

# Beclin1 inhibition enhances paclitaxel-mediated cytotoxicity in breast cancer *in vitro* and *in vivo*

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**Abstract.** Beclin1, a key regulator of autophagy, has been demonstrated to be associated with cancer cell resistance to chemotherapy. Paclitaxel is a conventional chemotherapeutic drug used in the clinical treatment of breast cancer. However, the function and mechanism of Beclin1 in paclitaxel-mediated cytotoxicity in breast cancer are not well defined. The present study demonstrated that paclitaxel suppressed cell viability and Beclin1 expression levels in BT-474 breast cancer cells in a dose- and time-dependent fashion. Compared with the control, the knockdown of Beclin1 significantly enhanced breast cancer cell death via the induction of caspase-dependent apoptosis following paclitaxel treatment *in vitro* ( $P < 0.05$ ). In a BT-474 xenograft model, paclitaxel achieved substantial inhibition of tumor growth in the Beclin1 knockdown group compared with the control group. Furthermore, analysis of the publicly available Gene Expression Omnibus datasets revealed a clinical correlation between Beclin1 levels and the response to paclitaxel therapy in patients with breast cancer. Collectively, the present results suggest that Beclin1 protects breast cancer cells from apoptotic death. Thus, the inhibition of Beclin1 may be a novel way to improve the effect of paclitaxel. Additionally, Beclin1 may function as a favorable prognostic biomarker for paclitaxel treatment in patients with breast cancer.

## Introduction

Breast cancer is a common malignant tumor threatening women's health globally. According to the latest data from GLOBOCAN 2012, produced by the International Agency for Research on Cancer, an estimated 1.7 million new breast

cancer cases were diagnosed and 520,000 cases of mortality from the cancer occurred in 2012 globally (1). Despite emerging novel treatments options, including noninvasive surgeries (2,3), targeted therapy (4,5), chemotherapy (6,7) and radiotherapy (8,9), a chemotherapy regimen composed of paclitaxel remains the main treatment for breast cancer management (10,11). Paclitaxel is isolated from the bark of *Taxus brevifolia*, a Pacific yew, and mechanistically interferes with tubulin to stabilize microtubule composition resulting in G<sub>2</sub>/M cell cycle arrest and the subsequent apoptosis of the rapidly proliferative cancer cells (12-14). However, patients with breast cancer become insensitive to paclitaxel following a cancer-free period (15,16). Thus, there is an urgent requirement to uncover the mechanisms affecting therapeutic responsiveness and develop novel and efficacious therapeutic agents, with suitable markers for an effective chemotherapy method.

Autophagy, termed by Christian de Duve in 1963 (17), is an evolutionary, ancient and highly conserved catabolic process which degrades intracellular long-lived proteins and organelles in lysosomes (18). In the past three decades, a series of evolutionarily-conserved autophagy-associated (ATG) genes have been verified, suggesting that autophagy represents a genetically controlled process (19). Beclin1, the first identified ATG gene in mammalian cells (20), is an ortholog of the Atg6/vacuolar protein sorting-associated protein (Vps)30 protein in yeast and serves a pivotal function in autophagosome formation. It does this by interacting with Vps34 (a phosphoinositide 3-kinase class III isoform) (21), a signaling mechanism which suppresses MCF-7 cell proliferation and clonogenicity *in vitro*, causing the inhibition of tumor formation *in vivo* in nude mice (22). Beclin1 was originally considered to be a candidate for tumor suppression (22-24). Furthermore, previous reports have stated that the overexpression of Beclin1 increased the chemosensitivity of cervical cancer cells to paclitaxel (25,26). However, certain evidence also indicates that higher expression levels of Beclin1 function to protect tumor cells from the cytotoxic effects of paclitaxel in treated neuroblastoma, ovarian cancer, nasopharyngeal carcinoma, non-small cell lung cancer (NSCLC) and endometrial carcinoma cells (27-31). Therefore, whether the mechanisms of Beclin1 are cytotoxic or cytoprotective for paclitaxel therapy may be associated with the type of cancer.

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Thus far, the function of Beclin1 in the paclitaxel treatment of breast cancer has not yet been fully elucidated.

The purpose of the present study was to investigate the function and mechanism of Beclin1 in paclitaxel-mediated cytotoxicity in breast cancer cells, and to determine the association between Beclin1 levels and response to paclitaxel therapy in patients with breast cancer.

## Materials and methods

**Cell cultures and reagents.** Human breast cancer cell lines BT-474 and MDA-MB-231 and the embryonic kidney cell line 293T were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Paclitaxel was purchased from Selleck Chemicals (Houston, TX, USA). Polybrene and puromycin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

**Cell Counting Kit-8 (CCK-8) assay of paclitaxel-treated cells.** Cells ( $1.0 \times 10^4$  cells/well BT-474 cells or  $3.0 \times 10^3$  MDA-MB-231 cells) were seeded into 96-well plates. After 24 h, the cells were exposed to different concentrations of paclitaxel (0.001, 0.01, 0.1, 0.5, 1, 5, 10 and 20  $\mu$ M for BT-474 cells, or 0.001, 0.002, 0.005, 0.01 and 0.02  $\mu$ M for MDA-MB-231 cells) for 72 h at 37°C, unless otherwise stated. Each concentration was repeated in quadruplicate wells. Subsequently, cell viability was measured by adding 10  $\mu$ l CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) to each well. Plates were incubated at 37°C for 1 h and absorbance was evaluated using a Bio-Rad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm wavelength. The percentage of viable untreated control cells was set as 100%.

**Determination of cellular apoptosis in paclitaxel-treated cells by flow cytometry.** Subsequent to 0.5 or 0.002  $\mu$ M paclitaxel treatment for 72 h at 37°C for BT-474 or MDA-MB-231 cells, respectively, they were collected and stained using the Annexin V-fluorescein isothiocyanate (FITC) kit (Miltenyi Biotec, Inc., Cambridge, MA, USA) according to the manufacturer's protocol. Briefly, cells were resuspended in binding buffer (100  $\mu$ l) and stained with Annexin V-FITC (10  $\mu$ l) for 15 min at room temperature in the dark. Subsequent to adding propidium iodide solution (5  $\mu$ l), the apoptosis rate was immediately determined by calculating the percentage of cells that were positive for Annexin V, as measured using the FACS Calibur flow cytometer and CellQuest™ software (version 6.0; BD Biosciences, Franklin Lakes, NJ, USA). All experiments were performed three times.

**Western blot analysis.** The BT-474 and MDA-MB-231 cell lysates were subjected to western blot analysis. Anti-Beclin1 (cat. no. sc-11427; 1:2,000 dilution) antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-caspase-3 (cat. no. 9665; 1:1,000 dilution), anti-cleaved caspase-3 (cat. no. 9664; 1:1,000 dilution), anti-poly ADP

ribose polymerase (PARP; cat. no. 9542; 1:1,000 dilution), anti-cleaved PARP (cat. no. 5625; 1:1,000 dilution) and anti-Actin (cat. no. 4967; 1:1,000 dilution) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Western blot analysis was performed as previously described (32). Actin was used as a protein loading control. The optical density of each protein band was quantified using ImageJ software version 1.52 (National Institutes of Health, Bethesda, MD, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA extracted from BT-474 and MDA-MB-231 cells was isolated using TRIzol (Life Technologies; Thermo Fisher Scientific, Inc.) and 2  $\mu$ g total RNA was reverse transcribed using Superscript™ III transcriptase (Life Technologies; Thermo Fisher Scientific, Inc.). For RT-qPCR, cDNA was amplified using THUNDERBIRD SYBR qPCR Mix (Toyobo Life Science, Osaka, Japan) in a Chromo4™ system (Bio-Rad Laboratories, Inc.). PCR was initiated with a 10-min denaturation step at 95°C, followed by a 40-step cycle at 95°C for 15 sec and 60°C for 60 sec. The primers used were as follows: Beclin1 forward, 5'-TCT CGCAGATTCATCCCC-3' and reverse, 5'-TCTTCGGCT GAGGTTCTCCAT-3'; GAPDH forward, 5'-ACGGATTG GTCGTATTGGG-3' and reverse, 5'-TGATTTTGGAGG GATCTCGC-3'. The  $2^{-\Delta\Delta C_q}$  method was used to calculate the relative gene expression (33). GAPDH was used as an internal control for normalization.

**RNA interference knockdown of Beclin1 and virus packaging and infections.** An RNA interference technique was used to knockdown Beclin1 in breast cancer cells, as previously described (34,35). The backbone vector used for generating the lentivirus was pGC-LV, purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The sense strands of the short hairpin RNAs (shRNAs) were as follows: shControl, 5'-TTC TCCGAACGTGTCACGT-3'; shBeclin1#1, 5'-CAGTTTGGC ACAATCAATA-3'; and shBeclin1#2, 5'-CCCGTGGAAATGG AATGAGATT-3'.

