USP18 promotes breast cancer growth by upregulating EGFR and activating the AKT/Skp2 pathway

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Abstract. Recent studies have suggested that ubiquitin-specific peptidase (USP)18 may act as an oncogene in various types of cancer. Although the role of USP18 in breast cancer cell lines has been elucidated, the underlying mechanisms and clinical role of USP18 in breast cancer are currently not well understood. The bioinformatics analysis and experimental results of the present study demonstrated that aberrant promoter methylation led to increased expression of USP18 in breast cancer. In addition, correlation analysis suggested that a negative correlation between methylation and USP18 mRNA expression was observed in The Cancer Genome Atlas database. USP18 promoted cell proliferation, colony formation and cell cycle progression *in vitro*. Furthermore, the Gene Set Enrichment Analysis results demonstrated that USP18 may be negatively associated with apoptosis in patients with breast cancer. Bioinformatics analysis results indicated that USP18 was also revealed to be associated with the protein kinase B (AKT) signaling pathway and mammary tumorigenesis *in vivo*. In addition, the results indicated that USP18 may promote the epidermal growth factor (EGF)-mediated EGF receptor (EGFR)/AKT/S-phase kinase-associated protein 2 (Skp2) pathway by upregulating EGFR and Skp2 in a AKT/forkhead box O3-dependent manner in breast cancer. The results of bioinformatics analysis revealed that increased USP18 expression was associated with a higher TNM stage and unfavorable prognosis in clinical patients. USP18 was also significantly enhanced in patients with human epidermal growth factor receptor 2-positive breast cancer; furthermore, Kaplan-Meier curve demonstrated that combining USP18 and Skp2 expression improved prognostic capability in breast cancer. Taken together, these results suggested that USP18 may serve a key role in breast cancer development by upregulating EGFR and subsequently activating the AKT/Skp2 feedback loop pathway. The role of USP18 in breast cancer provides a novel insight into the clinical application of the USP18/AKT/Skp2 pathway.

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Abbreviations: USP18, ubiquitin-specific peptidase 18; Skp2, S-phase kinase-associated protein 2; EGFR, epidermal growth factor receptor; FoxO3, forkhead box O3

Key words: USP18, EGFR, protein kinase B, Skp2, breast cancer

Introduction

Breast cancer is one of the most aggressive types of cancer worldwide; in recent decades, the incidence rate of breast cancer has increased and it accounts for >458,000 annual cases of mortality worldwide. In addition, breast cancer is the fifth most common cause of cancer-associated mortality worldwide (1). Although breast cancer has been studied extensively, the causes of tumorigenesis remain unclear. Adjuvant treatments have had a limited impact on the survival of patients with breast cancer; breast cancer is a heterogeneous disease sustained by complex growth pathways and adjuvant treatments do not always guarantee optimal results (2,3). Therefore, improved understanding of the molecular mechanisms underlying breast cancer progression is required, in order to identify novel molecular therapeutic targets.

Ubiquitin-specific peptidase (USP)18 is known as an interferon (IFN)-stimulated gene 15 (ISG15) isopeptidase, and a negative regulator of type I and type III IFN signaling (4). Previous studies have reported that USP18 has an important role in tumorigenesis. USP18 exerts tumor-promoting functions in various types of cancer; USP18-deficient mice exhibited a decrease in tumor growth in a murine model of breast cancer, and the absence of USP18 in the MCF-7 breast cancer cell line resulted in an increase in the induction of apoptosis by chemotherapy and IFN-α treatment (4,5). Furthermore, downregulation of USP18 reduces acute promyelocytic leukemia cell growth and induces apoptosis (6,7), whereas silencing USP18 in T98G glioblastoma cells enhances IFN-induced apoptosis (8). Conversely, increased expression of USP18 is associated with enhanced proliferation in kidney cancer (9). In a previous clinical study, USP18 was revealed to be upregulated in lung cancer (10); furthermore, reduced USP18 expression is associated with significantly longer cancer-specific survival in muscle invasive bladder cancer (11). These findings suggest that USP18 may act as an oncogene in

various types of cancer; however, the clinical role of USP18 and the molecular mechanisms underlying the effects of USP18 in breast cancer remain to be elucidated.

Although the role of USP18 *in vitro* and *in vivo* has been elucidated, the underlying mechanisms and clinical role of USP18 in breast cancer are currently not well understood. In order to study the potential upregulation, and the underlying mechanism, of USP18 in breast cancer, the present study analyzed the promoter methylation and mRNA expression levels of USP18 in clinical patients. Furthermore, the relationship between promoter methylation and USP18 expression was examined by 5-aza-2-deoxycytidine (5'Aza) treatment and correlation analysis. The function of USP18 was validated by cell function experiments and bioinformatics analyses. To determine the tumor-promoting mechanism of USP18, the present study identified associated signaling pathways from microarray data obtained from the Gene Expression Omnibus (GEO) database; the protein kinase B (AKT)/S-phase kinase-associated protein 2 (Skp2) signaling pathway was revealed to be associated with USP18. In addition, the present results revealed that the regulatory effects of USP18 on Skp2 expression may be dependent on AKT/forkhead box O3 (FoxO3) expression in breast cancer. Increased USP18 levels were also associated with increasing TNM stage and unfavorable prognosis in breast cancer. Furthermore, USP18 was significantly upregulated in patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer, and combining USP18 and Skp2 expression improved prognostic capability in breast cancer. All of these results indicated that USP18 may serve a key role in breast cancer development by upregulating epidermal growth factor receptor (EGFR) and subsequently activating the AKT/Skp2 feedback loop pathway. In addition, these results may help to elucidate the pathogenesis of breast cancer, and provide a novel potential therapeutic target and prognostic marker for patients with breast cancer.

Materials and methods

Cell culture, transfection and treatments. Human breast cancer cell lines were purchased from the Cell Resource Center of Beijing Xiehe (Beijing, China) and cultivated at 37˚C in an atmosphere containing 5% CO_2. MCF-7 and MDA-MB-231 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare, Logan, UT, USA) and 100 U/ml penicillin (Thermo Fisher Scientific, Inc.). Cells were seeded in 6-well plates and were grown overnight, in order to reach 60-70% confluence prior to transfection. Transfection of MCF-7 and MDA-MB-231 cells with Myc-USP18 plasmid (250 ng/μl, 8 μl; RC202374; OriGene Technologies, Inc., Beijing, China) or vector plasmid (500 ng/μl, 4 μl) was conducted at room temperature for 30 min using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Prior to detecting USP18 expression, cells were treated with 2 μM 5'Aza (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 3 days at 37˚C; medium and 5'Aza were replenished every day.

A total of 24 h post-transfection, breast cancer cells were serum starved overnight, and were then treated with 100 ng/ml EGF (Sigma-Aldrich; Merck KGaA) for 5 min at 37˚C, in order to detect the effects of USP18 overexpression on the EGF-mediated AKT signaling pathway. Furthermore, LY294002 stock solution (10 mM; Merck KGaA) was diluted with dimethyl sulfoxide, and 24 h post-transfection, cells were treated with 10 μM LY294002 for 24 h at 37˚C.

Prediction of CpG islands, methylation-specific polymerase chain reaction (PCR) (MSP) and combined bisulfite restriction analysis. The CpG island of the USP18 gene was predicted using The University of California Santa Cruz (UCSC) Genome Browser database (http://genome.ucsc.edu) and the bisulfite-conversion-based methylation PCR primers were designed using the MethPrimer database (http://www.urogene.org/methprimer/). Genomic DNA was extracted for methylation analysis from MCF-7 and MDA-MB-231 cells in culture using the Genomic DNA Miniprep kit (Sigma-Aldrich; Merck KGaA). Genomic DNA (1 μg) was then modified with sodium bisulfite using the DNA Bisulfite Conversion kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. MSP was run in a total volume of 20 μl; MSP reactions were subjected to initial incubation at 95˚C for 5 min, followed by 35 cycles at 94˚C for 20 sec, annealing at 60˚C for 30 sec and 72˚C for 20 sec. Final extension was performed at 72˚C for 5 min. MSP products were separated by 2% agarose gel electrophoresis and were visualized following ethidium bromide (Thermo Fisher Scientific, Inc.) staining. The following primers were used: Unmethylated forward, 5'-TGTTTTTTATTTAGTGGAAAATGA-3' and reverse 5'-ACCAAAAACCAAACTAAAACCAA-3'; and methylated forward, 5'-CGTTTTTTATTTAGTGGAAAACGA-3' and reverse, 5'-GAAAACCGAACTAAAACCGAA-3'. Normal blood lymphocyte DNA acted as a negative control and was obtained from peripheral blood of three healthy volunteers (two female volunteers aged 45 and 46, and one male volunteer aged 37). The present study was approved by the Research Ethics Committee of Qilu Hospital of Shandong University (Qingdao, China) and was conducted according to the Declaration of Helsinki. All volunteers provided written informed consent.

