

SBEM confers paclitaxel resistance in breast cancer via DUSP16-mediated MAPK/AMPK pathway activation

LIANG LIU¹, NING LU², XIAOMEI LIU³, QINGTING YI³,
SHENGNAN LI³, JIE WEN³, HUIJUAN SUN³ and YUANMING DU³

¹Graduate School, Xinjiang Medical University, Urumqi, Xinjiang Uygur Autonomous Region 830000, P.R. China; ²Department of Oncology, Xinjiang Military Command General Hospital of PLA, Urumqi, Xinjiang Uygur Autonomous Region 830000, P.R. China;

³Department of Oncology, Qingdao West Coast New Area People's Hospital, Qingdao, Shandong 266000, P.R. China

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Abstract. Small breast epithelial mucin (SBEM) is upregulated in primary breast tumors and metastatic lymph nodes and has been implicated in chemoresistance. While it has been identified as a potential biomarker for monitoring hematogenous micrometastasis in patients with breast cancer undergoing adjuvant chemotherapy, the molecular mechanisms by which SBEM confers this resistance remain unclear. Therefore, the present study aimed to elucidate the mechanisms by which SBEM regulates paclitaxel (PTX) resistance in breast cancer. To this aim, breast cancer cell lines with SBEM overexpression and knockdown were developed from parental drug-resistant strains. Additionally, AMPK activator 13 was used to investigate the involvement of the AMPK pathway in the SBEM-mediated effects. The results indicated that SBEM overexpression promoted cell viability and enhanced resistance to PTX in breast cancer cells. Conversely, SBEM knockdown significantly increased apoptosis, with a three-fold increase compared with the controls, and restored PTX sensitivity in drug-resistant cells. Mechanistically, SBEM was found to interact with dual-specificity phosphatase 16 (DUSP16) and upregulate its expression. Additionally, SBEM downregulation inhibited AMPK signaling activity, thereby suppressing cancer cell viability. In conclusion, abnormal activation of the AMPK signaling pathway was shown to contribute to PTX resistance in breast cancer. SBEM enhanced DUSP16 expression and activated the AMPK signaling pathway, thereby conferring resistance to PTX.

Introduction

Breast cancer is one of the most prevalent malignant tumors among women and poses a significant threat to both health and survival (1). In recent decades, the burden of breast cancer has intensified in developing countries due to rising incidence rates and persistently high mortality (2). By 2040, it is projected that population growth and aging alone will contribute to >3 million new breast cancer cases and 1 million related deaths annually (3). Current treatment modalities include surgery, targeted therapies, immunotherapy, radiotherapy and chemotherapy (4). Among the chemotherapeutic agents, paclitaxel (PTX) plays a crucial role in breast cancer management (5). However, the development of PTX resistance during treatment remains a major clinical challenge, often leading to therapeutic failure (6). Understanding the mechanisms underlying PTX resistance and identifying effective reversal strategies is therefore of great clinical importance.

Small breast epithelial mucin (SBEM) is a secreted protein, the gene expression of which has been associated with breast cancer prognosis (7). Elevated SBEM expression has been linked to poorer clinical outcomes, suggesting its potential as a prognostic biomarker (8). Research indicates that SBEM contributes to the invasion and metastasis of breast cancer cells (9), potentially by regulating extracellular matrix degradation, modulating cell adhesion and altering the tumor microenvironment (10). However, the exact biological role of SBEM in breast cancer remains unclear. SBEM has been identified as a potential predictive biomarker for adjuvant chemotherapy in breast cancer (11). PTX, a drug frequently employed in combination chemotherapy regimens, still faces limitations in its clinical application. This is due to notable individual variations in drug sensitivities (5). To the best of our knowledge, no studies have clearly revealed the association between SBEM and the response to PTX, especially the role of SBEM in predicting PTX sensitivity.

The mitogen-activated protein kinase (MAPK) signaling pathway plays a central role in the initiation, progression and metastasis of breast cancer (12). Dysregulated MAPK activation promotes tumor cancer cell proliferation, invasion, metastasis and resistance to multiple chemotherapeutic agents (13), including PTX. Numerous studies have confirmed

Correspondence to: Professor Ning Lu, Department of Oncology, Xinjiang Military Command General Hospital of PLA, 359 Youhao North Road, Shayibake, Urumqi, Xinjiang Uygur Autonomous Region 830000, P.R. China
E-mail: jinshatanwo@aliyun.com

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that abnormal MAPK signaling is a key mechanism driving PTX resistance (14–16). MAPK activity is primarily regulated by a family of proteins known as dual-specificity phosphatases (DUSPs) (17), also referred to as MAPK phosphatases (MKPs), which are involved in signal transduction, cell growth regulation and metabolism (18). Among this protein family, MKP7, also known as dual specificity phosphatase 16 (DUSP16), is primarily cytoplasmic and inhibits the activity of both p38 and JNK MAPKs (19,20). DUSP16 also regulates ERK and p38MAP activation and has been shown to influence AMPK signaling (21–23). Additionally, abnormal DUSP16 expression is associated with tumorigenesis, progression and metastasis (24). Despite this, the regulatory relationship between SBEM and DUSP16 as well as their combined influence on the AMPK signaling pathway remains largely unexplored.