To produce infectious viruses,  $3 \times 10^6$  293T cells were seeded on 100-mm plates and the day after cotransfected with lentiviral backbone plasmid and packaging plasmids (pHelper 1.0 and pHelper 2.0). All transfections were performed using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The vector pGC-LV was used as an empty vector control to assess transfection efficiency. After 48 h transfection, the medium was collected, purified, aliquoted and stored at -80°C. Virus titer was determined with a Lenti-X™ p24 Rapid Titer kit (cat. no. 632200; Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocols. For infections, BT-474 and MDA-MB-231 breast cancer cells plated in 60-mm plates for 24 h prior to infection were incubated with lentivirus at a multiplicity of infection of 20 in the presence of polybrene (8  $\mu$ g/ml) for 48 h at 37°C. After, the media containing 1  $\mu$ g/ml puromycin was added to select stably infected cell populations.

**Colony formation assay.** BT-474 (4,000 cells/well) or MDA-MB-231 (500 cells/well) cells were plated in triplicate

into 6-well plates for ~24 h in a humidified atmosphere of 5% CO<sub>2</sub> overnight at 37°C, followed by exposure to paclitaxel for 72 h at 37°C. Subsequently, cells were cultured for up to 3 weeks (BT-474) or 2 weeks (MDA-MB-231), during which time fresh medium was added every 3 days. For the colony formation assay, plates were fixed using 4% paraformaldehyde for 10 min at room temperature, stained with 0.1% crystal violet for 15 min at room temperature and counted under a light microscope (magnification, x40; Nikon Corporation, Tokyo, Japan). Colonies with >50 cells were counted per well.

***In vivo therapeutic analysis in xenograft models.*** A total of 16 six-week-old BALB/c female nude mice were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained under specific pathogen-free conditions (constant temperature, 24–26°C; humidity, 40–70%; 12/12 h light/dark cycle; free access to food and water). BT-474-shBeclin1#1 or BT-474-shControl cells (1×10<sup>7</sup> per mouse) were injected subcutaneously into the right flank of the corresponding mice previously implanted with 17β-estradiol pellets. The tumor volumes were monitored twice weekly. When the tumor volumes reached ~100 mm<sup>3</sup>, the animals were divided randomly into four groups of 4 mice each. Mice were then intraperitoneally injected with either control vehicle or paclitaxel (10 mg/kg) twice a week for a total of 8 times (4 weeks). Tumor size was monitored using calipers and calculated using the formula: Length × width<sup>2</sup>/2. The rate of tumor inhibition was evaluated by the formula: (volume of the untreated group - the volume of the paclitaxel treatment group)/volume of the untreated group × 100%. All animal experiments were performed following the National Institutes of Health guide for the care and use of laboratory animals (36), and reviewed and ethically approved by the Ethics Committee of Navy General Hospital (Beijing, China). No significant animal weight loss was observed during the *in vivo* experiments. When the control tumors reached 1,000 mm<sup>3</sup>, all mice were sacrificed by cervical dislocation subsequent to being anesthetized with sodium pentobarbital intraperitoneally (60 mg/kg). Subsequently, the tumors were collected, fixed in 10% formalin for 48 h at room temperature and immunohistochemically analyzed.

***Immunohistochemistry (IHC) staining.*** Mouse tumor tissues were embedded in paraffin and sectioned into 5 μm slices. Serial sections were dewaxed in xylene and hydrated with graded concentrations of ethanol (100, 95, 90, 80 and 70%). Epitopes were retrieved using microwave in boiling 0.01 M sodium citrate buffer (pH 6.0) for 10 min at 98°C. Slides were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min, blocked with 5% goat serum for 1 h at room temperature and incubated with primary antibodies against Beclin1 (cat. no. sc-11427; 1:100 dilution; Santa Cruz Biotechnology, Inc.) and cleaved caspase-3 (cat. no. 9664; 1:200 dilution; Cell Signaling Technology, Inc.) at 4°C overnight. The signal was detected following incubation with a commercial immunoglobulin G horseradish peroxidase-conjugated secondary antibody polymer detection system (cat. no. PV-9001; OriGene Technologies, Inc., Beijing, China) for 20 min at 37°C and 3′3-diaminobenzidine substrate (OriGene Technologies, Inc.). Finally, the slides were counterstained with 0.5% hematoxylin (Beyotime Institute of

Biotechnology, Shanghai, China) for 2 min at room temperature and observed by a light microscopy (magnification, x200; Eclipse Ti-U; Nikon Corporation). Slides incubated without the primary antibody served as the negative controls.

***Analysis of microarray data.*** Gene expression data (accession nos. GSE22513 and GSE25066) were retrieved from the National Centre for Biotechnology Information Gene Expression Omnibus (GEO) database (37). Beclin1 levels were analyzed in patients with pathologic complete response (pCR), which was defined as the lack of any invasive cancer, and non-pCR, which was defined as any viable tumor in breast or lymph nodes (partial response) or disease progression. pCR and non-pCR were determined from the primary pathologic slide selected at the time of surgery by a breast pathologist. Details of patients and microarray data were described previously (38,39). Expression data from GSE22513 were RMA summarized, quantile normalized and baseline transformed, making use of the median of all samples, using GeneSpring GX software (version 10.0.2; Agilent Technologies, Inc., Santa Clara, CA, USA). Data from GSE25066 were normalized using the MAS5 algorithm, mean centered to 600 and log<sup>2</sup> transformed.

***Statistical analysis.*** SPSS version 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Statistical significance of the data was analyzed using a two-sample Student's t-test (for two groups) or a one-way analysis of variance followed by Bonferroni's post hoc test (for multiple groups). Data were presented as mean ± standard deviation (SD). The error bars represent SD values from three independent experiments unless otherwise indicated. P<0.05 was considered to indicate a statistically significant difference.

## Results

***Paclitaxel inhibits the viability of BT-474 breast cancer cells via the induction of apoptosis.*** Initially, the effect of paclitaxel on BT-474 breast cancer cells was examined by exposing cells to various concentrations of the drug for 72 h. As the paclitaxel dose increased, the viability of BT-474 cells significantly decreased, demonstrating a dose-dependent drug effect (P<0.01; Fig. 1A). Next, a drug concentration of 0.5 μM was selected, which was known to result in an ~50% inhibition of cell viability, in order to analyze the time effect. As presented in Fig. 1B, paclitaxel significantly suppressed BT-474 cell proliferation in a time-dependent manner, as revealed by using a CCK-8 assay (P<0.001). In order to further determine whether paclitaxel-induced cytotoxicity was associated with apoptosis, an Annexin V-FITC apoptotic assay was performed, and programmed cell death was analyzed using the bright green FITC fluorescence detection of flow cytometry. The results revealed that 0.5 μM paclitaxel induced Annexin V-positive cells in 6.20±1.19, 10.54±1.32, 19.42±1.41 and 32.81±2.43% of total cells after 0, 24, 48 and 72 h of incubation (Fig. 1C and D), respectively, demonstrating that apoptosis was enhanced with time, with significant differences observed at the 48 and 72 h mark compared with untreated cells (P<0.001). Furthermore, apoptotic proteins were detected using western blot analysis.