GEO datasets and differential expression analysis. Microarray data were obtained from seven GEO datasets that were accessed from the National Centers for Biotechnology Information GEO database (http://www.ncbi.nlm.nih.gov/geo/), which serves as a public repository for gene expression datasets. The accession numbers were as follows: GSE66695, GSE72653, GSE61500, GSE23938, GSE2528, GSE61499 and GSE61500. Differentially expressed genes were determined using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/), which is an interactive web tool that compares two groups of samples under the same experimental conditions and can analyze almost any GEO series.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) analyses. Total RNA was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized using a reverse transcription kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. qPCR was performed using SYBR Green master mix on a HT7500 system (Applied Biosystems;

Thermo Fisher Scientific, Inc.). The following primers were used: USP18 forward, 5'-AACGTGCCCTTGTTTGTCCAA-3' and reverse 5'-GAGTCCTTCACCCGGATCGTA-3'; Skp2 forward, 5'-ATGCCCCAATCTTGTCCATCT-3' and reverse 5'-CACCGACTGAGTGATAGGTGT-3'; and GAPDH forward, 5'-CTGGGCTACACTGAGCACC-3' and reverse 5'-AAGT GGTCGTTGAGGGCAATG-3'. qPCR was conducted as follows: 95˚C for 10 min, followed by 40 amplification cycles at 95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 30 sec. The FC (fold change) of gene expression was calculated using the quantification cycle (Cq) method with the following formula: $FC = 2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = (Cq_{target} - Cq_{reference})$ sample - $(Cq_{target} - Cq_{reference})$ control (12).

Western blotting. Cells were solubilized in lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM Na_3VO_4, 1 mM DTT and 10 mM NaF]. Protein samples were obtained from whole cell lysates, mixed with 2X loading buffer [1:1; 20 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 20% glycerol and 0.016% bromophenol blue] and incubated at 99˚C for 15 min. Protein content determination within cell lysates was conducted using the bicinchoninic acid (BCA) method (Pierce BCA protein assay kit; Thermo Fisher Scientific, Inc.). After quantification, protein samples (~10 μl/300 μl whole cell lysates, ~20 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, the blots were blocked in blocking buffer [5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween (TBST)] for 1 h at room temperature, and were then incubated with primary antibodies (1:1,000) in blocking buffer overnight at 4˚C. The blots were washed three times with TBST and were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit (A4914) immunoglobulin G secondary antibodies (1:3,000; Sigma-Aldrich; Merck KGaA) in blocking buffer. Finally, the blots were washed three times with TBST and visualized by enhanced chemiluminescence (ECL) using an ECL kit (Applygen Technologies, Inc., Beijing, China). The following primary antibodies were used in the present study: Anti-Myc (#2278, 1:3,000), anti-USP18 (#4813, 1:1,000), anti-AKT (#4685, 1:1,000), anti-phosphorylated (p) AKT_308 (#4056, 1:500), anti-pAKT_473 (#3787, 1:1,000), anti-EGFR (#4267, 1:1,000), anti-Skp2 (#2652, 1:1,000) and anti-GAPDH (#5174, 1:5,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA).

The Cancer Genome Atlas (TCGA) data. The mRNA (RNA Seq v2) and protein (reverse phase protein array) data were downloaded from https://www.synapse.org, and the clinical information for patients in TCGA_breast cancer (BRCA) dataset was downloaded from cBioPortal database (www.cbioportal.org); these data were used to analyze differential mRNA expression, correlation, prognosis and gene set enrichment. Methylation data and the results of correlation analysis between methylation percentage and mRNA level were downloaded from MethHC database (http://methhc.mbc.nctu.edu.tw/php/index.php). The protein expression data were obtained from The Cancer Proteome Atlas database (http://www.tcpa-portal.org/tcpa/).

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK8) assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The transfected cells were plated in 96-well plates (2,000 cells/well) and cell proliferation was detected every 24 h. Briefly, 10 μl CCK8 solution was added to each well and incubated for 1 h at 37˚C. The solution was then measured spectrophotometrically at 450 nm.

Colony formation assay. A total of 2 days post-transfection with Myc-USP18 or empty vectors, 500 cells were subsequently seeded into each well of 6-well plates with full culture medium. After 7-10 days, cells were fixed with 70% ethanol for 10 min and stained with 0.5% crystal violet solution. Nikon Eclipse 600 photomicroscope (Nikon Corporation, Tokyo, Japan) was used to observe colony formation and to capture images. The experiment was conducted in three independent triplicates.

Gene Set Enrichment Analysis (GSEA). The association between phenotypes, pathways and USP18/Skp2 expression was analyzed using GSEA v2.2 (http://www.broad.mit.edu/gsea/). GSEA calculates a gene set Enrichment Score that estimates whether genes from a pre-defined gene set (obtained from the Molecular Signatures Database, MSigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp) are enriched among the highest- or lowest-ranked genes or distributed randomly. Default settings were used. Thresholds for significance were determined by permutation analysis (1,000 permutations). false discovery rate (FDR) was calculated. A gene set was considered significantly enriched when the FDR score was <0.05.

Cell cycle analysis. Cells transfected with USP18 or empty vectors were fixed in 70% ethanol for 12 h at 4˚C, treated with 0.1 mg/ml RNase A for 30 min at 37˚C, and stained with 50 mg/ml propidium iodide for 30 min at 37˚C in the dark (PI; BD Biosciences, Franklin Lakes, NJ, USA). The cells were then analyzed by flow cytometry using FACSCalibur (BD Biosciences) (13), and cell cycle distributions were calculated using CellQuest software version 5.1 (BD Biosciences). These experiments were performed three times in triplicate.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analyses. To explore the biological function of USP18 *in vivo*, the Database for Annotation Visualization and Integrated Discovery database (https://david.ncifcrf.gov/home.jsp) was used to map the KEGG pathways and GO terms associated with the differentially expressed genes in USP18 knockout (KO) mice (GEO dataset, GSE61500).

Protein-protein interaction (PPI) network construction. Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) is a database of known and predicted protein interactions, which may aid in the comprehensive description of cellular mechanisms and functions. A PPI network of the important differentially expressed genes with GO annotations in USP18 KO mice was constructed using the STRING database.

