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SP1 promotes triple-negative breast cancer progression by targeting USP5

Shi-Yi Wu[1†], Zi-Mei Peng[1†], Feng-Yi Deng[1], Jin-Yong Xiong[1], Pu-Ying Luo[2], Xiao-Jian Han[3] and Zhen Zhang[1,4*]

Abstract

Background Triple-negative breast cancer (TNBC) is characterized by the absence of targeted therapies and a dismal prognosis, necessitating a critical exploration of the molecular mechanisms driving TNBC pathogenesis and the identification of novel therapeutic targets. While dysregulated USP5 expression has been observed in various malignancies, its specific functions and mechanisms in TNBC remain poorly understood.

Methods The study utilized a combination of TCGA database analysis, immunohistochemistry staining (IHC), quantitative RT-PCR, and western blotting assay to investigate the expression of USP5 and SP1 in TNBC. Furthermore, the study examined the role of the SP1-USP5 axis and the USP5 inhibitor periplocin in TNBC progression through CCK-8 assay, colony formation assay, EDU incorporation assay, and tumor xenograft experiments. Additionally, the study explored the underlying mechanisms involved in the regulation of USP5 expression in TNBC using luciferase assay, ChIP-qPCR, quantitative RT-PCR, and western blotting assay. In order to ascertain potential inhibitors of USP5 activity, a combination of the Molecular Operating Environment (MOE) multi-functional docking platform, cellular thermal shift assay, and in vitro USP5 activity assay were utilized.

Results In the current investigation, it was observed that the expression of USP5 was elevated in TNBC and was significantly correlated with decreased overall survival rates among patients. The upregulation of USP5 was found to be mediated by the transcription factor SP1 through its binding to the USP5 promoter, consequently facilitating the progression of TNBC. Notably, the natural compound periplocin was identified as a promising inhibitor of USP5, demonstrating potential efficacy in impeding the advancement of TNBC.

Conclusions Our research findings indicate that the SP1-USP5 signaling pathway is significantly involved in the advancement of TNBC, and periplocin's ability to target USP5 presents a potential therapeutic approach for managing TNBC. These results offer valuable insights for the development of novel treatment strategies for TNBC patients.

Keywords TNBC, USP5, SP1, Cancer progression

†Shi-Yi Wu and Zi-Mei Peng contributed equally to this work.

*Correspondence:
Zhen Zhang
nkzhenzhang@163.com
[1]Institute of Clinical Medicine, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, 152 Aiguo Road, Nanchang, Jiangxi 330006, China
[2]Jiangxi Maternal and Child Health Hospital, Nanchang, Jiangxi, China
[3]Institute of Geriatrics, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, Nanchang, Jiangxi, China
[4]Jiangxi Province Key Laboratory of Immunity and Inflammation, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, Nanchang, Jiangxi, China

Background

Breast cancer is the predominant neoplastic ailment among women, with a rising incidence. It is categorized into luminal, HER2-overexpressing (HER2+), and triple-negative subtypes [1]. Despite constituting only 10–15% of breast cancer cases, triple-negative breast cancer (TNBC) is responsible for over one-third of breast cancer-related fatalities [2]. Due to the absence of specific treatments and restricted therapeutic choices, the prognosis for TNBC patients is notably inferior in comparison to other breast cancer subtypes. Hence, it is imperative to elucidate the molecular mechanisms associated with TNBC, pinpoint potential therapeutic targets, and devise personalized targeted treatment strategies.

Ubiquitin-specific protease 5 (USP5), alternatively referred to as Isopeptidase T (ISOT), is a member of the USPs subfamily and is implicated in various diseases through its ability to cleave the isopeptide bond between Ubiquitin and the target protein, notably in cancer. For instance, USP5 serves as a key regulatory factor in the progression of colorectal cancer and lung cancer by deubiquitinating and stabilizing PD-1. Conditional knockout of USP5 has been shown to enhance the production of effector cytokines and impede tumor growth [3]. Furthermore, research indicates that USP5 is significantly upregulated in lung cancer, and its suppression inhibits cancer advancement by modulating the PARP1-mediated mTOR signaling pathway [4]. In colorectal cancer, USP5 is prominently expressed in tumor tissues and facilitates tumor growth and resistance to chemotherapy by deubiquitinating TUFM [5]. Moreover, our previous study has demonstrated that the USP5-mediated deubiquitination and stabilization of PFKP were essential for cancer cell aerobic glycolysis and TNBC progression [6]. However, the mechanisms of USP5 expression in TNBC remain unclear.

Recently, there has been a growing interest in naturally occurring phytochemicals due to their potential anticancer effects [7]. Periplocin, a natural cardiotonic steroid commonly found in Periploca forrestii, has been shown to play a significant role in preventing various diseases. Research indicates that periplocin can inhibit osteoclastogenesis and provide protection against osteoporosis by targeting low-density lipoprotein receptor-related protein 4 (LRP4) [8]. Additionally, periplocin has been found to reduce cell viability and induce apoptosis in TNF-α-induced rheumatoid arthritis fibroblast-like synoviocytes by inhibiting the NF-κB signaling pathway, suggesting a promising treatment option for rheumatoid arthritis [9]. Furthermore, numerous studies have demonstrated the tumor-suppressing properties of periplocin. Specifically, periplocin has been shown to impede the progression of hepatocellular carcinoma by inducing G2/M arrest, facilitating apoptosis, and inhibiting myeloid-derived suppressor cell accumulation through suppression of the AKT/NF-κB pathway [10]. And periplocin has been shown to restore sensitivity to gemcitabine in gemcitabine-resistant pancreatic cancer cells through the regulation of Nrf2-mediated signaling pathways, suggesting that a combination of periplocin and gemcitabine targeting Nrf2 may be a promising therapeutic approach for gemcitabine-resistant pancreatic cancer [11]. However, the specific anticancer effects and mechanisms of periplocin in TNBC have not been elucidated.

The objective of this study is to investigate the expression, functions, and regulatory mechanisms of USP5 in TNBC. Moreover, the study aims to develop small molecule inhibitors targeting USP5 and assess their inhibitory effects on the progression of TNBC.

Methods

Database analysis

Gene expression data for USP5 were obtained from the TCGA BRCA cohort through the UCSC Xena browser [12], followed by an analysis of USP5 expression profiles in TNBC and normal samples. Additionally, the UCSC ENCODE [13], JASPAR [14], and PROMO [15, 16] platforms were utilized to predict the transcription factors involved in the regulation of USP5 transcription.

Tissue microarray and IHC staining

Tissue microarrays containing 172 cases of human TNBC and 130 cases of para-carcinoma tissues were obtained from Shanghai Superbiotek Pharmaceutical Technology Co., Ltd. Written informed consent for using clinical information and tissue samples was obtained from all patients. The samples were stained with a 1:200 dilution of an anti-USP5 antibody (Proteintech, 10473-1-AP) and a 1:200 dilution of an anti-SP1 antibody (Proteintech, 21962-1-AP) using the IHC Detection System (ZSGB-BIO, Beijing, China) according to the manufacturer's protocol. The IHC score was determined based on the percentage of positively stained cells and staining intensity. The quantity score is categorized on a scale from 0 to 4, where 0 indicates no immunostaining; 1 corresponds to 1–14% of the areas exhibiting positivity; 2 denotes 15–49% positivity; 3 represents 50–74% positivity; and 4 signifies ≥ 75% positivity. The intensity of staining is assessed as follows: 0 for no coloration, 1 for light yellow, 2 for light brown, 3 for brown, and 4 for dark brown. The overall score for each tissue sample is determined by summing the intensity and quantity scores, resulting in a possible range of 0 to 8. Samples with an immunohistochemistry (IHC) score greater than 4 are classified as exhibiting high expression, whereas those with an IHC score of 4 or less are classified as exhibiting low expression.