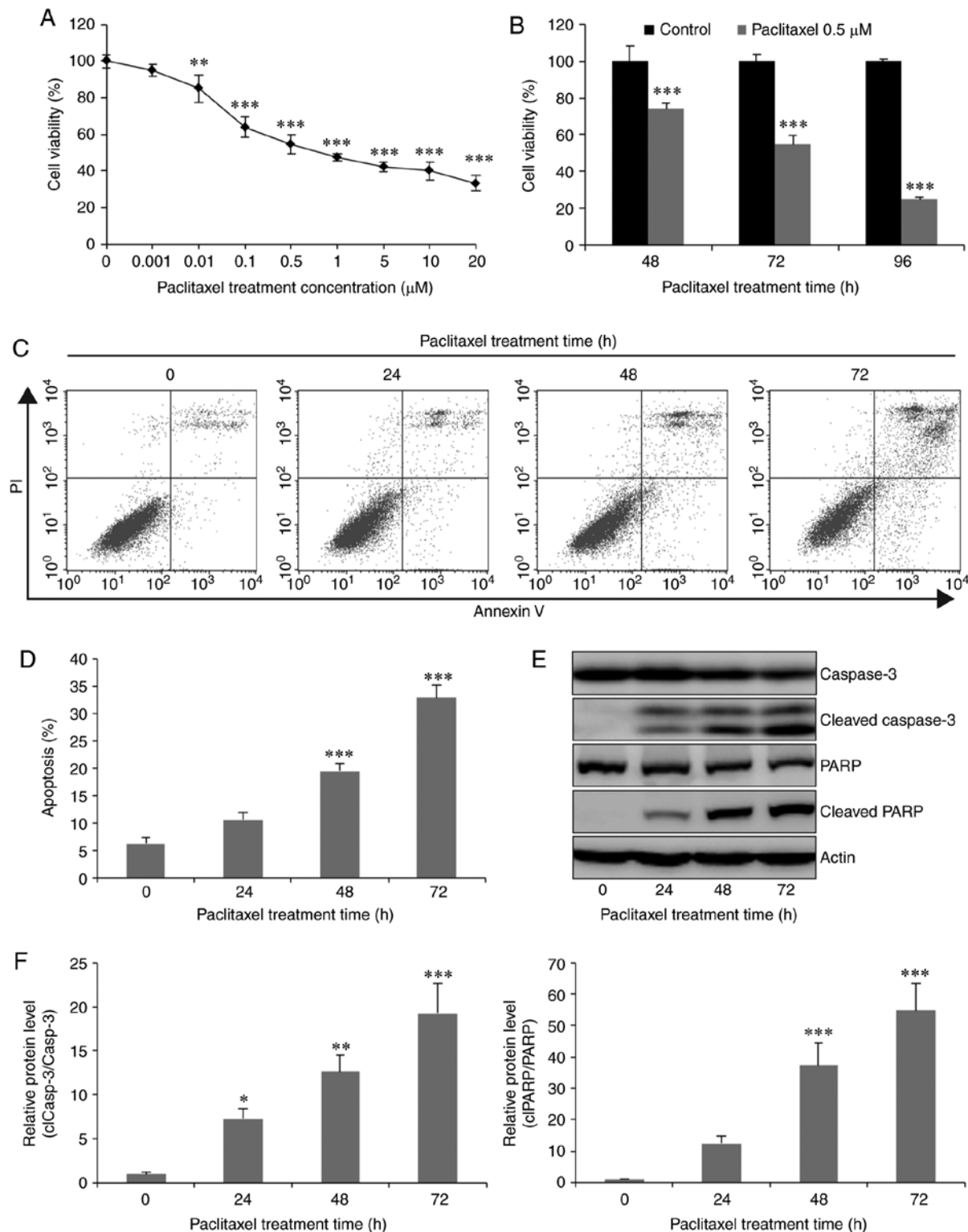


Figure 1. Cytotoxicity effects of paclitaxel in BT-474 breast cancer cells. (A) Paclitaxel suppressed cell viability in a dose-dependent fashion. BT-474 cells were exposed to paclitaxel at a series of concentration gradients for 72 h, and cell viability was determined using a CCK-8 assay ( $n=4$ ). (B) Paclitaxel inhibited cell viability in a time-dependent fashion. BT-474 cells were exposed to 0.5  $\mu\text{M}$  paclitaxel for 48, 72 and 96 h. Subsequently a CCK-8 assay was performed to detect cell proliferation ( $n=4$ ). (C) Effect of paclitaxel on apoptotic death in BT-474 cells. Cells were exposed to 0.5  $\mu\text{M}$  paclitaxel for 24, 48 and 72 h. An apoptosis assay was performed, using an Annexin V-fluorescein isothiocyanate kit and detected using flow cytometry. (D) Quantification of paclitaxel-induced apoptosis ( $n=3$ ). (E) Expression levels of apoptotic proteins examined using western blot analysis using specific anti-apoptotic antibodies, as indicated in the blots. BT-474 cells were exposed to 0.5  $\mu\text{M}$  paclitaxel for 24, 48 and 72 h. The blots were probed with an actin antibody to serve as the protein normalization control. (F) Quantitative analysis of the protein levels of cleaved caspase-3 and PARP. \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$  vs. untreated cells. Casp-3, caspase-3; cCasp-3, cleaved caspase-3; cPARP, cleaved PARP; PARP, poly ADP-ribose polymerase; CCK-8, Cell Counting Kit-8; PI, propidium iodide.

It was observed that the significantly increased cleavage of caspase-3 and PARP occurred 24 h after paclitaxel treatment

in BT-474 cells compared with untreated cells for caspase-3 ( $P<0.05$ ; Fig. 1E) and after 48 h for PARP ( $P<0.001$ ; Fig. 1F).

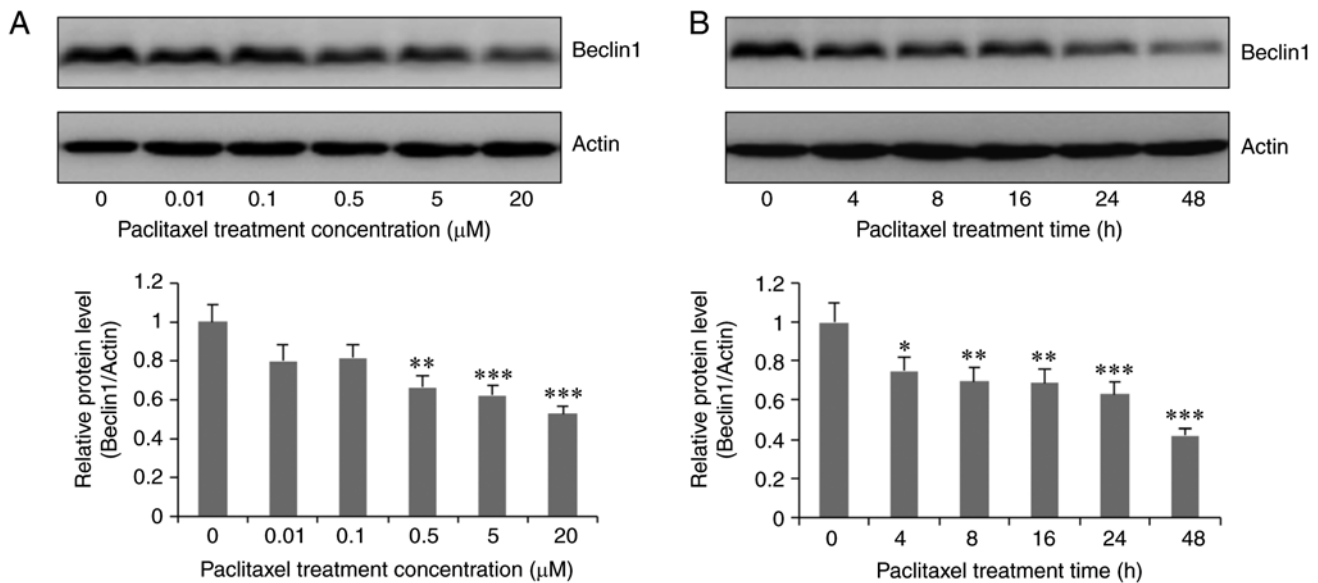


Figure 2. Paclitaxel suppresses the expression of Beclin1 in BT-474 breast cancer cells. (A) Paclitaxel dose-dependently inhibited Beclin1 expression levels. BT-474 cells were exposed to paclitaxel at different concentration gradients for 24 h, and then the expression of Beclin1 was detected using western blot analysis with Beclin1 polyclonal antibody (above). Quantitative analysis of Beclin1 protein levels are presented below. (B) Paclitaxel time-dependently decreased Beclin1 expression. BT-474 cells were exposed to 0.5  $\mu$ M paclitaxel for 4, 8, 16, 24 and 48 h, and then Beclin1 expression was determined using western blot analysis (above). Quantitative analysis of Beclin1 protein levels are presented below. Actin was probed with a specific antibody to serve as a protein normalization control. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. untreated cells.

These results suggest that paclitaxel-induced apoptosis occurs through activation of caspases.