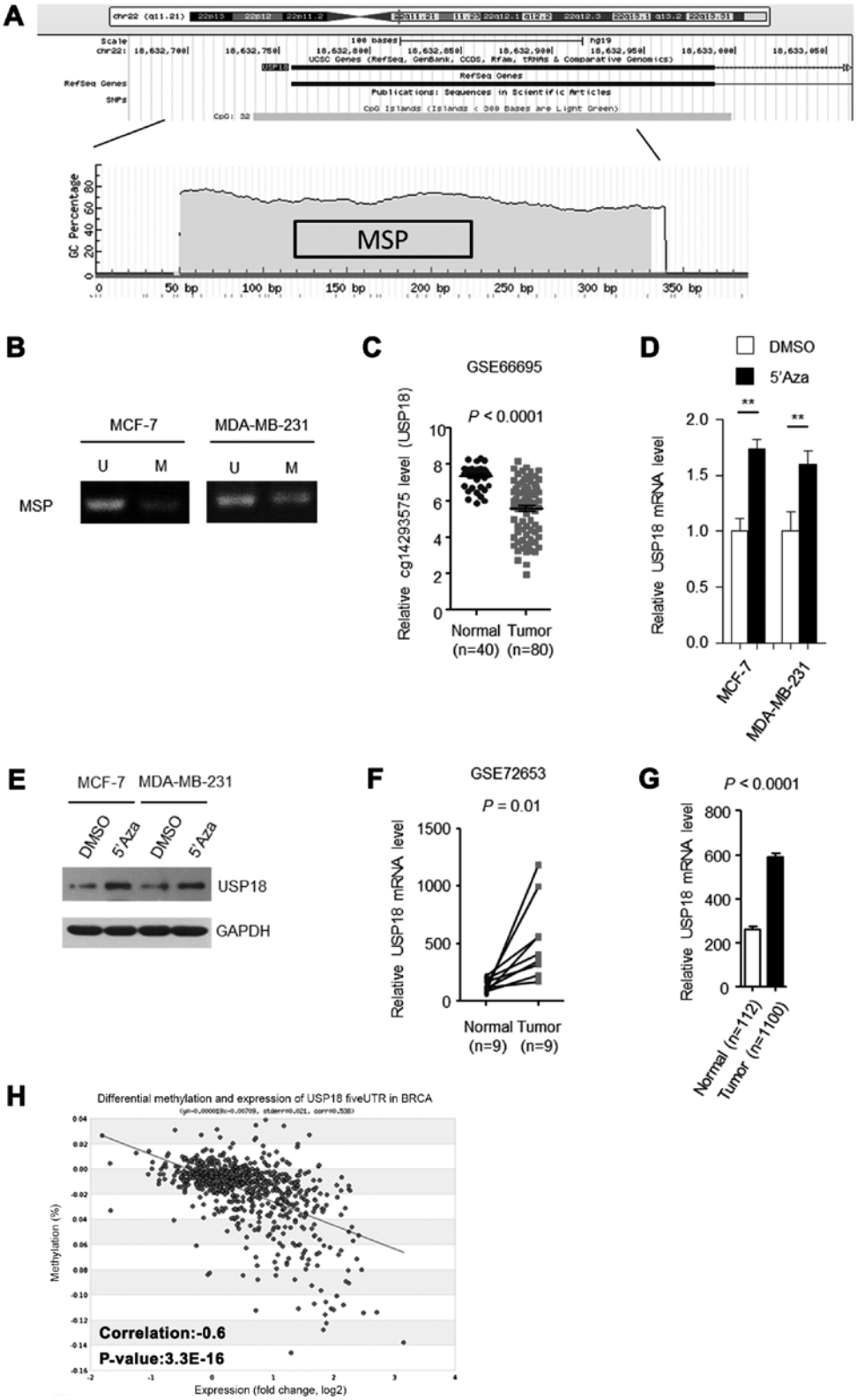

Figure 1. Transcriptional upregulation of USP18 is associated with promoter hypomethylation. (A) Schematic diagram of the gene sequence of USP18. The diagram presents visualization of annotations mapped to genomic coordinates and CpG islands in the USP18 gene (top). The GC percentage of CpG islands and the target region for MSP detection are also denoted (bottom). (B) Methylation status of USP18 in breast cancer cell lines. The CpG islands of USP18 were investigated by MSP. (C) Methylation status of USP18 in patients with breast cancer. Promoter methylation of USP18 was significantly reduced in breast tumor samples compared with in normal tissues (tumor, n=80 vs. normal, n=18; P<0.0001). Error bars represent standard error of the mean. Significance between the two populations was determined with a Student's t-test. (D) USP18 mRNA expression was increased following 5'Aza treatment in breast cancer cell lines. USP18 mRNA expression was detected using quantitative polymerase chain reaction. **P<0.01. (E) 5'Aza treatment resulted in increased USP18 protein expression in breast cancer cell lines, as determined by western blotting. GAPDH served as a loading control. (F) USP18 mRNA expression in breast tumor and matched normal tissues from a GEO dataset (GSE72653, n=9, paired sample t-test, P=0.01). (G) USP18 expression in breast cancer was validated in TCGA_BRCA dataset (tumor, n=1,100 vs. normal, n=112; P<0.0001). (H) Negative correlation between methylation and mRNA expression of USP18 in TCGA_BRCA dataset. Line indicates the linear regression of methylation percentage and mRNA expression (log). 5'Aza, 5-aza-2-deoxycytidine; BRCA, breast cancer; DMSO, dimethyl sulfoxide; M, methylated; MSP, methylation-specific PCR; TCGA, The Cancer Genome Atlas; U, unmethylated; USP18, ubiquitin-specific peptidase 18.