Cell culture

The MDA-MB-231, SUM-159, MCF-10 A, and 293T cell lines were procured from the American Type Culture Collection (ATCC) and Asterand Bioscience. We ensured that all cell lines were authenticated by STR profiling and tested for mycoplasma infection. The MDA-MB-231 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 environment. SUM-159 and 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere. MCF-10 A cells were cultured in DMEM/F12 medium supplemented with 5% horse serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, and 100 ng/mL cholera toxin in a humidified incubator at 37 °C with 5% CO_2.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from cells utilizing TRIzol, followed by cDNA synthesis using a cDNA Synthesis SuperMix (TransGen, Beijing, China). The amplification of specific USP5 or SP1 products was conducted through quantitative PCR with a TransStart Green q-PCR SuperMix kit (TransGen, Beijing, China), with GAPDH serving as a normalization control. The primer sequences can be found in Table S1.

Immunoblotting assay

Total protein was extracted using RIPA lysis buffer (CWBIO, China), and protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Following the boiling of protein samples in SDS loading buffer at 99 °C for 10 min, the samples were analyzed using SDS/PAGE gels and subsequently transferred to a PVDF membrane (Millipore). The membranes were then incubated overnight at 4 °C with a 1:1000 dilution of an anti-USP5 antibody (Proteintech, 10473-1-AP), a 1:1000 dilution of an anti-SP1 antibody (Proteintech, 21962-1-AP), a 1:1000 dilution of an anti-PFKP antibody (Santa Cruz, sc-514824), and a 1:1000 dilution of an anti-β actin antibody (Santa Cruz, sc-47778) after being blocked for 1 h in 5% skimmed milk in TBS-T. Following this, the membranes underwent exposure to a secondary antibody at room temperature for a duration of 1 h and were subsequently visualized utilizing an ECL reagent.

Lentiviral knockdown and expression systems

The human complementary DNA (cDNA) fragment encoding the complete USP5 or SP1 sequence was generated through PCR and inserted into the pLV-EF1-MCS-IRES-Bsd vector (Biosettia). Specific shRNA targeting USP5 or SP1 was annealed and subcloned into the pLV-H1-EF1α-puro vector (Biosettia). HEK293T cells were then co-transfected with lentiviral vectors and packaging plasmids using Lipofectamine 2000 reagent (Invitrogen) in order to produce lentiviral particles. The primer sequences of USP5 and SP1 are listed in Table S1.

Cell proliferation assay

In the CCK-8 assay, cells were seeded at a density of 3×10^3 per well in 96-well plates and incubated for specified time points at 37 °C in a 5% CO_2 environment. Following the incubation period, 10 ml of CCK-8 reagent (Dojindo) was added and allowed to incubate for 2 h at 37 °C. Subsequently, the absorbance at 450 nm was measured.

For the colony formation assay, cells were seeded at a density of 1×10^3 per well in 6-well plates and cultured for a duration of 2 weeks at 37 °C in a 5% CO_2 environment. The resulting colonies were then fixed in 4% paraformaldehyde for 30 min and stained with 1% crystal violet for 15 min.

The EdU incorporation assay was conducted by utilizing the EdU Imaging Kits-Cy3 (APExBIO) in accordance with the manufacturer's instructions. Specifically, cells were exposed to 50 mM EdU for a duration of 2 h, followed by fixation in 4% paraformaldehyde for 15 min. Subsequent steps involved permeabilization with 0.5% Triton X-100 for 20 min, staining with a click reaction cocktail for 30 min, and nuclear staining with 5 mg/mL Hoechst 33,342 for 5 min. The quantification and visualization of EdU-positive cells were performed under a microscope.

Tumor xenograft experiments

The mouse experiments conducted in this study were ethically approved by the Ethics Committee at Jiangxi Provincial People's Hospital. Six-week-old female NOD-SCID mice were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd. Tumor cells of TNBC were suspended in phosphate-buffered saline (PBS) and subsequently inoculated into the mammary fat pads of the NOD-SCID mice. The tumors were visible approximately one week after engraftment, and then the size of tumor was measured every 3 days starting from the eighth day. After a period of 20 days, the mice were euthanized, and the tumors were excised, weighed, and documented through photography. In the periplocin treatment assay, approximately six days after engraftment, mice were randomly allocated into two groups and administered periplocin (20 mg/kg, once every 2 days, via intraperitoneal injection) or vehicle for a further 2 weeks. Subsequently, the tumors were collected, weighed, and photographed.

Luciferase assay

The human USP5 promoter sequences were amplified via PCR from human genomic DNA and inserted into

the pGL3-basic vector (Promega). Cells were then transfected with either wild-type or mutant human USP5 promoters in 24-well plates. Lysates were prepared 48 h post-transfection, and luciferase activity was quantified using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's guidelines. Luciferase activity was standardized against Renilla luciferase values. The primer sequences for the human USP5-WT and USP5-MUT promoters can be found in Table S1.

ChIP-qPCR

The ChIP assays were conducted in accordance with the manufacturer's instructions using the EZ-ChIP kit (Millipore). Cells were treated with a 1% formaldehyde solution for 10 min at room temperature, followed by quenching with 125mM glycine. Chromatin extracts containing DNA fragments were immunoprecipitated with an anti-SP1 antibody (Proteintech, 21962-1-AP). The enriched DNA from the ChIP was then subjected to quantitative PCR using specific primers detailed in Table S1.

Molecular Docking experiment

Molecular docking studies were conducted using the Molecular Operating Environment (MOE) software. Protein Data Bank (PDB) format files of USP5 were retrieved from the PDB database and subsequently imported into the MOE software for preprocessing, which involved the removal of water molecules and ligand impurities. The three-dimensional structures of small molecular drugs were sourced from the PubChem database. Utilizing a semi-flexible docking approach, USP5 was docked with periplocin within the MOE software. The docking parameters employed included Rescoring 1: London dG and Rescoring 2: GBVI/WSA dG. The docking results were ultimately visualized using the MOE software.

Cellular thermal shift assay

The cellular thermal shift assay was carried out as previously outlined in the literature [17]. In summary, cells were exposed to a high concentration of periplocin for a duration of three hours, followed by collection in PBS with a complete protease inhibitor cocktail. The cells were then individually incubated at temperatures of 40℃, 44℃, or 48℃ for a period of three minutes. Subsequently, the cells underwent freeze-thawing in liquid nitrogen, with the resulting supernatant being collected. The proteins were then analyzed using Western blotting assay with a USP5 antibody (Proteintech, 10473-1-AP).

In vitro USP5 activity assay

Ubiquitin-AMC assays were utilized to assess the deubiquitinase activity of USP5. The reaction mixture consisted of 50mM HEPES, 0.5mM EDTA, 0.1 M NaCl, 1mM DTT, 0.1 mg/ml BSA, 50nM USP5 protein, and 0.1μM periplocin, which was then incubated at 37℃ for 30 min. Subsequently, 250nM Ubiquitin-AMC was introduced, and the fluorescence intensity was monitored for an additional 30 min.

Statistical analysis

Data analysis was performed utilizing GraphPad Prism 9 (GraphPad Software Inc) and SPSS-22 (SPSS Inc). The data from the experiments were reported as means ± SEM. Spearman's rank correlation test was employed to assess the correlation of gene expression in tissue samples. Statistical differences were calculated using Student's t-test or the Mann-Whitney test, as appropriate. Survival analysis was conducted using the Kaplan-Meier and log-rank tests. Statistical significance was defined as a P-value < 0.05. The r-value was used to evaluate the correlation analysis.