*Paclitaxel suppresses Beclin1 expression and the knockdown of Beclin1 enhances paclitaxel-mediated cytotoxicity in breast cancer cells.* As the function of Beclin1 in paclitaxel-mediated cytotoxicity may be cancer type dependent (25-31), the present study initially detected the expression of Beclin1 following paclitaxel treatment by western blot analysis. It was revealed that paclitaxel down-regulated Beclin1 protein levels in BT-474 cells in a dose- and time-dependent manner, with significant differences observed at the 0.5  $\mu$ M dose ( $P < 0.01$ ) and 4 h of treatment ( $P < 0.05$ ) compared with the untreated cells (Fig. 2A and B). To assess the importance of Beclin1 alteration subsequent to paclitaxel treatment, the specific shRNA-mediated knockdown of the endogenous Beclin1 in BT-474 cells was performed. The effects of two different shRNAs on Beclin1 were evaluated using RT-qPCR and western blot analysis. The mRNA expression levels of Beclin1 in the shBeclin1 groups were demonstrated to be significantly lower in comparison with that in the empty vector control group ( $P < 0.001$ ; Fig. 3A). These results indicated that the transfection efficiency of shBeclin1 was high. Furthermore, the two shRNAs were able to significantly decrease the mRNA and protein expression levels of Beclin1, compared with the shControl group ( $P < 0.001$ ; Fig. 3A and B). Next, the present study assessed the responses of BT-474 cells to treatments with different concentrations of paclitaxel for 72 h using a CCK-8 assay. As presented in Fig. 3C, the curative effect of paclitaxel (at a concentration of 0.1  $\mu$ M or above) was significantly enhanced due to Beclin1 knockdown, compared with the control group that was infected with shControl in BT-474 cells ( $P < 0.05$ ). Furthermore, the time-dependent effect of

BT-474 cells responses to paclitaxel was analyzed using a fixed drug concentration of 0.5  $\mu$ M. Similar to concentration effects, the inhibition of Beclin1 resulted in the significant potentiation of cytotoxicity of paclitaxel in a time-dependent manner compared with the control group ( $P < 0.01$ ; Fig. 3D). To exclude effects associated with cell specificity, an additional breast cancer cell line, MDA-MB-231, was used to demonstrate the effects of Beclin1 silencing on paclitaxel response. As presented in Fig. 4, suppression of Beclin1 also enhanced paclitaxel-mediated cytotoxicity in MDA-MB-231 cells compared with the control cells. In accordance with the results from the CCK-8 assay, Beclin1 knockdown significantly enhanced the inhibition of colony formation by paclitaxel compared with the shControl group in BT-474 and MDA-MB-231 cells ( $P < 0.01$ ; Fig. 5). In summary, the results of the present study demonstrated that Beclin1 protected breast cancer cells from the cytotoxicity effect of paclitaxel.

*Inhibition of Beclin1 promotes breast cancer cell apoptosis under paclitaxel treatment.* As the present results and previous research has demonstrated that paclitaxel is able to induce apoptosis in breast cancer cells (40-42), the present study further aimed to identify whether Beclin1 knockdown (which resulted in the enhancement of the effect of paclitaxel) is associated with increased apoptosis. Analysis of apoptosis, performed using flow cytometry, discovered that Beclin1 knockdown alone was not able to significantly enhance apoptosis, compared with the shControl group in BT-474 and MDA-MB-231 cells (Fig. 6). However, the suppression of Beclin1 significantly enhanced paclitaxel-induced apoptosis in BT-474 and MDA-MB-231 cells compared with the shControl group ( $P < 0.05$ ; Fig. 6). Furthermore, the present study determined the expression of apoptosis-associated proteins by western blot analysis. In accordance with the



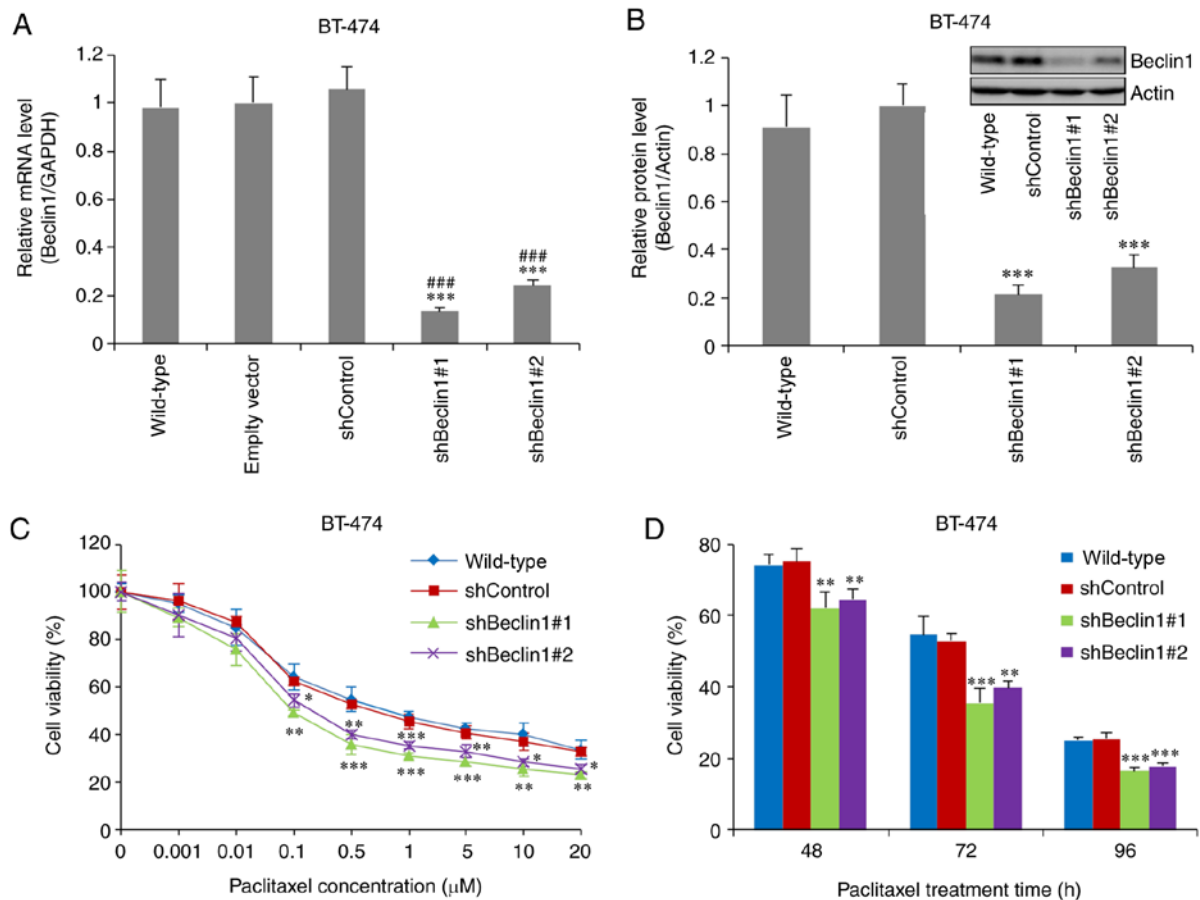


Figure 3. Beclin1 knockdown decreases BT-474 breast cancer cell viability following paclitaxel treatment *in vitro*. (A) Beclin1 mRNA expression was reduced by specific shRNAs in knockdown experiments. BT-474 cells were infected with shBeclin1-pGC-LV, shControl-pGC-LV or the empty vector control pGC-LV. The levels of Beclin1 mRNA was determined using a reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH. (B) Beclin1 protein expression was reduced using specific shRNAs. BT-474 cells were infected with shBeclin1-pGC-LV or shControl-pGC-LV. The expression of the Beclin1 protein was determined using western blot analysis with Beclin1 polyclonal antibody, and actin served as protein normalization control (above). Quantitative analysis of Beclin1 protein levels is presented below. (C) Knockdown of Beclin1 potentiated the cytotoxicity of paclitaxel in a dose-dependent manner. BT-474 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were exposed to paclitaxel at different concentration gradients for 72 h. Then a CCK-8 assay was employed to measure cell viability (n=4). (D) Knockdown of Beclin1 enhanced the cytotoxicity of paclitaxel in a time-dependent manner. BT-474 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were treated with 0.5  $\mu$ M paclitaxel for 48, 72 and 96 h. Then a CCK-8 assay was performed (n=4). \*\*\*P<0.001 vs. empty vector control pGC-LV. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. shControl group. sh, short hairpin RNA; CCK-8, Cell Counting Kit-8.

above-mentioned results, the cleavage of caspase-3 (P<0.05) and PARP (P<0.01) were significantly elevated in the Beclin1 knockdown plus paclitaxel treatment groups, compared with the paclitaxel-treated alone control group in breast cancer cells (Fig. 7). Collectively, these data suggested that Beclin1 inhibited caspase-dependent apoptosis induced by paclitaxel in breast cancer cells.