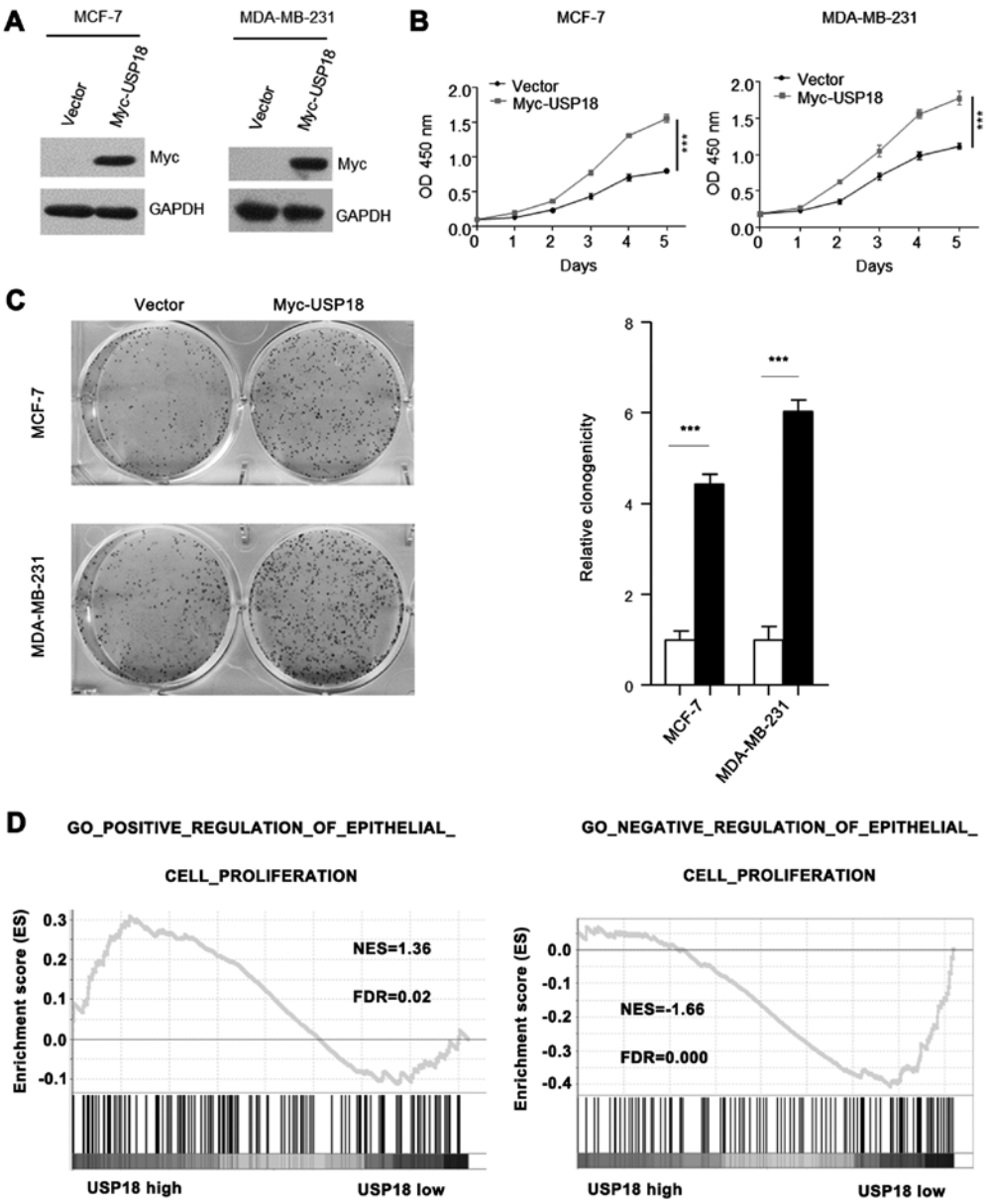

Figure 2. Effects of USP18 on cell proliferation and colony formation. (A) Overexpression of Myc in MCF-7 and MDA-MB-231 cells post-transfection with USP18 expression vectors was confirmed by western blotting. (B) CCK8 assays indicated that USP18 overexpression significantly promoted the growth of MCF-7 and MDA-MB-231 cells. (C) Colony formation ability of MCF-7 and MDA-MB-231 cells was significantly promoted by USP18 overexpression. (D) USP18 was revealed to be positively associated with cell proliferation in clinical patients. Gene Set Enrichment Analysis of The Cancer Genome Atlas data depicting enrichment of the epithelial cell proliferation gene set. Samples were divided into USP18 high and low groups. Positive regulation of the epithelial cell proliferation gene set was enriched in the USP18 high group. Conversely, negative regulation of the epithelial cell proliferation gene set was enriched in the USP18 low group. ***P<0.001. GO, Gene Ontology; OD, optical density; USP18, ubiquitin-specific peptidase 18.

Transcription factor binding prediction. JASPAR database (http://jaspar.binf.ku.dk/) was used to predict transcription factor binding sites in DNA sequences.

Statistical analysis. All experiments were conducted in three independent triplicates and data are presented as the means ± standard error of the mean. Statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Group distributions were compared using Student's t-test, or one-way analysis of variance followed by post hoc Bonferroni tests for the analysis of multiple comparisons. Correlations were analyzed using the Pearson correlation analysis. In addition, log-rank test for the generated Kaplan-Meier curves was conducted to evaluate the association between gene expression and survival rate. P<0.05 was considered to indicate a statistically significant difference.

Results

Low methylation in the promoter region of USP18 leads to its upregulation in breast cancer. Aberrant promoter methylation always affects transcriptional gene expression. Sequence analysis using the UCSC database (http://genome.ucsc.edu/index.html) revealed a CpG island located within the USP18 promoter region (Fig. 1A). As shown in Fig. 1B, the promoter

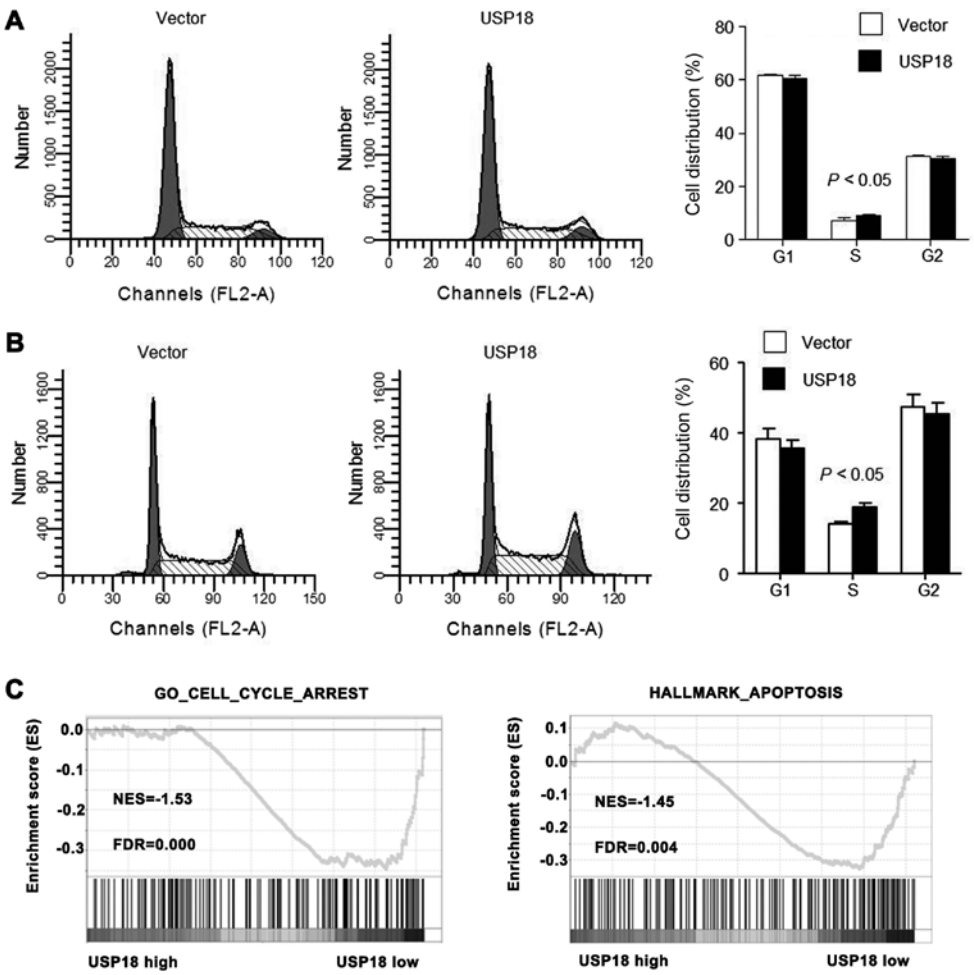

Figure 3. Effects of USP18 on cell cycle progression and apoptosis. (A and B) USP18 overexpression promoted breast cancer cell cycle progression. (C) USP18 was negatively associated with cell cycle arrest and apoptosis in patients with breast cancer. GO, Gene Ontology; USP18, ubiquitin-specific peptidase 18.

region of USP18 exhibited poor methylation in MCF-7 and MDA-MB-231 cells, and the USP18 methylation level was significantly downregulated in breast cancer compared with in normal tissues (Fig. 1C), and primer efficiency was verified by a negative control (normal blood lymphocyte DNA; data not shown). In addition, demethylation was induced by treatment with 5'Aza, which successfully increased the mRNA and protein expression levels of USP18 in MCF-7 and MDA-MB-231 cells (Fig. 1D and E). Subsequently, the expression levels of USP18 were detected in tumor tissues compared with in adjacent tissues using public datasets; the results indicated that USP18 was clearly enhanced in breast cancer tissues (Fig. 1F and G). To further evaluate whether a correlation existed between promoter DNA methylation and USP18 mRNA expression in breast cancer, the present study further detected USP18 promoter DNA methylation and mRNA expression data from the MethHC database (http://methhc.mbc.nctu.edu.tw/php/index.php). Linear regression analysis demonstrated a significant negative correlation between mRNA expression and promoter methylation in USP18 (Fig. 1H). These findings suggested that reduced methylation of USP18 in the promoter region may induce USP18 upregulation in breast cancer.

USP18 promotes breast cancer cell growth. To elucidate the biological function of USP18 in MCF-7 and MDA-MB-231 cells, the cells were transfected with a human USP18 vector. Myc protein expression was confirmed in Myc-USP18-transfected MCF-7 and MDA-MB-231 cells by western blotting (Fig. 2A). Subsequently, the effects of USP18 on cell proliferation were examined by CCK8 and colony formation assays. As shown in Fig. 2B, cell proliferation was significantly promoted by USP18 in MCF-7 and MDA-MB-231 cells (P<0.0001, Fig. 2B). In addition, USP18 expression promoted colony-forming ability in MCF-7 and MDA-MB-231 cells (P<0.0001, Fig. 2C). In order to evaluate the role of USP18 in clinical patients with breast cancer, GSEA results revealed that USP18 mRNA expression was positively correlated with the epithelial cell proliferation gene set (Fig. 2D). These results indicated the enhancing effects of USP18 on breast cancer growth.