Results

USP5 exhibits high expression levels in TNBC and is correlated with a poor prognosis in TNBC patients

In order to investigate the potential role of USP5 in TNBC progression, we analyzed TCGA datasets. Our findings, as illustrated in Fig. 1A, demonstrate that USP5 expression is markedly elevated in TNBC samples compared to normal breast tissues. To further confirm this observation, we conducted immunohistochemistry analyses to assess USP5 expression in human TNBC and adjacent para-carcinoma tissues. Our results indicate a significant upregulation of USP5 expression in TNBC tissues relative to para-carcinoma tissues (Fig. 1B and C). The 172 cases of TNBC tissues were stratified into low-USP5-expression and high-USP5-expression groups based on USP5 expression levels (Fig. 1D). Analysis revealed a significant association between high USP5 expression and advanced TNM stage, as depicted in Fig. 1E. Survival analysis indicated that patients with elevated USP5 expression exhibited poorer overall survival outcomes (Fig. 1F). Additionally, the mRNA and protein expression levels of USP5 were assessed in TNBC cell lines (MDA-MB-231 and SUM-159) and normal cell lines (MCF-10 A). The findings indicated that the expression of USP5 was comparatively low in MCF-10 A cells, but notably elevated in MDA-MB-231 and SUM-159 cells (Fig. 1G and H). These results collectively suggest that USP5 is highly expressed in TNBC and is correlated with unfavorable prognosis in TNBC patients.

Inhibition of USP5 reduces TNBC progression in *vitro* and in vivo

To investigate the impact of USP5 on TNBC advancement, USP5 was silenced in MDA-MB-231 and SUM-159 cells using lentivirus-mediated delivery of specific short

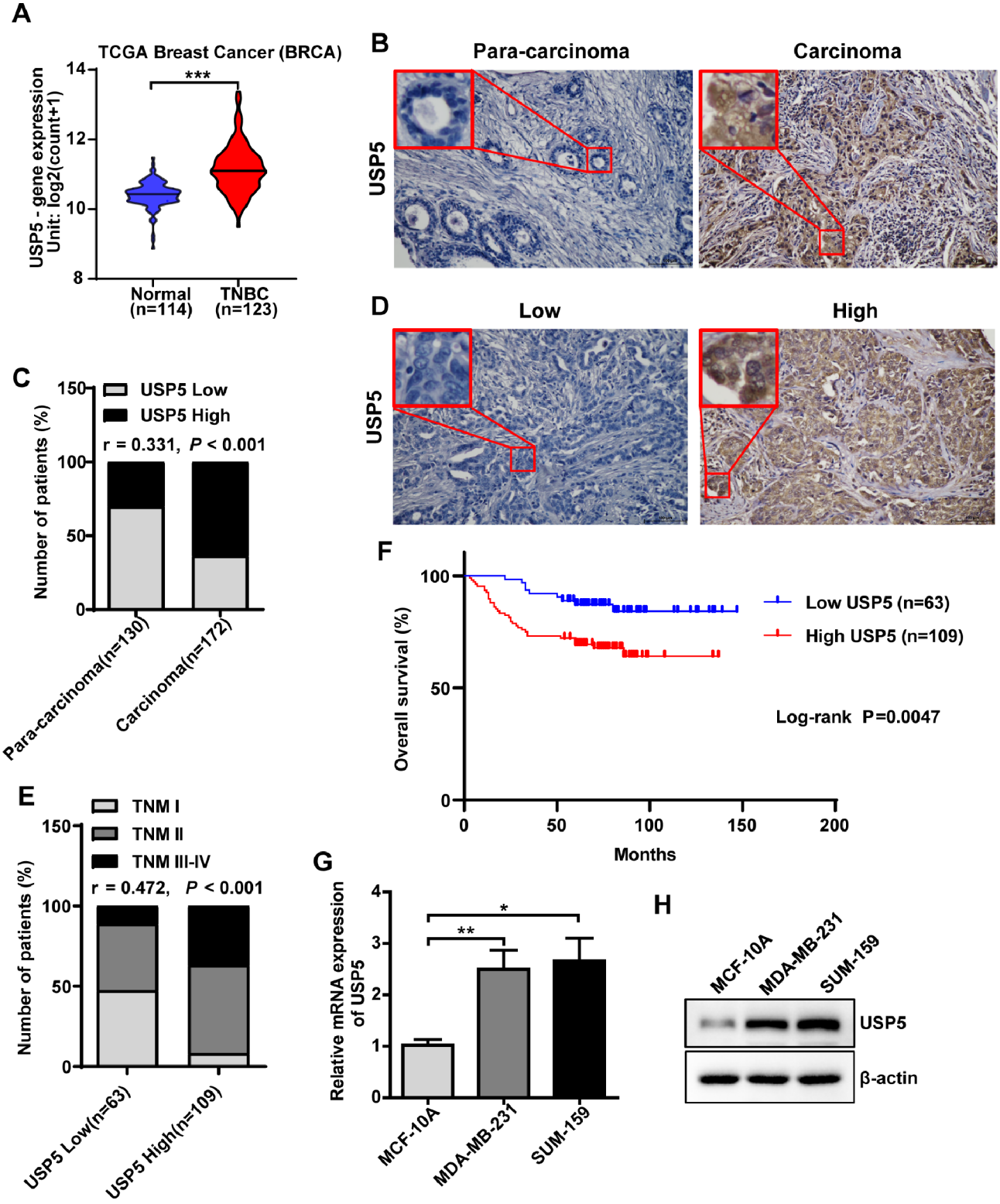

Fig. 1 USP5 is highly expressed in TNBC and is associated with poor prognosis in TNBC patients. (**A**) Expression of USP5 is higher in TNBC than in normal breast tissues in TCGA data. ***$P<0.001$. (**B**) USP5 expression was detected using an immunohistochemical technique in TNBC carcinoma and para-carcinoma tissues. (**C**) USP5 protein expression in carcinoma tissues ($n=172$) was significantly higher than that in para-carcinoma tissues ($n=130$). $r=0.331$, $P<0.001$. Scale bars, 100 μm. (**D**) Representative IHC images of USP5 in TNBC carcinoma tissues. Scale bars, 100 μm. (**E**) The expression of USP5 correlates positively with the TNM stage in human TNBC samples. $r=0.472$, $P<0.001$. (**F**) High expression of USP5 is correlated with poor overall survival of TNBC patients. Log-rank $P=0.0047$. (**G**, **H**) The USP5 mRNA (**G**) and protein (**H**) expression in normal breast epithelial cells (MCF-10 A) and TNBC cells (MDA-MB-231 and SUM-159). **$P<0.01$, *$P<0.05$

hairpin RNAs targeting USP5 (shUSP5-1 and shUSP5-2) or a control shRNA (shCtrl). The results obtained from the qRT-PCR and western blotting assays indicated a significant decrease in both mRNA and protein levels of USP5 following USP5 knockdown, as illustrated in Fig. 2A and B, and Supplementary Figure S1A, B. Subsequently, the impact of USP5 on the proliferative capacity of TNBC cells was assessed through CCK-8, clone formation, and EDU incorporation assays. The data presented in Fig. 2C-G and Supplementary Figure S1C-G demonstrated a marked reduction in cell proliferation abilities upon USP5 knockdown in MDA-MB-231 and SUM-159 cells.

Furthermore, the involvement of USP5 in TNBC progression was investigated in vivo using nude mice xenograft tumor models. In order to investigate the impact of USP5 knockdown on tumor growth, MDA-MB-231 cells were utilized to establish xenograft models in NOD-SCID mice. The results demonstrated a significant inhibition of tumor growth, size, and weight in the USP5 knockdown group (Fig. 2H-J).