**Suppression of Beclin1 enhances the antitumor efficacy of paclitaxel in a breast cancer xenograft model.** The results obtained above demonstrate that Beclin1 knockdown enhanced paclitaxel-mediated cytotoxicity by inducing apoptosis *in vitro*. The present study further aimed to reveal whether the therapeutic efficacy of paclitaxel may be improved following Beclin1 suppression *in vivo*. As presented in Fig. 8A, tumor growth in the Beclin1-knockdown group was significantly inhibited compared with the shControl group (P<0.05), subsequent to paclitaxel treatment in the BT-474 xenograft model. The rate of tumor inhibition of paclitaxel was also calculated. It was established that Beclin1 knockdown significantly improved the antitumor efficacy of paclitaxel, compared with

the shControl (P<0.05; Fig. 8B). Tumor specimens collected at the end of the experiments were analyzed using IHC staining (Fig. 8C). Suppression of Beclin1 in the shBeclin1#1 group was confirmed *in vivo* (Fig. 8C, upper row). Furthermore, IHC staining revealed that the cleaved caspase-3 positive cells in the Beclin1 knockdown tumor types were elevated compared with control tumor types in response to paclitaxel treatment (Fig. 8C, lower row). These results are consistent with the *in vitro* data. Altogether, these observations supported the hypothesis of Beclin1 serving a critical function in modulating the antitumor efficacy of paclitaxel in breast cancer cells *in vivo*.

**Association of Beclin1 level with response to paclitaxel therapy in patients with breast cancer.** To clarify whether levels of Beclin1 are associated with clinical response to paclitaxel therapy, the present study analyzed one GEO dataset (GSE22513) containing patients receiving neoadjuvant paclitaxel/radiation treatment (35). The results revealed that Beclin1 levels in non-pCR patients were significantly elevated, compared with pCR patients (P<0.001; Fig. 9A). Meanwhile,

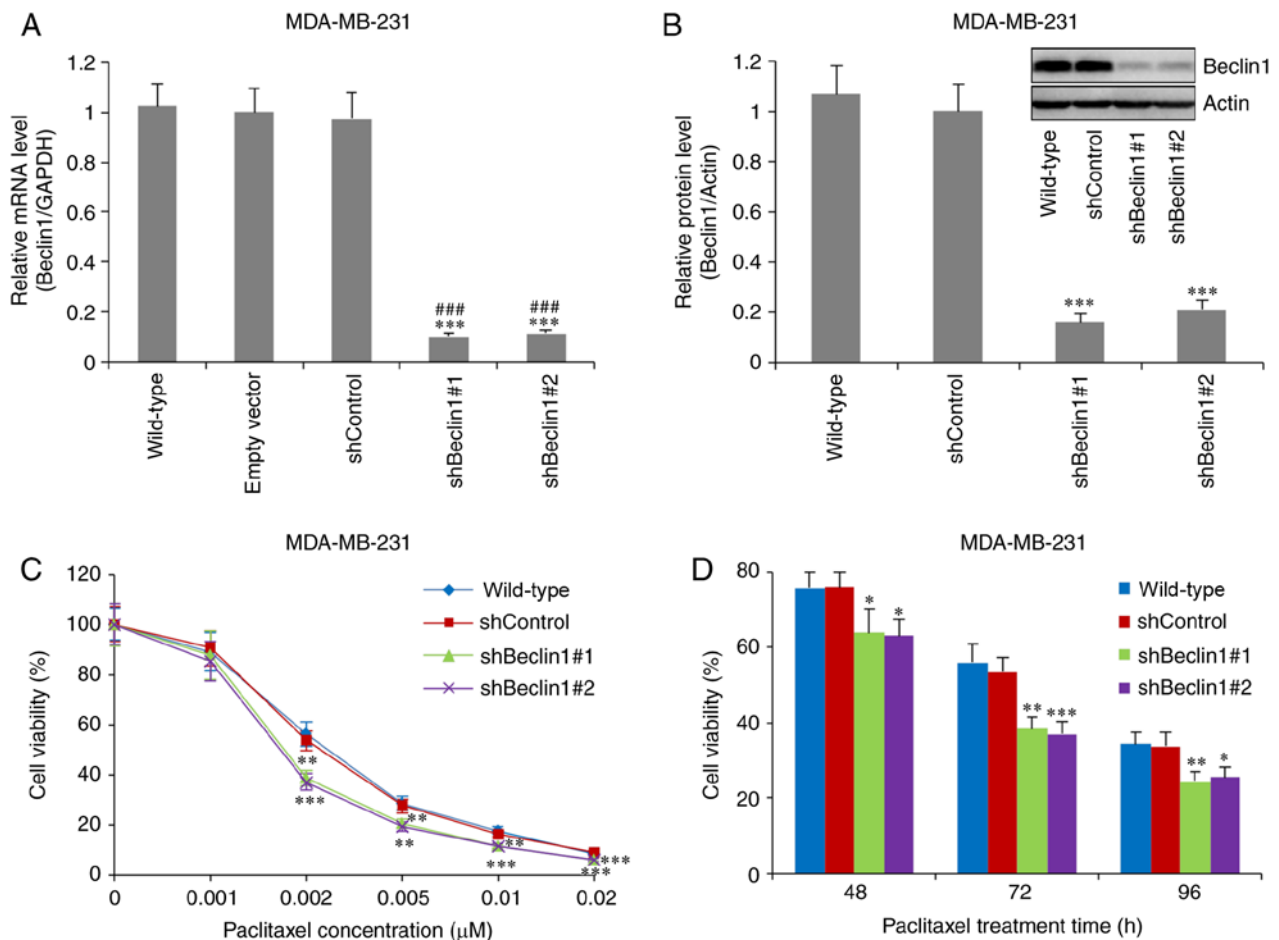


Figure 4. Beclin1 knockdown inhibits MDA-MB-231 breast cancer cell viability following paclitaxel treatment *in vitro*. (A) Beclin1 mRNA expression was reduced using specific shRNAs in knockdown experiments. MDA-MB-231 cells were infected with shBeclin1-pGC-LV, shControl-pGC-LV or the empty vector control pGC-LV. The expression levels of Beclin1 mRNA were determined using a reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH. (B) Beclin1 protein expression was reduced using specific shRNAs. MDA-MB-231 cells were infected with shBeclin1-pGC-LV or shControl-pGC-LV. The expression of the Beclin1 protein was determined using western blot analysis with Beclin1 polyclonal antibody and actin served as the protein normalization control (above). Quantitative analysis of Beclin1 protein levels is presented below. (C) Knockdown of Beclin1 potentiated the cytotoxicity of paclitaxel in a dose-dependent manner. MDA-MB-231 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were exposed to paclitaxel at different concentration gradients for 72 h. Then a CCK-8 assay was employed to measure cell viability (n=4). (D) Knockdown of Beclin1 enhanced the cytotoxicity of paclitaxel in a time-dependent manner. MDA-MB-231 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were treated with 0.002 μM paclitaxel for 48, 72 and 96 h. Then a CCK-8 assay was performed (n=4). ###P<0.001 vs. empty vector control pGC-LV. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. shControl group. sh, short hairpin RNA; CCK-8, Cell Counting Kit-8.

another GEO dataset (GSE25066), including patients treated with neoadjuvant chemotherapy containing paclitaxel, was analyzed (36). Compared to pCR patients, the expression levels of Beclin1 were significantly increased in non-pCR patients (P<0.001; Fig. 9B). These results suggest that Beclin1 may be a potential clinical predictor of the response of a patient to paclitaxel.

## Discussion

Paclitaxel is widely used to prolong the survival of patients with breast cancer, but resistance to the drug remains an obstacle to successful cancer treatment (43). Paclitaxel resistance may be attributed to numerous mechanisms, including apoptosis resistance resulting from the upregulation of anti-apoptotic proteins including B-cell lymphoma 2 (Bcl-2) (44), the increment of drug efflux owing to the upregulation of ATP-dependent transporters (45) and the altered expression or mutation of tubulin which affects drug-tubulin binding (46). These results have

prompted a search for novel strategies for the improvement of the therapeutic effect of paclitaxel in patients with breast cancer.