USP18 regulates cell cycle arrest and apoptosis in breast cancer cells. The present study further examined the effects of USP18 on cell cycle progression by flow cytometry. Following PI staining, the proportion of MCF-7 cells in S phase was increased from 7.15 to 9.22% in response to USP18 overexpression (Fig. 3A). Similar findings were observed in MDA-MB-231 cells in response to USP18 overexpression; the number of cells in S phase was elevated from 14.53 to 18.35% (Fig. 3B). GSEA results revealed that USP18 expression was negatively correlated with the cell cycle arrest gene set and

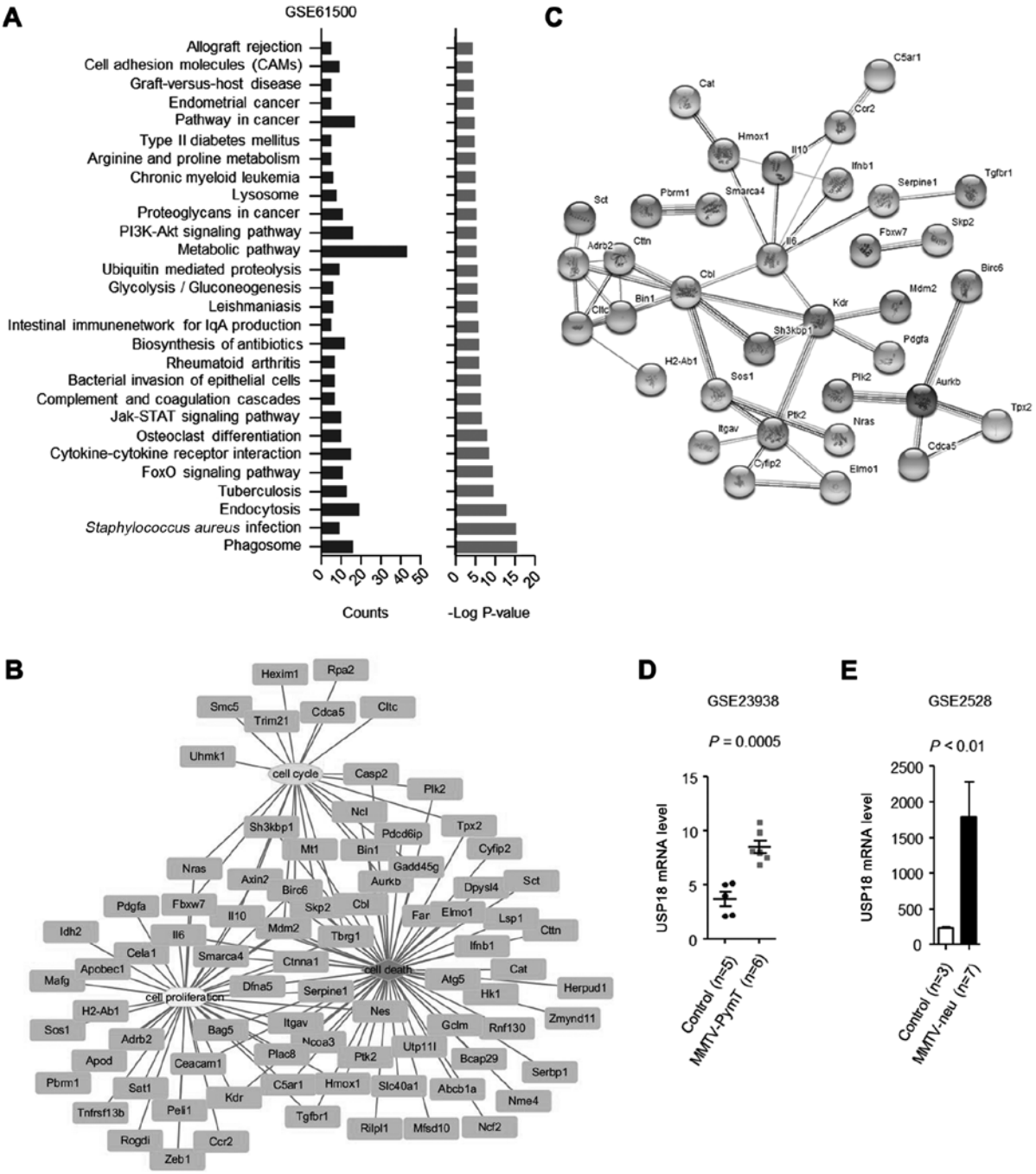

Figure 4. Functions of USP18 *in vivo*. (A) Kyoto Encyclopedia of Genes and Genomes pathway analysis in USP18 KO mice compared with in WT mice. Gene counts and significance (-log P-value) are presented (GSE61500). (B) GO analysis in USP18 KO mice compared with in WT mice. Significant associations among genes in the GO groups (cell death, cell cycle and cell proliferation) are presented. (C) Search Tool for the Retrieval of Interacting Genes database was used to mine the protein-protein interaction pairs of differentially expressed genes in the GO groups (cell death, cell cycle and cell proliferation). (D) USP18 was upregulated in an MMTV-PymT mouse mammary tumor model (GSE23938: Control, n=5; PymT, n=6; P=0.0005). (E) USP18 was upregulated in an MMTV-neu mouse mammary tumor model (GSE2528: Control, n=3; PymT, n=7; P<0.01). GO, Gene Ontology; KO, knockout; USP18, ubiquitin-specific peptidase 18; WT, wild-type.

the apoptosis gene set in patients with breast cancer (Fig. 3C). These results demonstrated that USP18 may participate in breast cancer development by promoting cell cycle progression and inhibiting apoptosis; these findings are similar to those presented in a previous study (14).

Physiological function analysis of USP18 in vivo. The present study demonstrated that USP18 may regulate cell cycle progression and apoptosis in breast cancer cells; therefore, further investigation was conducted to determine the physiological and pathological roles of USP18. KEGG pathway enrichment analysis and GO enrichment analysis were performed in USP18 KO mice and mouse mammary tumor models. As shown in Fig. 4A, the AKT/FoxO signaling pathway was one of the most dysregulated pathways, as determined by KEGG pathway analysis, in USP18 KO mice. In addition, as shown in Fig. 4B, the majority of the enriched GO terms belonged to the biological process category. Cell death, cell cycle and cell proliferation were the most relevant biological processes. Furthermore, to obtain PPI data, differentially