USP5 is regulated by transcription factor SP1 that binds to its promoter in TNBC cells

To elucidate the molecular mechanisms underlying USP5 regulation in TNBC, bioinformatics tools such as UCSC ENCODE, JASPAR, and PROMO were employed to predict potential transcription factors involved in USP5 transcription. The findings indicate that the transcription factor SP1 may serve as a significant transcriptional regulator of USP5 expression, as illustrated in Fig. S2A. SP1, a member of the zinc finger transcription factor family,

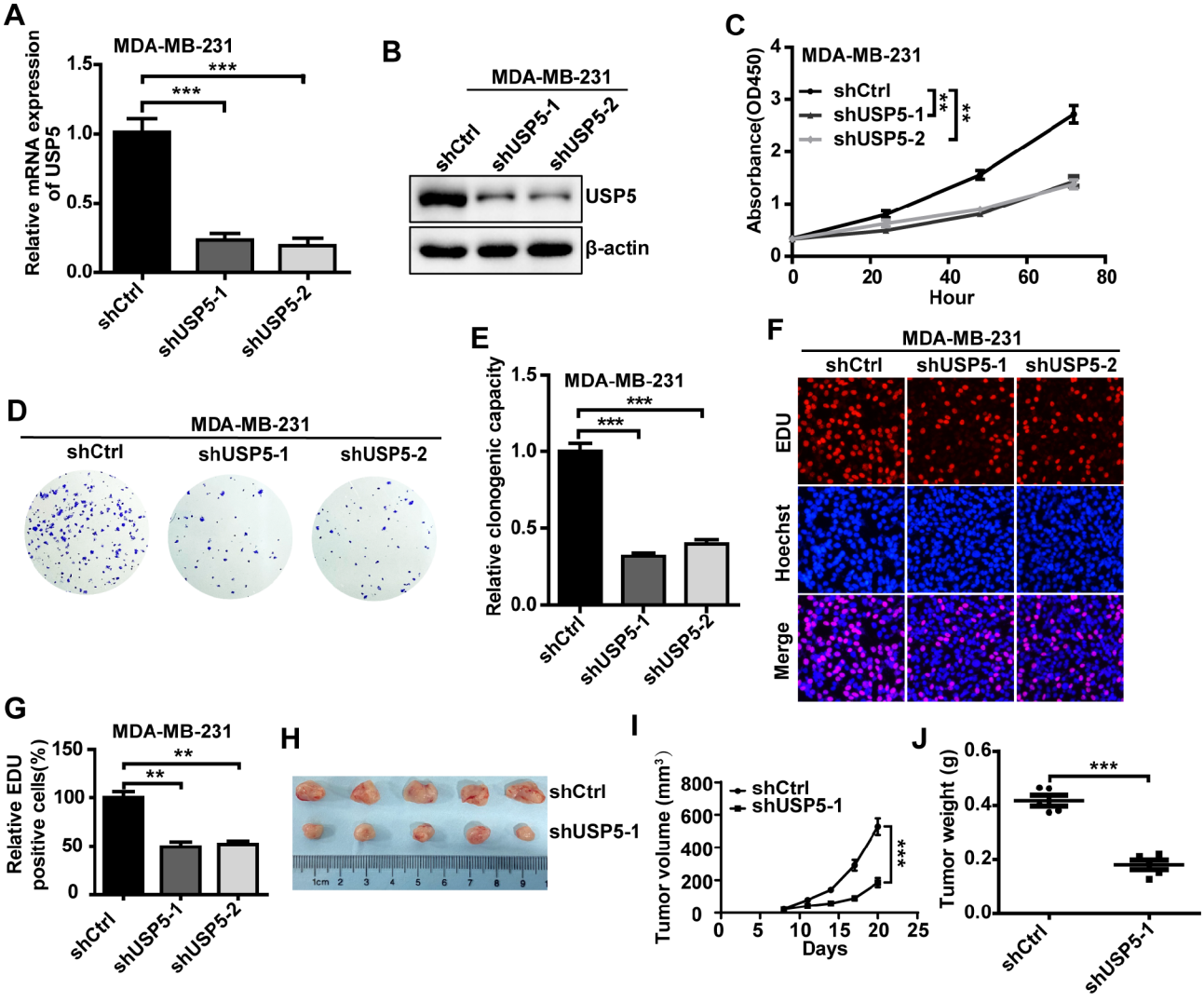

Fig. 2 Inhibition of USP5 reduces TNBC progression in vitro and in vivo. (**A**, **B**) Confirmation of USP5 knockdown by qRT-PCR (**A**) and Western blot (**B**) analysis in MDA-MB-231 cells. ***$P < 0.001$. (**C**-**G**) MDA-MB-231 cell proliferation following USP5 knockdown was evaluated using CCK8 assay (**C**), clonogenic assay (**D**, **E**), and EDU incorporation assay (**F**, **G**). ***$P < 0.001$, **$P < 0.01$. (**H**) Images of nude mice with xenograft tumors in each group. (**I**) USP5 knockdown reduces tumor growth of nude mice. ***$P < 0.001$. (**J**) USP5 knockdown reduces tumor weight of nude mice. ***$P < 0.001$

exerts its regulatory function by binding to conserved sites in the promoter regions of various genes [18]. Subsequent bioinformatic analysis conducted through the PROMO platform revealed the presence of two putative SP1 binding sites within the promoter region of USP5, as depicted in Fig. S2B. To investigate the potential role of SP1 in regulating USP5 transcription through these identified binding sites, a dual-luciferase reporter assay was initially conducted. The luciferase assay vectors were created containing wild-type or mutated SP1 binding site sequences of the USP5 promoter (Fig. 3A) and subsequently transfected into MDA-MB-231 cells along with an SP1 overexpression vector. The findings indicated that SP1 overexpression enhanced the luciferase activity of the wild-type USP5 promoter, whereas mutation of these binding sites led to a decrease in luciferase activity (Fig. 3B). Subsequently, Chip-qPCR was utilized to further substantiate this hypothesis, revealing that the overexpression of SP1 notably enhanced its binding to the identified binding sites in the USP5 promoter region (Fig. 3C and D). This observation underscores the significant role of these specific binding sites in the activation of USP5 transcription by SP1. Furthermore, qRT-PCR and western blotting analyses confirmed a decrease in both USP5 mRNA and protein levels in MDA-MB-231 and SUM-159 cells following treatment with SP1-specific shRNAs (Fig. 3E and F and Fig. S2C, D). These findings suggest that SP1 plays a role in activating USP5 transcription and promoting USP5 expression in TNBC.

SP1 promotes TNBC progression by regulating USP5 expression

Given the regulatory relationship between SP1 and USP5 expression, we hypothesized that USP5 may be involved in SP1-mediated TNBC progression. To investigate this hypothesis, we overexpressed USP5 in TNBC cells following SP1 knockdown. Our results demonstrate that exogenous overexpression of USP5 can reverse the decrease in USP5 levels caused by SP1 knockdown (Fig. 4A and Fig. S3A). Our prior research has established that the deubiquitination and stabilization of PFKP, mediated by USP5, are critical for the progression of