Beclin1, the first identified ATG gene in mammalian cells (20), is monoallelically deleted in 40-75% of breast, ovarian and prostate cancer types (47) and was initially considered to be a tumor suppressor (22-24). Rai *et al* (48) demonstrated that resveratrol was able to chemosensitize doxorubicin in combination through suppressing Beclin1 in breast cancer cells. Zhang and Li (49) reported that crocetin may increase autophagic cell death via the inhibition of the expression of Beclin1 in breast cancer cells during fluorouracil treatment. However, few reports have demonstrated an association between Beclin1 and paclitaxel treatment in breast cancer cells. Therefore, the effects of paclitaxel on breast cancer cells were initially examined. In previous research, Veldhoen *et al* (50) revealed that paclitaxel was able to inhibit the accumulation of LC3 and the formation of autophagosomes in breast cancer MCF-7 cells. The present study additionally demonstrated that

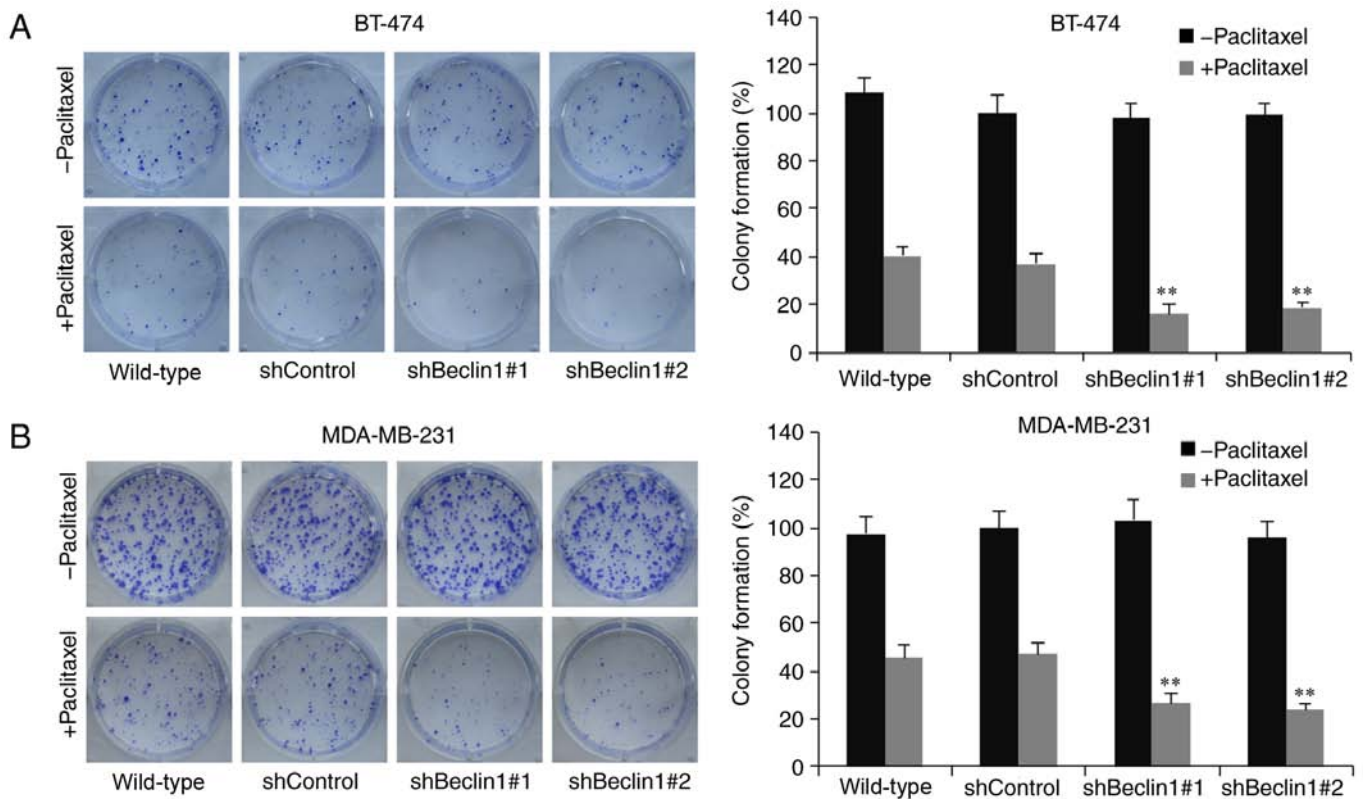


Figure 5. Beclin1 knockdown suppressed the colony formation of breast cancer cells following paclitaxel treatment *in vitro*. (A) BT-474 cells were infected with shBeclin1-pGC-LV or shControl-pGC-LV and incubated with 0.5  $\mu$ M paclitaxel for 72 h and then were cultured for up to 3 weeks, and (B) MDA-MB-231 cells were infected with shBeclin1-pGC-LV or shControl-pGC-LV and incubated with 0.002  $\mu$ M paclitaxel for 72 h and then were cultured for up to 2 weeks. Colonies were fixed, stained with crystal violet and counted. Indicated wells are representative of three similar wells (left). Quantitative data of colony formation was expressed as a histogram (right), and the percentage was normalized to shControl-pGC-LV cells. \*\*P<0.01 vs. shControl group. sh, short hairpin RNA.

Beclin1 expression was dose- and time-dependent suppressed in breast cancer BT-474 cells in the course of paclitaxel treatment. To further clarify the critical function of Beclin1 in paclitaxel-mediated cytotoxicity, Beclin1 expression was interrupted using specific shRNA. It was identified that Beclin1 knockdown substantially improved the therapeutic effect of paclitaxel in breast cancer, compared with the controls *in vitro* and *in vivo*. These results are similar to previous discoveries in neuroblastoma, ovarian cancer, nasopharyngeal carcinoma, NSCLC and endometrial carcinoma (27-31). In contrast to the present results, other reports revealed that the overexpression of Beclin1 promoted cell death in cervix carcinoma CaSki and HeLa cells treated with paclitaxel (25,26). It thus appears that the effect of Beclin1 on cell survival may be dependent on the cancer cell type. Although the reasons are unclear, individual genetic, epigenetic and metabolic backgrounds of specific types of cancer may be responsible for this observation. Therefore, it is suggestive that the mechanisms of Beclin1 affecting paclitaxel-induced cell death may be multifactorial, with one aspect of it being the inhibition of Beclin1 in breast cancer cells, which triggers paclitaxel-induced cell death. The present results indicate that Beclin1 may serve as a novel target for the improvement of the paclitaxel effect in the management of breast cancer.

Next, the present study preliminarily investigated how Beclin1 knockdown may enhance paclitaxel-mediated cytotoxicity. Paclitaxel ultimately kills breast cancer cells mainly through induction of apoptosis (51,52). Furthermore, Beclin1

contains a BH3 domain, which may interact with either Bcl-2 or B-cell lymphoma-extra large to inhibit apoptosis (53). Thus, one hypothesis may be that Beclin1 is involved in the interrupted apoptosis of breast cancer cells in response to paclitaxel treatment, which is enhanced subsequent to Beclin1 knockdown. In the present study, paclitaxel-induced apoptosis was confirmed using Annexin V-FITC staining and examining the levels of cleaved caspase-3 and PARP in breast cancer cells. Compared with the controls, enhanced Annexin V-FITC staining and the increased cleavage of caspase-3 and PARP levels were observed following Beclin1 knockdown followed by paclitaxel treatment. What was established in the present study is that Beclin1 protects breast cancer cells from caspase-dependent apoptotic death.

Although the inhibition of Beclin1-augmented paclitaxel-induced caspase-dependent apoptosis in breast cancer cells, Beclin1 knockdown alone was unable to affect the survival and apoptosis of breast cancer cells *in vitro* and *in vivo*. A similar phenomenon was discovered in cisplatin-treated human hypopharyngeal cancer cells in xenograft mice (54). These observations may be attributed to the down-regulated expression of Beclin1 in breast and hypopharyngeal cancer types (22,55). That is, that breast and hypopharyngeal cancer cells may grow and survive without over-reliance on Beclin1 under normal conditions. Accordingly, there was no anti-tumor activity observed with the inhibition of Beclin1 alone, compared with control shRNA, revealed by control experiments.