The primary objective of the present study was to explore whether SBEM, by interacting with DUSP16 and activating the AMPK signaling pathway, contributes to PTX resistance in patients with breast cancer. Through a series of experiments and analyses, the study aimed to determine if such a mechanism exists and leads to the observed PTX resistance. This research also provides novel insights into future treatment strategies and the improvement of patient prognosis.

Materials and methods

Molecular docking. The 3D structure of the SBEM protein was predicted using AlphaFold software 2 (<https://alphafold.ebi.ac.uk>). PrankWeb (<https://prankweb.cz/>), a machine learning-based tool employing a Random Forest algorithm trained on known binding sites, was used to predict potential active sites (25). Through multi-sequence alignment analysis using BioXM 2.7.1, MUCL1 (UniProt ID: Q96DR8) and MUCL3 (UniProt ID: Q3MIW9) were examined to identify conserved regions. Subsequently, the 2D structure of the SBEM protein was analyzed using PyMOL 2.5 (<https://pymol.sourceforge.net>). Briefly, based on the protein structure confidence, 2D structure stability, active site prediction and sequence alignment, Met1 was selected as the lattice center and a docking box was established. The specific box information was as follows: center_x=6.439; center_y=-0.223; center_z=-12.452; size_x=73.15; size_y=73.15; and size_z=73.15. The SDF format of PTX was obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>) and converted to the PDB format using Open Babel (<http://openbabel.org/index.html>). The protein structure was dehydrated, hydrogenated and its charge calculated before being converted to the PDBQT format using AutoDockTools 1.5.7 (<https://autodocksuite.scripps.edu/adt/>). The ligand was also hydrogenated, its torsional degrees of freedom were determined and it was converted to the PDBQT format. The docking box coordinates were defined and molecular docking was performed using AutoDock Vina (<https://vina.scripps.edu/>). Visualization and 3D analysis diagrams were generated using PyMOL 2.1.0.

Cell culture. Human breast cancer cell lines (SUM190PT, BT474, AU565, SKBR3 and MCF7) and the normal mammary epithelial cell line MCF10A were purchased from Qingqi (Shanghai) Biotechnology Development Co., Ltd. All cell lines were authenticated by short tandem repeat profiling to prevent

cross-contamination. Cells were thawed from liquid nitrogen, resuspended and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Beyotime Biotechnology; cat. no. C0891) supplemented with 10% fetal bovine serum (Beyotime Biotechnology; cat. no. C0235) and 1% penicillin-streptomycin. The cells were placed in a cell incubator (Beyotime Biotechnology; cat. no. E2392) at 37°C with 5% CO₂.

Cell transfection. Exponentially growing SUM190PT and SKBR3 cells were treated with 2 ml trypsin digestion solution (Wuhan Servicebio Technology Co., Ltd.; cat. no. G4011) and incubated at 37°C in a 5% CO₂ atmosphere (Suzhou Jiemei Electronics Co., Ltd.; cat. no. CI-191C) for 3 min. Following incubation, 3 ml high-glucose DMEM (Wuhan Servicebio Technology Co., Ltd.; cat. no. G4524) was added and the cells were centrifuged at 1,200 × g for 3 min at 25°C. The supernatant was discarded and the pellet was resuspended in 3 ml DMEM. The cells were then seeded into 6-well plates at a density of 1 × 10⁵ cells/well and incubated overnight. The following day, small interfering RNA (siRNA) and overexpression vectors were mixed with Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, Inc.; cat. no. L3000001) and incubated at room temperature for 30 min. The transfection mixture was then added to the wells according to the experimental requirements and incubated at 37°C for 5 h. siSBEM was synthesized by GenScript Biotech Corporation, with a synthesis amount of 2.5 nmol diluted to 100 μmol/l. The overexpression vector backbone employed in this study was pcDNA3.1(+) (Thermo Fisher Scientific, Inc.; cat. no. V79020). For cell transfection, 4 μg of the pcDNA3.1(+) vector, at a concentration of 1 μg/μl, was introduced into the cells. The siRNA interference and overexpression primer sequences are shown in Tables SI and SII, respectively.