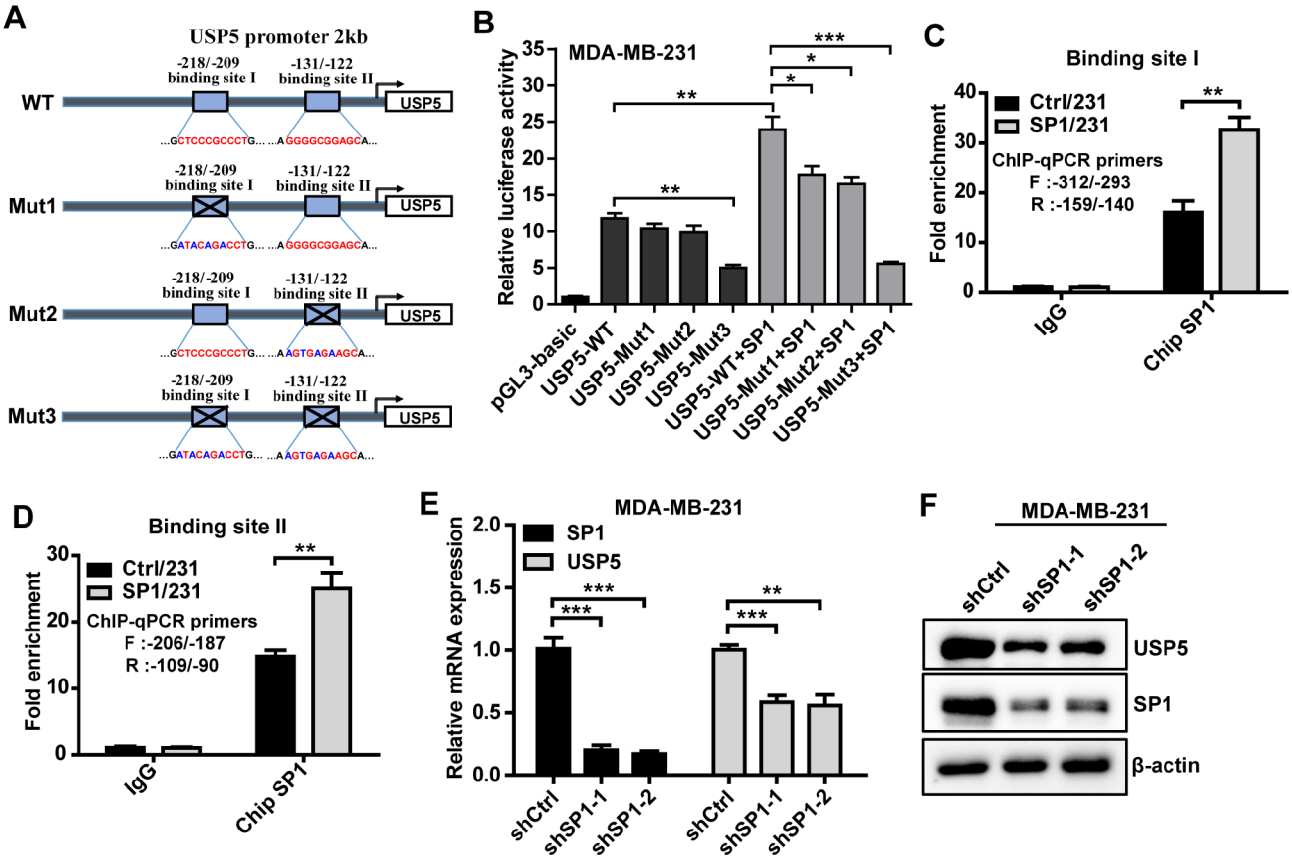

Fig. 3 USP5 is regulated by transcription factor SP1 that binds to its promoter in TNBC cells. (**A**) The diagram showed the SP1 binding sites located in the promoter region of USP5. (**B**) MDA-MB-231 cells were co-transfected with pGL3-basic, pGL3-USP5-WT, or pGL3-USP5-MUT promoter vector and pLV-Ctrl or pLV-SP1 expression vector, and the luciferase activity was determined using a dual luciferase reporter assay. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. (**C**, **D**) ChIP-qPCR analysis of SP1 binding to USP5 promoter in MDA-MB-231 cells. **$P < 0.01$. (**E**, **F**) The USP5、SP1 mRNA (**E**) and protein (**F**) expression in control and SP1 knockdown MDA-MB-231 cells. ***$P < 0.001$, **$P < 0.01$

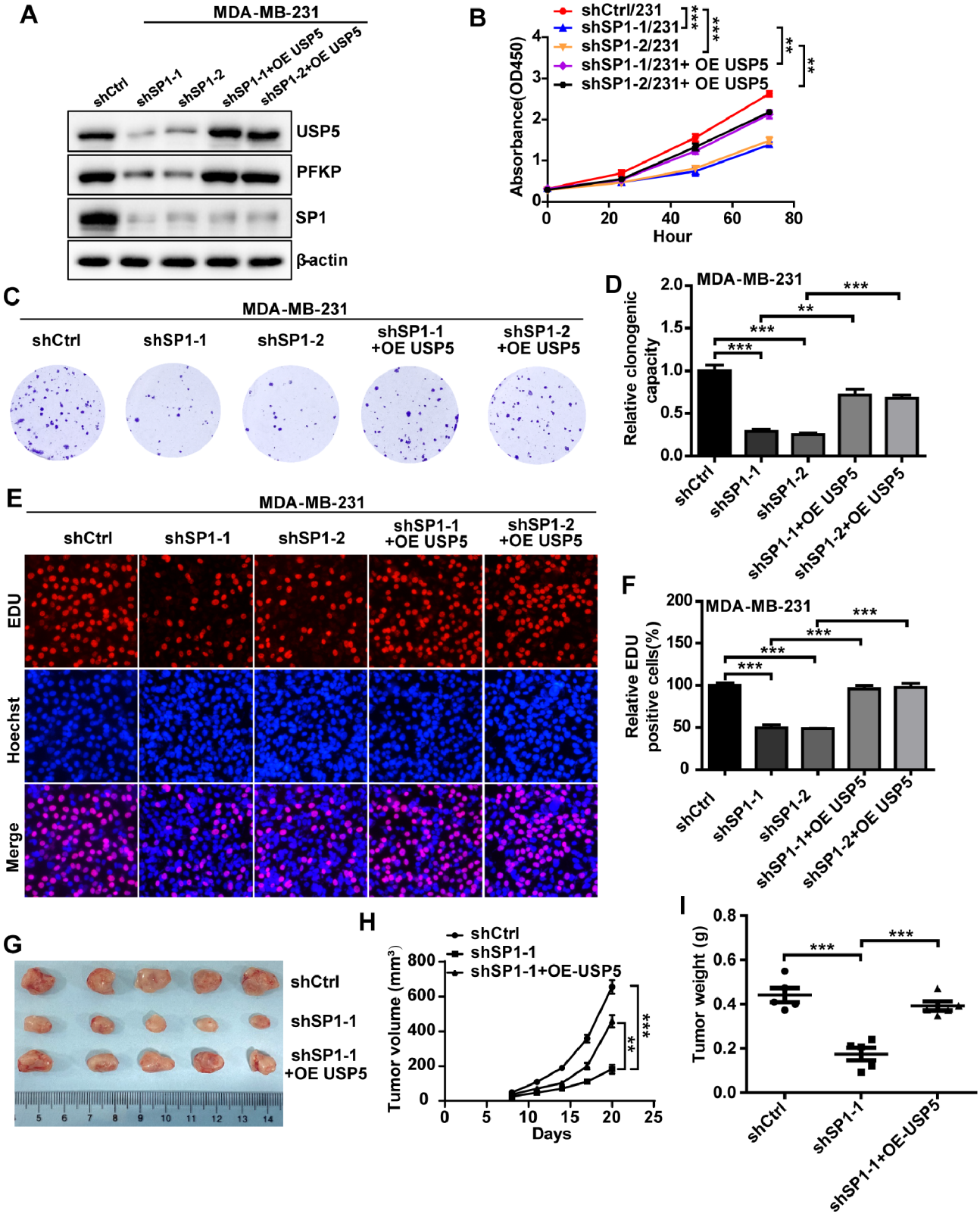

Fig. 4 SP1 promotes TNBC progression by regulating USP5 expression. (**A**) The protein expression of SP1 and USP5 in shCtrl/231 and shSP1/231 cells in the presence or absence of overexpressed USP5. (**B**-**F**) MDA-MB-231 cell proliferation following SP1 knockdown in the presence or absence of overexpressed USP5 was evaluated using CCK8 assay (**B**), clonogenic assay (**C**, **D**), and EDU incorporation assay (**E**, **F**). ***$P < 0.001$, **$P < 0.01$. (**G**) Images of nude mice with xenograft tumors in each group. (**H**) Tumor growth curve of nude mice in each group. ***$P < 0.001$, **$P < 0.01$. **(I)** Tumor weight of nude mice in each group. ***$P < 0.001$