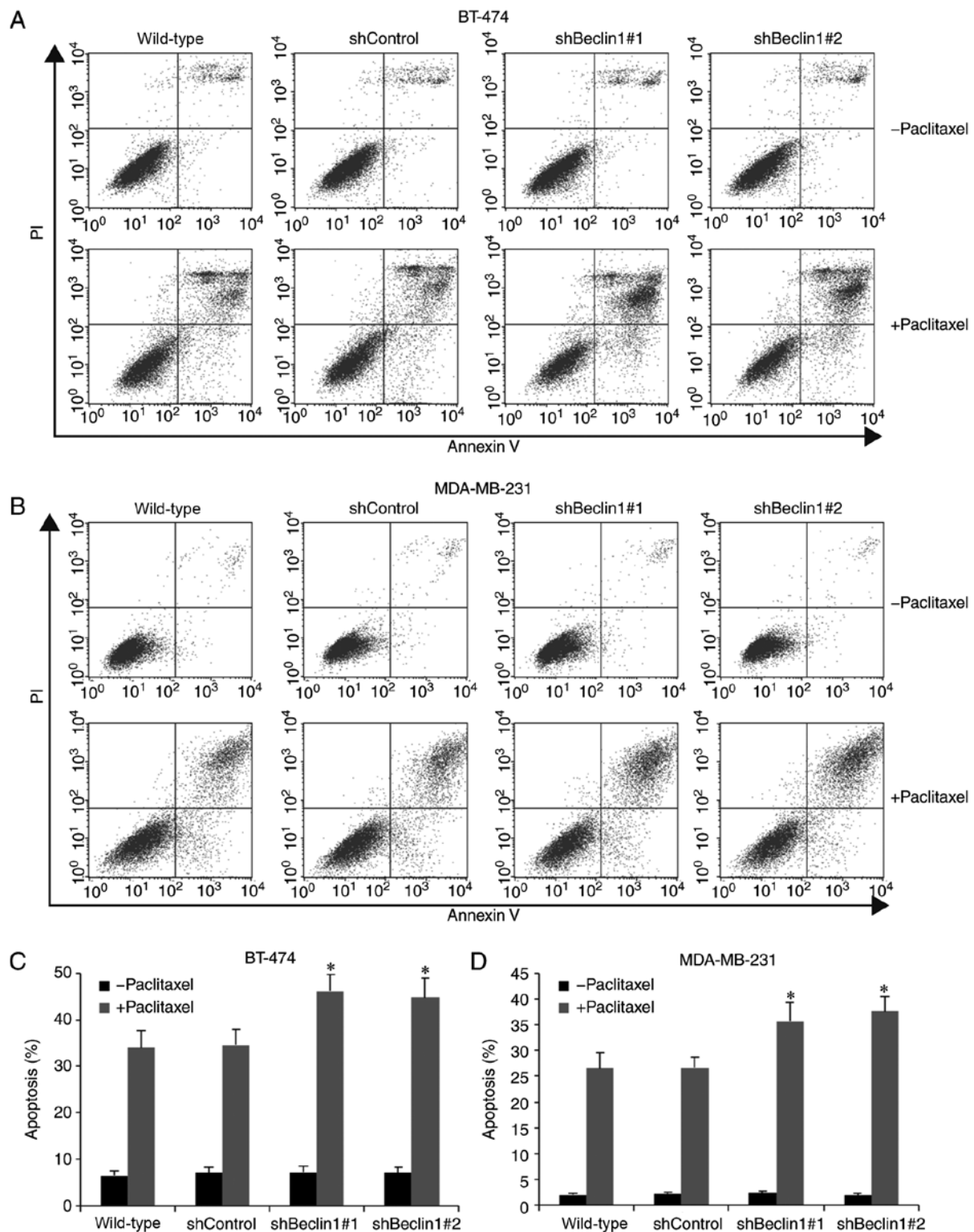


Figure 6. Beclin1 knockdown promotes paclitaxel-inducing apoptotic death in breast cancer cells. (A) BT-474 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were exposed to 0.5  $\mu$ M paclitaxel for 72 h, and (B) MDA-MB-231 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were exposed to 0.002  $\mu$ M paclitaxel for 72 h. A cellular apoptosis assay was performed using an Annexin V-fluorescein isothiocyanate kit and monitored using flow cytometry, following paclitaxel treatment. (C) Quantification of the apoptosis rate of BT-474 cells following Beclin1 knockdown and paclitaxel treatment (n=3). (D) Quantification of the apoptosis rate of MDA-MB-231 cells following Beclin1 knockdown and paclitaxel treatment (n=3). \*P<0.05 vs. shControl group treated with paclitaxel. sh, short hairpin RNA; PI, propidium iodide.

Despite the fact that the majority of patients with early diagnosed cancer may benefit from surgery, radiation therapy and chemotherapy, patients with advanced breast cancer typically have poor prognosis (56). So, it is urgent to identify

molecular or genetic markers which may predict whether patients with breast cancer are likely to benefit from chemotherapy. Zhang *et al* (28) demonstrated that the high expression of Beclin1 was substantially correlated with the chemoresis-

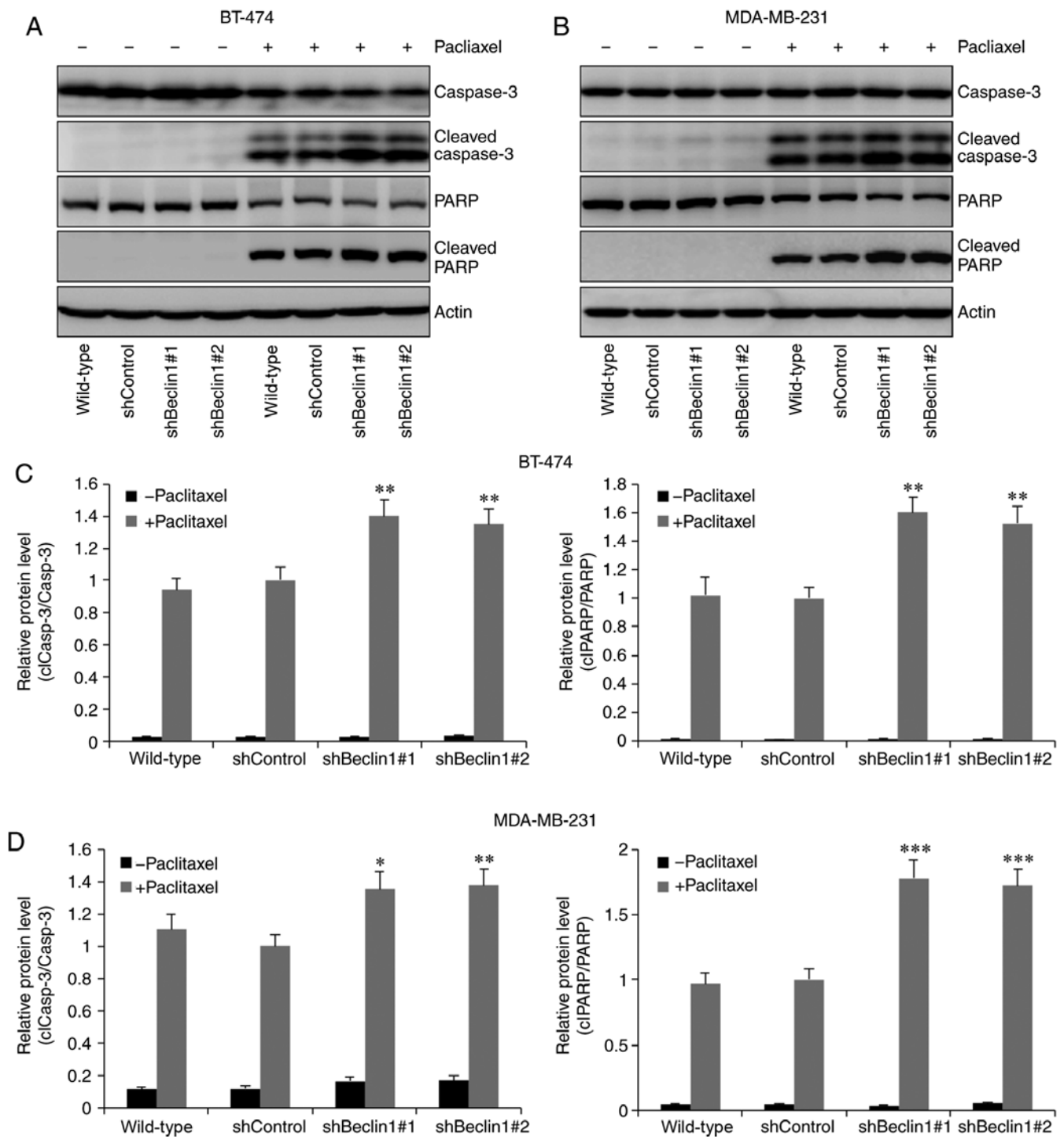


Figure 7. Knockdown of Beclin1 alters the expression of apoptotic proteins induced by paclitaxel in breast cancer cells. (A) BT-474 or (B) MDA-MB-231 cells infected with shBeclin1-pGC-LV or shControl-pGC-LV were treated with 0.5  $\mu$ M or 0.002  $\mu$ M paclitaxel, respectively, for 72 h. Expression of apoptotic proteins was evaluated using western blot analysis with specific anti-apoptotic protein antibodies, as indicated in the blot. The blot was probed with an actin antibody to serve as a protein normalization control. (C) Quantitative analysis of the relative protein levels of cleaved caspase-3 and PARP in BT-474 cells. (D) Quantitative analysis of the relative protein levels of cleaved caspase-3 and PARP in MDA-MB-231 cells. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. shControl group treated with paclitaxel. sh, short hairpin; Casp-3, caspase-3; cIcasp-3, cleaved caspase-3; cIPARP, cleaved PARP; PARP, poly ADP-ribose polymerase.

tance of patients with ovarian cancer. This is consistent with the results of the present study, where a positive association between Beclin1 expression and recurrence of breast cancer treated with paclitaxel-containing therapy was identified by analyzing GEO datasets. To the best of our knowledge, the present data is the first to demonstrate the clinical relevance of Beclin1 expression and paclitaxel sensitivity.