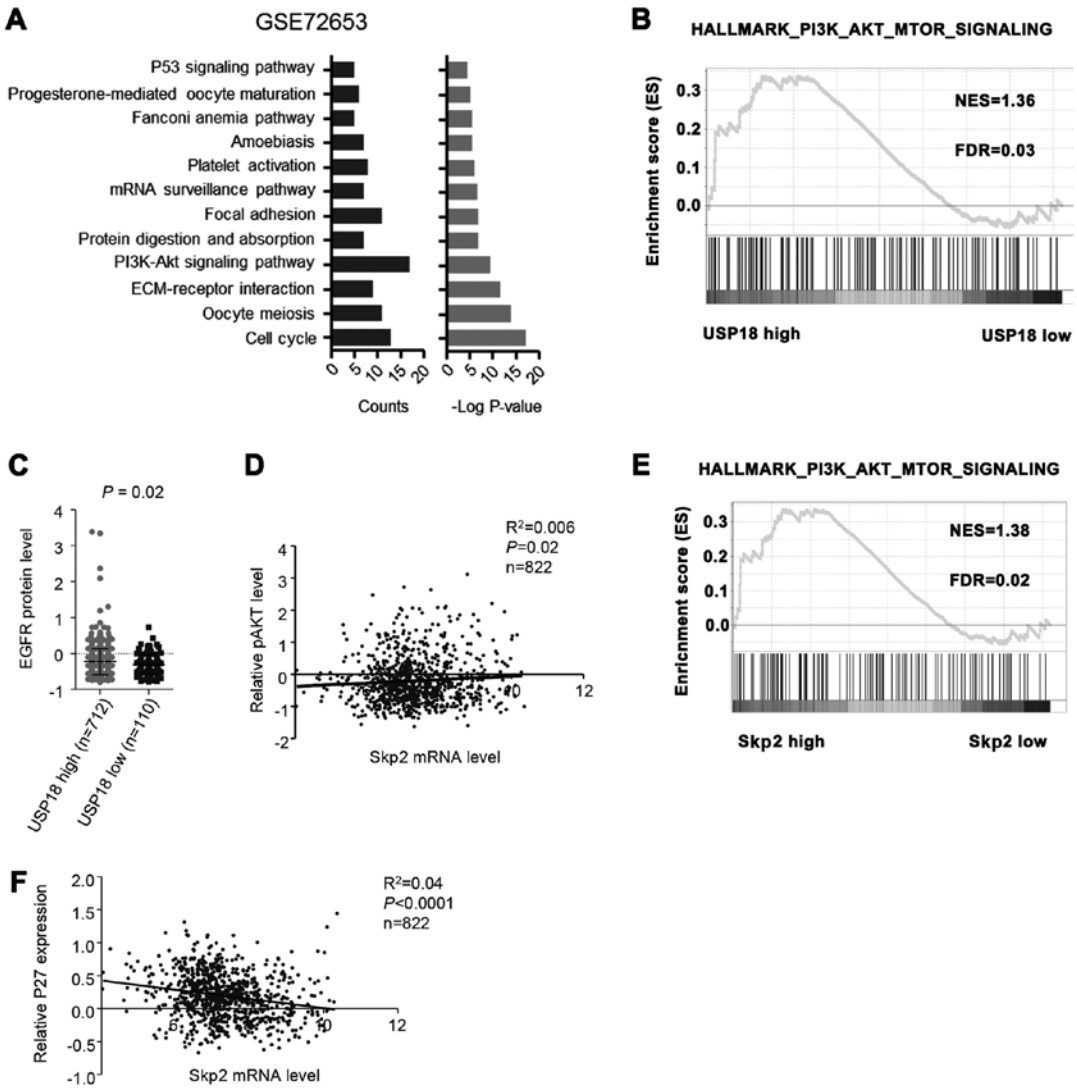

Figure 5. USP18 is positively associated with the AKT/Skp2 axis in clinical patients. (A) Kyoto Encyclopedia of Genes and Genomes pathway analysis in breast cancer compared with normal tissues. The results indicated that the AKT signaling and cell cycle pathways may be crucial in breast cancer (GSE72653). (B) USP18 was positively associated with the activated AKT pathway in clinical patients. GSEA demonstrated that the activated AKT pathway gene set was enriched in the USP18 high group from TCGA_BRCA dataset. (C) USP18 expression was positively associated with EGFR protein expression in clinical patients from TCGA dataset. EGFR protein expression was significantly higher in the USP18 high group (n=712) compared with in the USP18 low group (n=110) (P=0.02). (D) Skp2 mRNA expression was positively correlated with pAKT expression (R^2=0.006, n=822, P=0.02). (E) Skp2 was positively associated with the activated AKT pathway in clinical patients. GSEA demonstrated that the activated AKT pathway gene set was enriched in the Skp2 high group from TCGA_BRCA dataset. (F) Skp2 expression was negatively correlated with P27 protein expression in clinical patients from TCGA dataset (R^2=0.04, n=822, P<0.0001). AKT, protein kinase B; BRCA, breast cancer; EGFR, epidermal growth factor receptor; GSEA, Gene Set Enrichment Analysis; pAKT, phosphorylated-AKT; Skp2, S-phase kinase-associated protein 2; TCGA, The Cancer Genome Atlas; USP18, ubiquitin-specific peptidase 18.

expressed genes, which were involved in cell death, cell cycle and cell proliferation biological processes, were uploaded to the STRING database. These samples with a PPI score >0.7 were used to construct the PPI network (Fig. 4C). Skp2, transforming growth factor β receptor 1, interleukin 6, polo-like kinase 2, Cbl proto-oncogene, MDM2 proto-oncogene, kinase insert domain receptor and aurora kinase B were the main nodes in this network. In addition, these genes were enriched in the KEGG pathway and GO terms. To further elucidate the function of USP18 in tumor progression, MMTV-PymT (GSE23938) and MMTV-neu (GSE2528) mouse mammary tumor models were used. As shown in Fig. 4D and E, USP18 was significantly upregulated in mouse models of mammary tumors. These results demonstrated that USP18 may serve an important role in breast cancer progression.

USP18 expression is positively associated with the AKT/Skp2 pathway in breast cancer. To investigate the main pathways in breast cancer, KEGG pathway analysis was conducted on differentially expressed genes in breast cancer tissues compared with matched normal tissues. As shown in Fig. 5A, the AKT signaling pathway was the most significantly enriched pathway, and the cell cycle process was dysregulated in clinical patients. KEGG pathway analysis validated that the AKT signaling pathway serves an important role in the occurrence of breast cancer. To evaluate the association between USP18 and the AKT pathway in breast cancer, GSEA was conducted; the results revealed that USP18 expression was positively correlated with the AKT signaling pathway (Fig. 5B). In previous studies (14,15), USP18 was reported to upregulate EGFR, which is the main upstream kinase of AKT that promotes activation of