TNBC [6]. To investigate whether SP1-mediated expression of USP5 subsequently influences PFKP expression, we examined the protein levels of PFKP in TNBC cells with SP1 interference, both with and without the restoration of USP5 expression. As illustrated in Fig. 4A and Supplementary Fig. S3A, western blot analyses revealed that SP1 knockdown resulted in the downregulation of PFKP protein expression in MDA-MB-231 and SUM-159 cell lines. However, this downregulatory effect was notably attenuated upon the restoration of USP5 expression, suggesting that SP1 modulates USP5-mediated PFKP expression in TNBC cells. Subsequent experiments utilizing CCK-8, clone formation, and EDU incorporation assays demonstrated a notable decrease in proliferation of MDA-MB-231 and SUM-159 cells following SP1 knockdown. Conversely, co-overexpression of USP5 with SP1 knockdown resulted in a significant restoration of proliferation capacity, surpassing that of SP1 knockdown alone (Fig. 4B-F and Fig. S3B-F). To further validate these findings, nude mice xenograft tumor models were employed to assess the in vivo impact of the SP1-USP5 axis on TNBC progression. Consequently, MDA-MB-231 cells depleted of SP1, with or without exogenous overexpression of USP5, were implanted into the mammary fat pads of NOD-SCID mice for evaluation of tumor growth. The findings revealed that tumor growth, size, and weight were reduced in the SP1 knockdown group; however, this effect was attenuated by the reintroduction of USP5 expression (Fig. 4G-I). The combined in vitro and in vivo data suggest that SP1 may facilitate cancer progression by enhancing USP5 expression in TNBC, underscoring the importance of the SP1-USP5 signaling pathway in driving TNBC progression.

SP1 is highly expressed and positively correlated with USP5 expression in TNBC

In order to ascertain the involvement of the SP1-USP5 signaling pathway in the progression of TNBC, immunohistochemistry was conducted to assess the expression of SP1 in human TNBC and para-carcinoma tissues. The findings indicated a significant upregulation of SP1 expression in TNBC tissues compared to para-carcinoma tissues (Fig. 5A and B). Subsequently, the 172 cases of TNBC tissues were categorized into low-SP1-expression and high-SP1-expression groups based on SP1 expression levels (Fig. 5C). The results depicted in Fig. 5D demonstrated a strong association between high SP1 expression and advanced TNM stage. Moreover, the survival analysis revealed that patients exhibiting elevated SP1 expression had a diminished overall survival rate (Fig. 5E). Furthermore, an investigation into the relationship between SP1 and USP5 in the 172 cases of TNBC tissues was conducted. The findings demonstrated a significant positive correlation between the expression levels of SP1 and USP5 (Fig. 5F). Importantly, TNBC patients with concurrent high levels of SP1 and USP5 in their tumors exhibited a shorter overall survival compared to those with low levels of both proteins (Fig. 5G), underscoring the pivotal role of the SP1-USP5 signaling pathway in TNBC progression and could be a potential therapeutic target.

Periplocin acts as a USP5 inhibitor and inhibits TNBC progression

Subsequently, we conducted a search for small molecules with the potential to inhibit USP5 activity by utilizing the Molecular Operating Environment (MOE) multi-functional docking platform to screen a natural compound library. Our analysis revealed periplocin as a promising compound that could bind to the USP5 protein, with residues Glu (400), Gln (409), and Lys (836) forming hydrogen bonds with periplocin, as illustrated in Fig. 6A, B. Additionally, the cellular thermal shift assay illustrated the efficacy of periplocin in stabilizing USP5 in MDA-MB-231 cells, suggesting direct targeting of USP5 by periplocin (Fig. 6C). To determine an appropriate concentration of periplocin for subsequent experiments, the IC50 values of periplocin were assessed in MCF-10 A, MDA-MB-231, and SUM-159 cell lines. As illustrated in Supplementary Fig. S4A, the IC50 values for periplocin were found to be 103.5 nM and 115.0 nM in MDA-MB-231 and SUM-159 cells, respectively, which are approximately half of the IC50 value observed in MCF-10 A cells (205.1 nM). Consequently, periplocin concentrations of 100 nM, which are close to and below the IC50 values for TNBC cells, were selected for further experimentation. Subsequently, the fluorescent ubiquitin-AMC assay, a dependable artificial fluorescent DUB substrate, was conducted to assess the impact of periplocin on USP5 activity. Figure 6D demonstrates that USP5 exhibited the ability to cleave Ub-AMC, resulting in the release of free AMC fluorescence. However, the impact of periplocin on this activity was notably diminished, suggesting a potent inhibitory effect of periplocin on USP5. Subsequently, the influence of periplocin on the proliferative capacity of TNBC cells was assessed using the CCK-8, clone formation, and EDU incorporation assays. The findings presented in Fig. 6E-I and Fig. S4B-F indicate that periplocin significantly inhibited cell proliferation in shCtrl/231 and shCtrl/159 cells, with a diminished effect observed in shUSP5/231 and shUSP5/159 cells. Additionally, in vivo xenograft tumor models demonstrated that periplocin effectively suppressed tumor growth, size, and weight by targeting USP5 (Fig. 6J-L). Notably, the administration of periplocin did not result in significant changes in the body weight of treated mice, suggesting the safety of this therapeutic approach (Fig. S4G). In conclusion, the data presented in this study indicate that periplocin functions as a USP5 inhibitor, effectively