In conclusion, the present *in vitro* and *in vivo* results demonstrate that Beclin1 suppression combined with paclitaxel treatment is superior to paclitaxel-alone treatment in breast cancer cells. High level of Beclin1 expression may predict the recurrence of breast cancer in paclitaxel-containing therapy. Although the *in vitro* and *in vivo* results may not directly translate to clinical responses due to the heteroge-

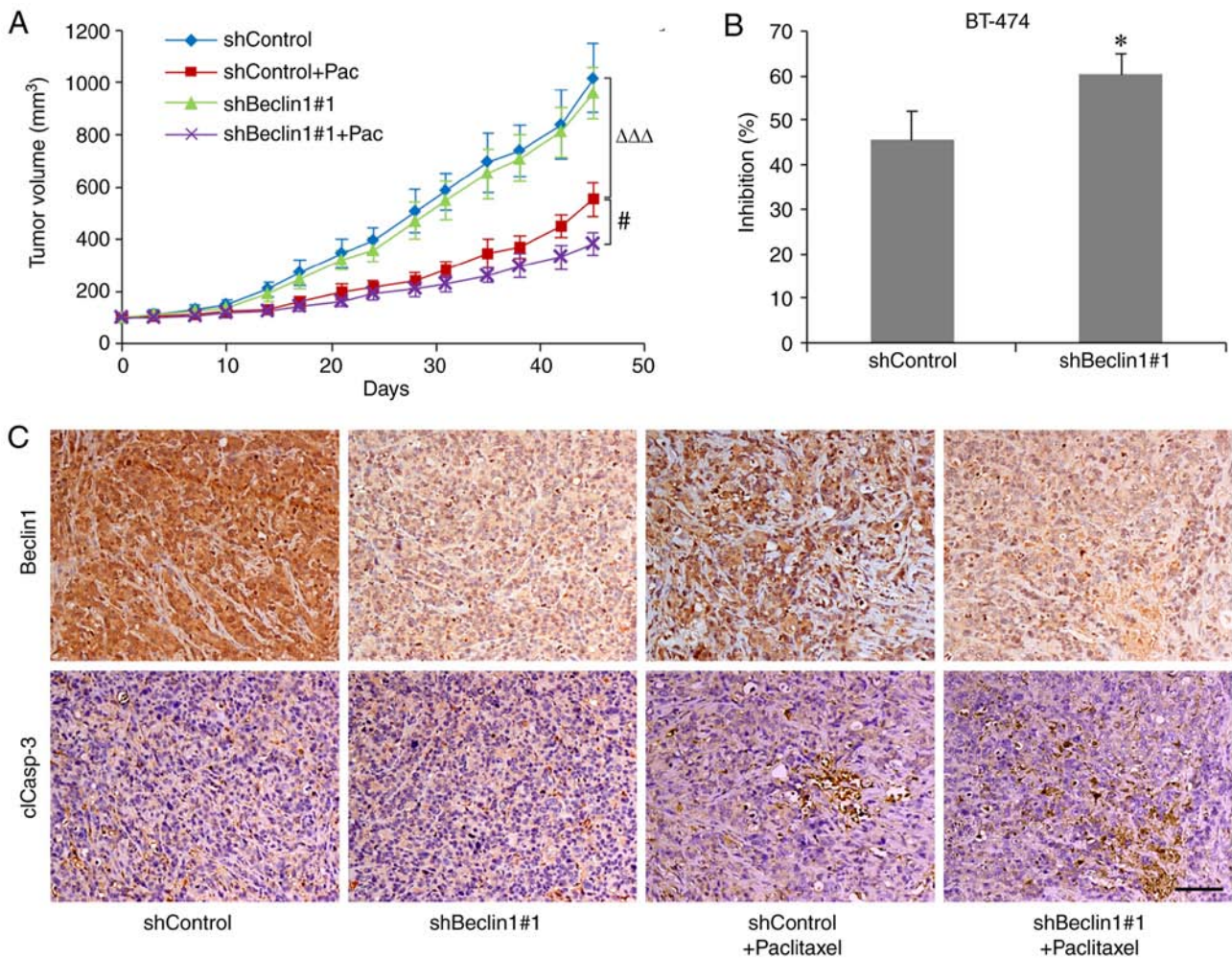


Figure 8. Beclin1 knockdown enhances the cytotoxicity of paclitaxel in BT-474 breast cancer cells *in vivo*. (A) Tumor volumes of shBeclin1#1 or shControl BT-474 breast cancer xenografts following paclitaxel treatment. Paclitaxel (10 mg/kg) was administered twice a week (for a total of 8 times in 4 weeks) by intraperitoneal injection when tumor volumes reached ~100 mm<sup>3</sup>. Tumor volumes were monitored twice a week. \* $P < 0.05$  shBeclin1 vs. shControl group treated with paclitaxel.  $\Delta\Delta\Delta P < 0.001$  shControl group treated with paclitaxel vs. untreated group. (B) Rate of tumor inhibition of paclitaxel in shBeclin1#1 or shControl BT-474 breast cancer xenografts. \* $P < 0.05$  vs. shControl group. (C) Representative micrographs of immunohistochemical staining of Beclin1 (upper row) and cleaved caspase-3 (lower row) in xenografts. Scale bar: 100  $\mu$ m. sh, short hairpin RNA; cleaved caspase-3; Pac, paclitaxel.

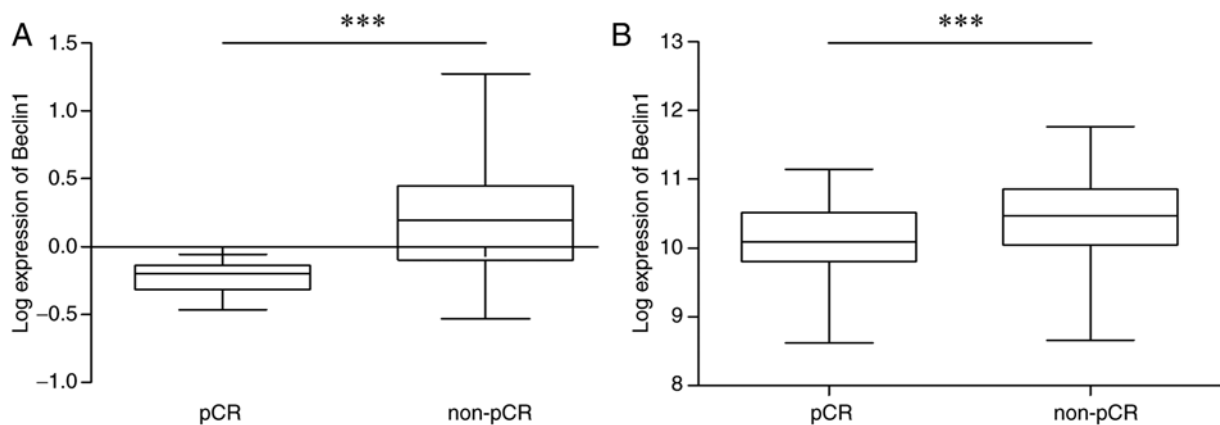


Figure 9. Expression of Beclin1 is associated with paclitaxel therapy in breast cancer. The publicly available Gene Expression Omnibus datasets (A) GSE22513 and (B) GSE25066 were employed to analyze Beclin1 levels and paclitaxel response in patients with breast cancer. The patients were divided into two groups (pCR and non-pCR groups). The association between Beclin1 expression level and pCR or non-pCR was assessed using a two-sample t-test. \*\*\* $P < 0.001$  vs. pCR group. pCR, pathologic complete response.

neity of cancer types, the present discoveries indicate that Beclin1 may be potentially used as a target for therapy or a

predictor for prognosis in patients with breast cancer treated with paclitaxel.

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## Availability of data and materials

All data (except the Gene Expression Omnibus datasets) analyzed during this study are included in the published article.

## Authors' contributions

LJZ conceived and designed the experiments. CLW, JFL, YL, YXW, KFF, XJY, QP and XXC performed the experiments. CLW and LJZ analyzed the data and wrote the paper. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All animal experiments were performed according to the National Institutes of Health guide for the care and use of laboratory animals and were reviewed and approved by the Ethics Committee of Navy General Hospital (Beijing, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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