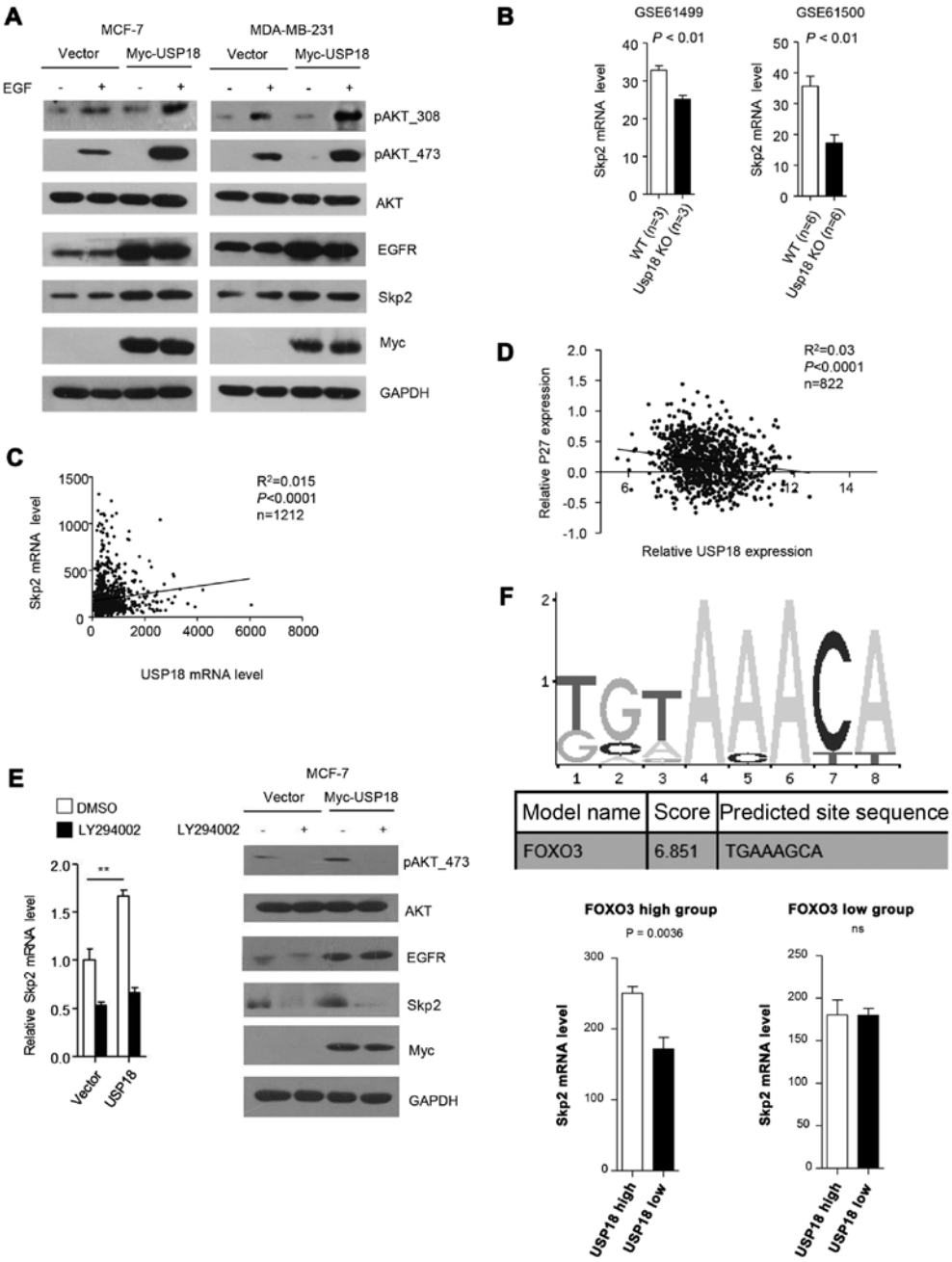

Figure 6. USP18 increases Skp2 mRNA expression by promoting AKT activation. (A) As determined by western blotting, USP18 overexpression promoted EGF-induced AKT activation, and upregulated EGFR and Skp2 protein expression in MCF-7 and MDA-MB-231 cells. (B) Skp2 mRNA expression was downregulated in USP18 KO mice (GSE61499, n=3, P<0.01; GSE61500, n=6, P<0.01). (C) Expression levels of Skp2 were positively correlated with USP18 expression in clinical patients from TCGA dataset (R^2=0.015, n=1,212, P<0.0001). (D) P27 protein expression was negatively associated with USP18 expression in clinical patients from TCGA dataset (R^2=0.03, n=822, P<0.0001). (E) Relative mRNA expression levels were analyzed by reverse transcription-quantitative polymerase chain reaction analysis using purified RNA from cells 24 h following LY294002 treatment; protein expression was analyzed by western blotting. (F) Promoter region of Skp2 was revealed to contain a FoxO3 binding site. The upstream 1,000 bp DNA sequence of Skp2 was used to predict binding using the JASPAR database. The binding motif and binding score are presented. Skp2 expression was positively associated with USP18 expression in the FoxO3 high group (P=0.0036). **P<0.01. AKT, protein kinase B; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; FoxO3, forkhead box O3; pAKT, phosphorylated-AKT; Skp2, S-phase kinase-associated protein 2; TCGA, The Cancer Genome Atlas; USP18, ubiquitin-specific peptidase 18.

AKT. Consistent with the results of the present study, and those of previous studies (14,15), it was demonstrated that the protein expression levels of EGFR were upregulated in the USP18 high expression group from TCPA_BRCA (reverse phase protein array) dataset (Fig. 5C). Furthermore, pAKT expression was positively correlated with expression of the downstream target gene Skp2 in TCPA_BRCA dataset. It is well known that Skp2 is a key cell cycle regulatory factor (Fig. 5D). In addition, GSEA results demonstrated that activation of the AKT signaling pathway was positively correlated with Skp2 expression (Fig. 5E). In addition, Skp2 was negatively associated with p27 protein expression, which is the specific substrate of Skp2 and a cell cycle inhibitor in TCPA_BRCA dataset (Fig. 5F). These results demonstrated that USP18 may have an important role in clinical breast cancer progression, which is associated with the AKT/Skp2 pathway.

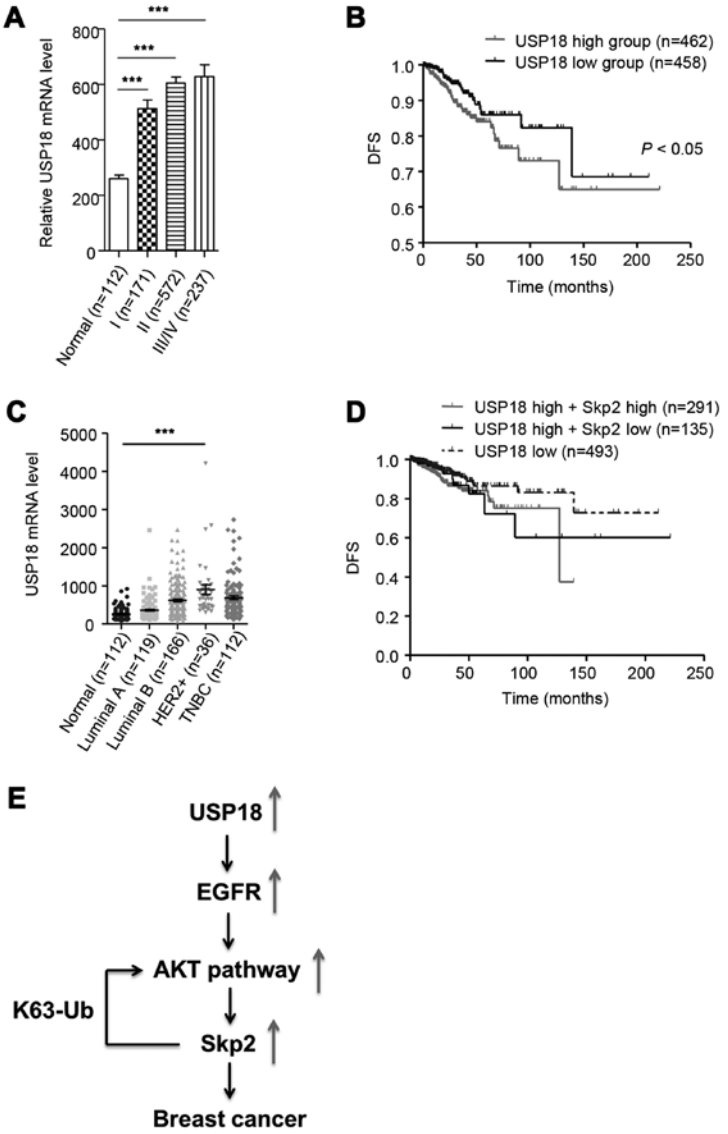

Figure 7. Clinical significance of USP18/Skp2 in breast cancer. (A) USP18 expression was positively associated with TNM staging in breast cancer. (B) Kaplan-Meier curve for the DFS of patients with relatively high (n=462) and low (n=458) USP18 mRNA expression. The curve indicated a shorter DFS time (P<0.05) with high USP18 mRNA expression. (C) Scatter plot of the expression levels of USP18 in breast cancer tissues from various breast cancer subtypes, and in normal tissues, from TCGA dataset (normal, n=112; luminal A, n=119; luminal B, n=166; HER2-enriched, n=36; TNBC, n=112). (D) Kaplan-Meier survival analysis of TCGA dataset for the relationship between survival time and USP18/Skp2 expression in breast cancer. (E) Schematic diagram showing the proposed feedback regulatory loop in breast cancer. ***P<0.001. AKT, protein kinase B; EGFR, epidermal growth factor receptor; DFS, disease-free survival; HER2, human epidermal growth factor receptor 2; Skp2, S-phase kinase-associated protein 2; TCGA, The Cancer Genome Atlas; TNBC, triple negative breast cancer; USP18, ubiquitin-specific peptidase 18.

USP18 increases EGFR expression and activates the AKT/Skp2 pathway. To confirm the effects of USP18 on EGFR expression and the AKT/Skp2 pathway in breast cancer cells, MCF-7 and MDA-MB-231 cells were transiently transfected with USP18 and empty vector plasmids. As shown in Fig. 6A, USP18 overexpression upregulated the protein expression levels of EGFR and Skp2, and increased EGF-mediated AKT phosphorylation in MCF-7 and MDA-MB-231 cells. Following 5 min of EGF stimulation, the activation levels of AKT in USP18-transfected cells were higher than those in empty vector plasmid-transfected cells. In addition, Skp2 mRNA expression was significantly downregulated in USP18 KO mice (Fig. 6B). In order to further evaluate the association between USP18 and Skp2 in breast cancer, USP18 and Skp2 mRNA expression, and p27 protein expression data were retrieved from TCGA dataset. Linear regression analysis demonstrated a significant positive correlation between USP18 and Skp2 expression (Fig. 6C), and a significant negative correlation between USP18 and P27 in TCPA_BRCA dataset (Fig. 6D). Notably, the effects of USP18 on Skp2 expression were attenuated following treatment with the phosphoinositide 3-kinase inhibitor LY294002 (Fig. 6E). In a previous study, FoxO3, which is a negative regulator of Skp2, was reported to be negatively regulated by AKT (16). As presented in Fig. 6F, the results of a bioinformatics analysis demonstrated that the promoter region of Skp2 contains a FoxO3 binding site. In addition, among patients with high FoxO3 expression, USP18 was positively associated with Skp2 expression; however, among patients with low FoxO3 expression, USP18 was not correlated with Skp2 expression, thus indicating that USP18 may upregulate Skp2 in a FoxO3

(AKT)-dependent manner in breast cancer. These findings suggested that USP18 may increase EGFR expression and promote AKT/Skp2 pathway activation in breast cancer.