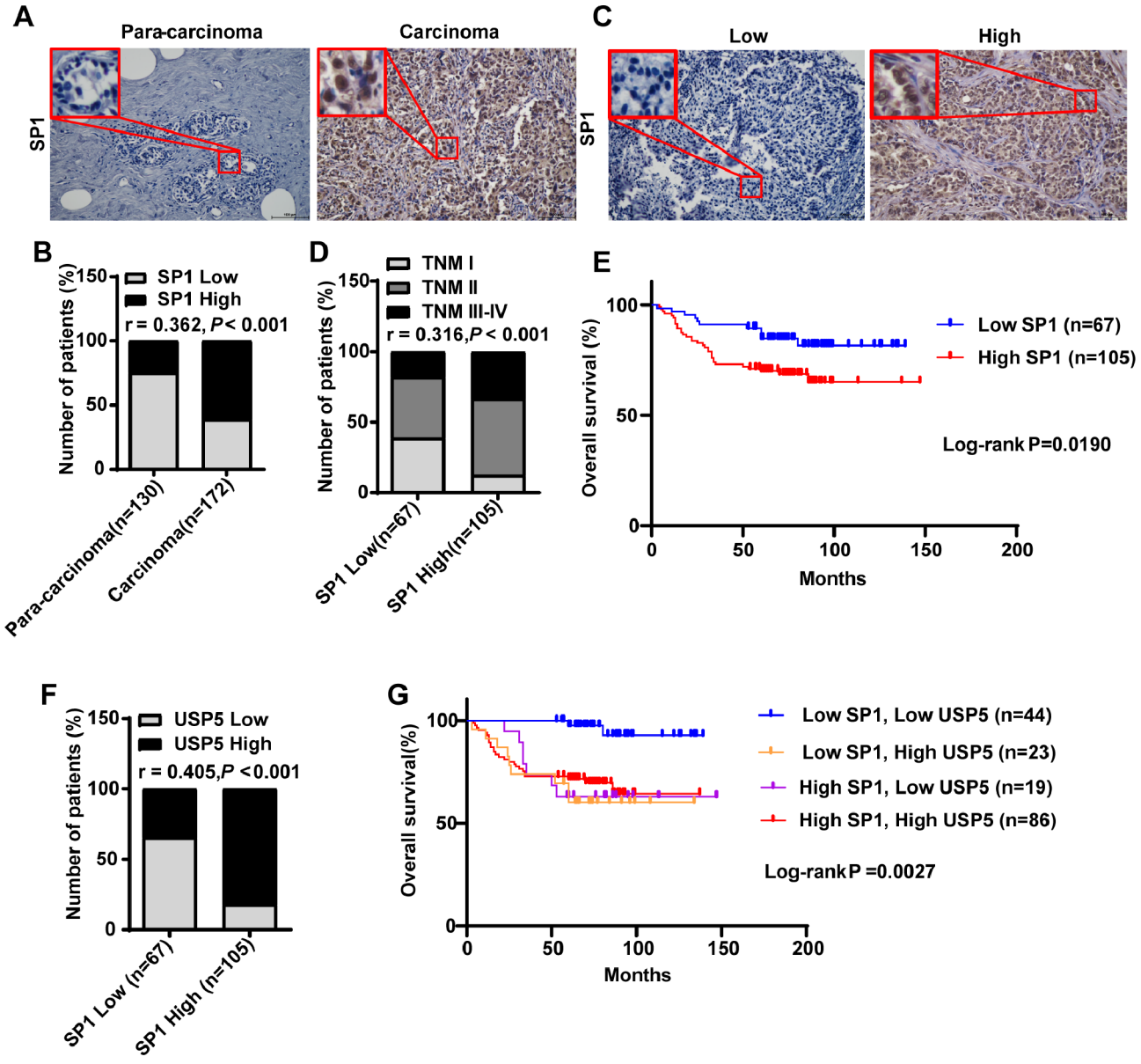

Fig. 5 SP1 is highly expressed and positively correlated with USP5 expression in TNBC. (**A**) SP1 expression was detected using an immunohistochemical technique in TNBC carcinoma and para-carcinoma tissues. (**B**) SP1 protein expression in carcinoma tissues ($n=172$) was significantly higher than that in para-carcinoma tissues ($n=130$). $r=0.362$, $P<0.001$. Scale bars, 100 μm. (**C**) Representative IHC images of SP1 in TNBC carcinoma tissues. Scale bars, 100 μm. (**D**) The expression of SP1 correlates positively with the TNM stage in human TNBC samples. $r=0.316$, $P<0.001$. (**E**) High expression of SP1 is correlated with poor overall survival of TNBC patients. Log-rank $P=0.019$. (**F**) The expression of SP1 and USP5 are positively correlated in TNBC samples. $r=0.405$, $P<0.001$. (**G**) Kaplan–Meier curves show that the overall survival of patients with high SP1 and USP5 expression in TNBC tumors was shorter than that of those with low SP1 and USP5 expression. Log-rank $P=0.0027$

impeding the progression of TNBC. This suggests that periplocin may hold promise as a therapeutic agent for the treatment of TNBC.

Discussion

The ubiquitin-proteasome system (UPS), a widely observed post-translational modification in eukaryotes, plays a crucial role in regulating various biological processes such as cell division, signal transduction, and development [19]. The UPS is composed of ubiquitin (Ub), E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, E3 ubiquitin ligase, 26 S proteasome, and deubiquitinase [20]. Dysregulation of UPS components has been increasingly associated with human diseases, particularly cancer, highlighting the significance of the UPS in therapeutic advancements [21]. For instance, bortezomib, a general proteasome inhibitor, and lenalidomide, an inhibitor of the CRL4 (CRBN) E3

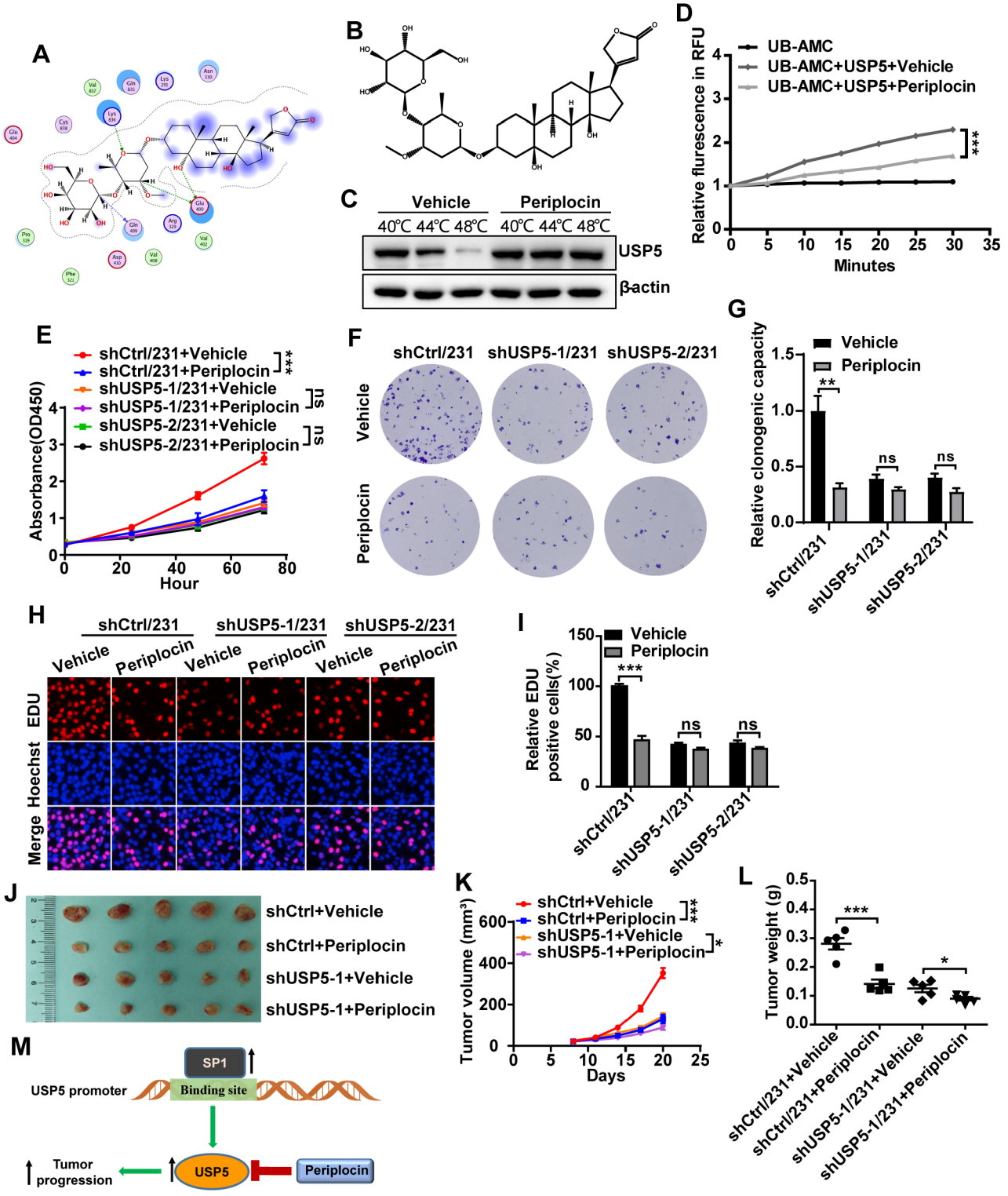

Fig. 6 Periplocin acts as a USP5 inhibitor and inhibits TNBC progression. (**A**) The mode of periplocin binding with USP5 was generated using Molecular Operating Environment (MOE) software. (**B**) Structural formula of periplocin. (**C**) Representative immunoblots from a cellular thermal shift (CETSA) assay showing the stabilization of USP5 by periplocin. (**D**) USP5-mediated UB-AMC hydrolysis treatment with vehicle or periplocin (100nM). (**E**-**I**) shCtrl/231 and shUSP5/231 cells proliferation following 100nM periplocin treatment was evaluated using CCK8 assay (**E**), clonogenic assay (**F**, **G**), and EDU incorporation assay (**H**, **I**). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. (**J**) Images of nude mice with xenograft tumors in each group. (**K**) Tumor growth curve of nude mice in each group. ***$P < 0.001$, *$P < 0.05$. (**L**) Tumor weight of nude mice in each group. ***$P < 0.001$, *$P < 0.05$. (**M**) Schematic diagram of periplocin inhibits triple-negative breast cancer progression by targeting SP1-USP5 axis (drawn using ChemDraw software)

