Metastasis is one of the classic features of cancer. It is a multi-stage process including

remodeling the local tumor microenvironment (TME), EMT, then the tumor cells invade the blood or lymph, survive in circulation, exude, and grow in a new microenvironment [25]. EMT is a key step in the process of cancer invasion and metastasis, characterized by decreased epithelial markers and increased expression of interstitial markers, promoting cell invasion and spread to distant areas, enhancing angiogenesis and mediating immune escape of tumor cells to promote tumor progression [26, 27].

TGF-β signaling is thought to be one of the main triggers of EMT process. SMAD-dependent pathway is an important transmission mode of TGF-β signaling pathway. SMAD family members are divided into three groups, namely (1) R-SMADs (R-SMADs), including SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8(also known as SMAD9), each of which regulates a specific signaling pathway; (2) Co-SMADs(Co-mediators SMADs), including SMAD4, mediate signal transduction of different members of the transforming growth factor-β family; (3) the antagonistic or inhibitory SMADs, including SMAD6 and 7, which negatively modulates the SMAD-dependent signaling pathway by degrading the receptor or binding to the R-SMADs competitively active isomer receptor complex. Multiple studies have shown that TGF-β signal can drive EMT by regulating the expression of related target genes, such as SNAIL, SLUG, HMGA2, matrix metalloproteinases (MMPs), etc [10, 28–30].

SMAD7 has been shown to interact with R-SMADs and regulate there polyubiquitination and degradation. It has also been shown to disrupt the assembly of the TGF-β-induced SMAD-DNA complex. It interacts with the SMAD response element in the TGF-β response gene through its MH2 domain and antagonizes the execution of TGF-β signaling [31].

Protein ubiquitination is a type of post-translational modification, leading to the combination of ubiquitin protein (Ub) and the ubiquitin binding domain on the protein. This modification can occur on a variety of cellular proteins [32], and is therefore involved in a variety of cellular biological processes [33]. DUBS stabilizes proteins by inducing protein degradation by dissociating the connections between Ub parts or between Ub and substrates [34, 35]. Ubiquitination and deubiquitination regulate many aspects of human cell biology and physiology. Studies have found that dysregulation of these processes can affect a variety of diseases, including cancer and infectious diseases [36].

More and more studies have shown that DUBs play a carcinogenic or inhibitory role at a multidimensional level in tumors [36]. Among them, the role of DUBs in tumor mainly includes proteasome degradation, DNA repair, apoptosis and metastasis. More than 100 DUBs have been found in nine families, of which OTUD3 belongs to the OTUs family [36]. Our previous studies demonstrated the deubiquitinating effect of OTUD3 on PTEN and P53, revealing the relevant regulatory mechanisms of its inhibition of breast cancer occurrence, cancer cell proliferation and apoptosis [16, 37]. However, it is not clear whether it also regulates breast cancer metastasis.

Through bioinformatics studies, we found that OTUD3 is generally low expression or even absent in a variety of human tumors, suggesting that OTUD3 has tumor suppressive effects in a variety of tumors. Our further analysis in breast cancer found that deletion of the OTUD3 gene is present in approximately 50% of tumors and is associated with poor patient outcomes (Fig. 5E, G). In TCGA-Breast cancer database, through the correlation analysis between mRNA expression of OTUD3 and SMAD7, we found that OTUD3 and SMAD7 expression were positively correlated, and this result was also consistent with the immunohistochemical results of our breast cancer samples.

The function of ubiquitin relies primarily on seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) and an N-terminal methionine (MET1), which facilitates the formation of complex ubiquitination connections [38]. Each connection coordinates a different signaling pathway to determine the fate of the substrate protein [39]. The most typical of these are polyubiquitin chains linked by Lys48 or Lys63 [40]. In this study, we found that OTUD3 could remove the K48 chain of SMAD7, inhibit its degradation, stabilize SMAD7 protein levels, and thus antagonize TGF-β/SMAD signaling. Therefore, we believe that the anti-metastasis effect of OTUD3 depends on the expression of endogenous SMAD7. Although OTUD3 did not show significant inhibition of metastasis in SMAD7-deficient cells, our results cannot rule out the possibility that OTUD3 may also target other substrates. While OTUD3-wt could completely separate the K48-linked polyubiquitin chains from SMAD7 in DMSO-treated cells, this effect was not observed in MG132-treated cells, suggesting that certain DUBs that may be present in precursors play a role in the deubiquitination of SMAD7.

Conclusions

In summary, we found a new OTUD3 mechanism, as a deubiquitase of SMAD7 in vivo, by controlling the intrinsic SMAD7 activity, blocking the activation of TGF-β/SMAD pathway, thereby limiting EMT and tumor stem cell properties, and inhibiting tumor metastasis.

Abbreviations

BRCA	Breast cancer
IARC	International Agency for Research on Cancer
DAB	Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium

DUBs	Deubiquitinating enzymes
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial mesenchymal transition
gRNA	Guide RNA
IB	Immunoblot
IHC	Immunohistochemistry
IP	Immunoprecipitation
LAP	Latency-associated protein
MET1	Methionine
MMPs	Matrix metalloproteinases
OTUD3	Ovarian tumor domain-containing protein 3
PBS	Phosphate-buffered saline
RFS	Recurrence-free survival
SD	Standard deviations
sgRNAs	Single guide RNAs
shRNA	Short-hairpin
SMAD	SMAD family member
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TGF-βR	Transforming growth factor-β receptor
TME	Tumor microenvironment
Ub	Ubiquitin protein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12935-025-03822-x.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Chenchen Geng and Ke Dong designed and conducted the experiments and analyzed data. Chenchen Geng, Ke Dong and Junhua An did bioinformatics experiments and cytology experiments. Chenchen Geng, Ziqian Liu and Qianqian Zhao performed ubiquitination and deubiquitination experiments, immunohistochemical assay and organized figures. Yanrong Lv got funding, designed and supervised the study, and was responsible for the manuscript.

Funding
This research was funded by Natural Science Foundation of Shandong (No. ZR2021MH211) and Qilu Hospital of Shandong University (Qingdao) Research Foundation (No. QDKY2021RX04).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Qilu Hospital of Shandong University (Qingdao) Research Ethics Committee (Approval no. KYLL-2021028, date of approval: 28 January 2021). Informed consent was obtained from all subjects involved in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 23 August 2024 / Accepted: 8 May 2025
Published online: 17 May 2025

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