Clinical significance of the USP18/AKT/Skp2 pathway in breast cancer. In order to gain further support for the role of USP18 in breast cancer progression, TCGA dataset was used. Notably, the expression levels of USP18 were positively associated with increasing TNM stages of breast tumors (Fig. 7A). To further analyze the prognostic value of USP18 in breast cancer tissues, a Kaplan-Meier analysis was conducted on TCGA dataset. As shown in Fig. 7B, high USP18 expression was significantly associated with a worse disease-free survival (log-rank $P<0.05$) of patients. In addition, the expression levels of USP18 in breast cancer tissues from various breast cancer subtypes were analyzed from TCGA dataset. As shown in Fig. 7C, compared with in matched normal tissues, USP18 was significantly upregulated in HER2-positive patients, which is consistent with the role of Skp2, as reported in a previous study (17). In addition, the expression of USP18 in HER2-positive breast cancer was increased compared with in other types of breast cancer. Further stratification of the patient groups, based on the expression of USP18/Skp2, demonstrated that patients with high expression of both USP18 and Skp2 had the worst survival rate (Fig. 7D). These results suggested that USP18/Skp2 may act as a potential biomarker in breast cancer.

Discussion

It has previously been reported that USP18 exerts it functions via two functional domains, one of which is responsible for isopeptidase activity toward ISG15 (18,19), the other of which inhibits IFN signaling and Janus kinase/signal transducer and activator of transcription activation by blocking the type I IFN receptor 2 subunit (4,20,21). Previous studies have suggested that USP18 serves its role in an isopeptidase-independent manner by removing ISG15 mediated-ISGylation. For example, USP18 inhibits nuclear factor (NF)-κB essential modulator (NEMO) ubiquitination and NF-κB signaling by masking the UBAN domain of NEMO (22). USP18 also recruits USP20 to promote the innate antiviral response via deubiquitination of stimulator of IFN genes/MITA (23). In addition, USP18 upregulates gene expression by inhibiting relative microRNA (miR) expression (14). Ubiquitin-specific protease-like 1 is a small ubiquitin-like modifier-deconjugating enzyme, which has been reported to exert stabilization of Cajal bodies in an isopeptidase-independent manner (24). In a previous study, USP18 KO mice were initially generated on a C57Bl/6 and 129 mixed background; these mice displayed neurological symptoms and hydrocephalus, and did not survive beyond 5 months (25). Compared with USP18 KO mice, $USP8^{C61A/C61A}$ mice do not exhibit brain abnormalities or increased lethality, but do exhibit enhanced viral resistance against vaccinia and influenza B viral infections. USP18 is one of the smallest members of the USP family, which does not comprise any other prominent domains besides the catalytic core (26); therefore, it is surprising that $USP18^{C61A/C61A}$ mice develop a phenotype so different from those lacking USP18. These findings suggested that non-enzymatic functions of USPs may be more common than generally assumed. Therefore, loss-of-function experiments and RNA interference (RNAi)-based screens inhibiting ubiquitin- and UBL-deconjugating enzymes (DUBs) must be cautiously interpreted.

In an RNAi screen of 106 genes associated with deubiquitination, silencing of USP18 reduced EGFR, which is frequently overexpressed or mutated in human cancer (27), without affecting the levels of other receptor tyrosine kinases (15). In addition, mechanistic studies demonstrated that depletion of USP18 led to upregulation of miR-7, which consequently suppressed EGFR mRNA translation and induced apoptosis (14,15). Another RNAi screen of 53 DUBs revealed that loss of USP18 enhanced bortezomib or etoposide-induced apoptosis (5); this finding was further supported by the finding that overexpression of USP18 suppressed IFN-α-, tumor necrosis factor-related apoptosis-inducing ligand- or bortezomib-induced apoptotic signaling in MCF7 breast cancer cells. Notably, a catalytically inactive mutant of USP18 exerted similar effects (5). In addition, reduction of mammary tumor growth was observed in Usp18 null mice (4). These studies indicated that USP18 may serve a tumor-promoting role in breast cancer via the non-enzymatic function.

Consistent with the results of a previous study, the present results indicated that cell cycle progression and the AKT signaling pathway were significantly dysregulated in patients with breast cancer (28). The EGFR downstream AKT/Skp2-associated pathway has a crucial role in regulating the cell cycle and cell proliferation in tumorigenesis (29). In addition, Skp2 overexpression has been observed in several types of human cancer, including breast cancer (30). The present results demonstrated that USP18 upregulated EGFR and promoted the AKT/Skp2 pathway in breast cancer cells, and was correlated with an unfavorable prognosis in patients with breast cancer. In a previous study, AKT-mediated Skp2 phosphorylation stabilized Skp2 expression and enhanced its E3 ligase activity (31). The activity of FoxO3 is negatively regulated by oncogenic kinases, such as AKT (32). Foxo3a activity is negatively regulated by oncogenic kinases, such as AKT, which are known to be activated in human cancer, thus raising the possibility that oncogenic kinases may trigger Foxo3a inactivation during cancer development. The results of the present study revealed that Skp2 may be regulated by USP18/AKT in a FoxO3-dependent manner in breast cancer. In addition, USP18 was significantly upregulated in HER2-positive breast cancer, which is consistent with the role of Skp2 in a previous study (17). Notably, Skp2 also promotes AKT K63-linked ubiquitination and EGF-mediated AKT activation, and serves as a prognostic marker in HER2-positive patients (17). The present finding revealed that combined expression of USP18 and Skp2 improved predictive prognostic capability in patients with breast cancer. These findings suggested that USP18 may promote the AKT/Skp2 pathway by upregulating EGFR; Skp2 may also serve as a positive feedback loop for AKT activity in breast cancer (Fig. 7E).

Recent evidence has demonstrated that dissemination may occur during the early stages of tumorigenesis and prior to the clinical manifestation of breast cancer (33). Over the last decade, aberrant DNA methylation has been reported to be a hallmark of cancer (34), which occurs very early in breast cancer development (35). Notably, in humans, USP18 expression is reduced in patients with multiple sclerosis (MS) compared with in healthy individuals (36). A genetic study revealed that MS is associated with one single-nucleotide polymorphism, rs9618216 (C/T),

which is located in the promoter region of USP18 (37). These data indicated that the promoter region of USP18 has a key role in USP18 regulation, and the present findings suggested that abnormal promoter methylation may affect USP18 transcriptional expression during the very early stages of breast cancer. In conclusion, these findings may provide a novel insight into the cancer-promoting effects of USP18 and its potential clinical application in breast cancer, thus suggesting that the USP18/AKT/Skp2 pathway may act as a crucial regulator in the occurrence and progression of breast cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HG conceived and designed the experiments; YT performed the experiments, analyzed the data and wrote the paper; GZ, XW and WC contributed reagents, materials and analytical tools. In addition, GZ performed GO, KEGG and PPI analyses, XW performed GSEA and methylation data analysis, and WC performed TCGA data analysis.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Qilu Hospital of Shandong University (Qingdao, China) and was conducted according to the Declaration of Helsinki. All volunteers provided written informed consent.

Consent for publication

All volunteers provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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