ubiquitin ligase complex, along with its analogs, have been employed in clinical settings for the management of haematologic malignancies [22, 23]. Furthermore, various small molecule inhibitors directed towards the E3 ubiquitin ligase MDM2 have progressed to clinical trials for the treatment of human cancers [24]. This study revealed periplocin as a potential inhibitor of deubiquitinase USP5, demonstrating its role in suppressing the progression of TNBC and suggesting its potential as a candidate for cancer treatment. Our findings align with prior research indicating the potential of targeting UPS components for anti-cancer drug development. However, further exploration is needed to investigate the therapeutic potential of targeting other UPS components.

Dysregulated USP5 expression has been observed in various malignancies, including lung adenocarcinoma [25], colorectal cancer [5], and hepatocellular carcinoma [26]. Nevertheless, the precise mechanisms underlying the accumulation of USP5 in tumors remain poorly elucidated. Limited research has investigated the molecular pathways contributing to the elevated expression of USP5 in cancer, such as the role of the 18 S rRNA methyltransferase METTL5 in facilitating USP5 mRNA translation in hepatocellular carcinoma [27]. Reactive oxygen species (ROS) have been shown to facilitate the dimerization of the USP5 protein, leading to increased protein stability and enzymatic activity in lung cancer [28]. This study aims to investigate the molecular mechanisms responsible for the upregulation of USP5 in TNBC. Our findings suggest that the transcription factor SP1 directly interacts with the USP5 promoter, thereby regulating its expression. This study sheds light on the mechanisms underlying USP5 accumulation in cancer.

In the present study, the findings depicted in Fig. 3B indicate that individual mutations at either SP1 binding site I or II on the USP5 promoter did not significantly affect luciferase activity. However, concurrent mutations at both SP1 binding sites I and II resulted in a marked downregulation of luciferase activity in the USP5 promoter within both parental and SP1-overexpressing MDA-MB-231 cells. These results suggest that both SP1 binding sites I and II play crucial roles in SP1-mediated regulation of USP5 transcription in TNBC cells. Moreover, the data imply that when one binding site is mutated, the other site remains functional. Simultaneous mutation of binding sites I and II of SP1 resulted in a significant downregulation of the luciferase activity associated with the USP5 promoter in TNBC cells; however, this activity did not decrease to the levels observed with the pGL3-basic vector. This observation suggests the involvement of additional transcription factors, such as MYCN [29] and EBF1 [5], in the regulation of USP5 transcription. Furthermore, given that SP1 is a general transcription factor known to regulate various oncogenes across different human cancers—such as FGF-BP1 in breast cancer [30], TIAM2S in hepatocellular carcinoma [31], and ADAM17 in glioma [32], USP5 may be a part of its tumorigenic function, as is also shown by our data.

Natural products and their derivatives are integral to the fields of chemistry and drug discovery, given their varied chemical structures and biological activities [33]. A growing body of research has highlighted the therapeutic potential of natural compounds in combating cancer. Nevertheless, the clinical utilization of these compounds is hindered by a lack of understanding regarding their mechanisms of action and specific therapeutic targets. This study has identified periplocin as a potential inhibitor of USP5, leading to the inhibition of TNBC progression by targeting USP5. These findings suggest new therapeutic strategies utilizing natural compounds for TNBC patients. Furthermore, our results align with previous research [10, 34, 35], indicating that periplocin treatment in mice did not result in any observable side effects, affirming the safety and efficacy of periplocin as a viable candidate for cancer therapy. Furthermore, periplocin has been found to modulate various signaling pathways. For instance, it demonstrates antitumor activity by influencing the NRF2-mediated signaling pathway in gemcitabine-resistant pancreatic cancer cells [11]. Additionally, another study identified periplocin as an Akt inhibitor in hepatocellular carcinoma [10]. In the current investigation, we observed that the inhibitory effect of periplocin on the progression of TNBC was diminished in cells with USP5 depletion, indicating that periplocin treatment mitigates cancer progression through the modulation of USP5. Previous research has demonstrated that USP5 can modulate the progression of Acute Myeloid Leukemia cells through the AKT/mTOR/4EBP1 signaling pathway [36]. Additionally, USP5 has been shown to stabilize HDAC2 via deubiquitination, thereby promoting its expression while concurrently inhibiting the expression of NRF2 [37]. These findings suggest that AKT and NRF2 may serve as downstream targets of USP5. Consequently, it can be inferred that periplocin may influence cancer progression by targeting USP5, potentially affecting the function of AKT, NRF2, or other related molecules, either directly or indirectly. This finding further elucidates the substantial reduction in TNBC progression inhibition observed with periplocin treatment subsequent to USP5 knockdown in the current study.

Conclusions

Due to the lack of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression in TNBC, the utilization of endocrine or anti-HER2 treatments is not feasible. Consequently, there is a pressing necessity to pinpoint potential therapeutic targets for TNBC.

This study reveals an elevation in USP5 expression in TNBC tissues and cells, with a correlation between USP5 expression and unfavorable overall survival rates in TNBC patients. Furthermore, the inhibition of USP5 through knockdown techniques significantly impedes the progression of TNBC both in vitro and in vivo. Mechanistically, the transcription factor SP1 was found to upregulate the expression of USP5 by binding to the USP5 promoter, thereby facilitating the progression of TNBC. Our investigation further revealed that periplocin, a natural compound, has the potential to inhibit USP5 and consequently impede TNBC progression both in vivo and in vitro (Fig. 6M). Overall, our research findings indicate that the SP1-USP5 signaling pathway is significantly involved in the advancement of TNBC, and periplocin has the potential to impede TNBC progression by targeting the SP1-USP5 axis. These results offer valuable insights for the development of novel therapeutic approaches for TNBC patients.

Abbreviations

TNBC	Triple-negative breast cancer
USP5	Ubiquitin-specific protease 5
SP1	SP1 transcription factor
TCGA	The Cancer Genome Atlas
TFs	Transcription factors
IHC	Immunohistochemistry
shRNAs	Short hairpin RNAs
MOE	Molecular Operating Environment

Supplementary Information

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Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Shi-Yi Wu, Zi-Mei Peng, and Zhen Zhang: conducting most of the experiments, acquiring data, and analyzing data. Feng-Yi Deng and Jin-Yong Xiong: assist in conducting in vitro experiments. Pu-Ying Luo and Xiao-Jian Han: assist in conducting in vivo experiments. Zhen Zhang: designing, supervising and advising the project; writing and revising the manuscript. All authors read and approved the final manuscript.

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

Declarations

Ethical approval
Studies on animals were approved by the Ethics Committee at the Jiangxi Provincial People's Hospital (approval number: KT2023-013).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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