Construction of PTX-resistant SUM190PT and SKBR3 cell lines. PTX-resistant breast cancer cells were developed based on previously described methods (26). Details of the drug-induction protocol are shown in Table SIII. To confirm the stability of the resistance phenotype, the PTX (MedChemExpress; cat. no. HY-B0015) concentration was gradually increased until a final concentration of 500 nmol/l was reached. The cells were then maintained at this concentration and subcultured for at least 10 generations. IC₅₀ values were measured at the 5th, 10th and 20th generations post-withdrawal of PTX. When the IC₅₀ values showed minimal variation and were significantly higher than those of the parental cell lines, the resistance model was deemed successfully established. The parental lines are denoted as SUM190PT and SKBR3, while their drug resistant counterparts are referred to as SUM190PT/PTX and SKBR3/PTX. Resistance success was confirmed by comparing cell viability and resistance coefficients before and after PTX exposure. Resistant cell lines were continuously cultured in 500 nmol/l PTX to maintain drug resistance.

Cell experimental grouping. To assess whether SBEM knock-down could reverse drug resistance in SUM190PT/PTX cells, the following groups were established: SUM190PT (control), SUM190PT + PTX, SUM190PT/PTX + PTX + si-negative control (NC) and SUM190PT/PTX + PTX + siSBEM. To evaluate whether SBEM overexpression induces PTX resistance,

SKBR3 cells, which have relatively low SBEM expression, were divided into: SKBR3 (control), SKBR3/PTX + PTX, SKBR3 + PTX + Vector and SKBR3 + PTX + oeSBEM. AMPK activator 13 (MedChemExpress; cat. no. HY-155363), an AMPK signaling pathway activator (27), was used to investigate the role of SBEM in regulating AMPK signaling. For this, SUM190PT/PTX cells were divided into four groups: Control, AMPK activator 13, siSBEM and AMPK activator 13 + siSBEM. Both SUM190PT and SKBR3 cells were treated with PTX at a concentration of 500 nmol/l for 48 h. Drug-resistant cell lines SUM190PT/PTX and SKBR3/PTX were maintained in medium containing 500 nmol/l PTX. Cells transfected with siRNA or overexpression plasmids were incubated at 37°C for 48 h. In all groups involving AMPK activator 13, the concentration was set at 5 μ mol/l with an incubation time at 37°C for 24 h (27).

Cell counting kit-8 (CCK-8) assay. Following transfection, breast cancer cells and drug-resistant cell lines in the exponential growth phase were harvested, digested and resuspended. Then, 3,000 cells per well were seeded into 96-well plates and incubated overnight. PTX was applied at varying concentrations (125, 250, 500, 1,000, 2,000, 4,000 and 8,000 nmol/l) and the cells were incubated for 48 h. Subsequently, 10 μ l CCK-8 solution (Wuhan Servicebio Technology Co., Ltd.; cat. no. G4103) was added to each well and incubated for an additional 3 h. Optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.; cat. no. A51119500C). IC₅₀ values and PTX resistance coefficients were calculated using GraphPad Prism version 9.5.0 (Dotmatics).

Total RNA extraction. Cell suspensions were collected and centrifuged at 1,200 x g for 3 min at 25°C. The supernatant was discarded and 1 ml RNA extraction reagent (Wuhan Servicebio Technology Co., Ltd.; cat. no. G3013) was added, followed by a 5-min incubation at room temperature. Next, 100 μ l chloroform (Wuhan Servicebio Technology Co., Ltd.; cat. no. G3014) was added and the mixture was shaken vigorously for 15 sec, then allowed to stand for 5 min at room temperature. The sample was centrifuged at 12,000 x g for 15 min at 4°C, and the aqueous phase was transferred to a new centrifuge tube. Then, 500 μ l isopropanol (Shanghai Macklin Biochemical Co., Ltd.; cat. no. 67-63-0) was added and incubated for 10 min at room temperature. After centrifugation at 12,000 x g for 15 min at 4°C, the supernatant was discarded. The RNA pellet was washed with 1 ml 75% ethanol (Shanghai Macklin Biochemical Co., Ltd.; cat. no. E809061), air-dried and resuspended in 50 μ l sterile water (Wuhan Servicebio Technology Co., Ltd.; cat. no. G4700). The RNA concentration was quantified for use in subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was conducted according to the instructions of the SweScript One-Step RT-PCR Kit (Wuhan Servicebio Technology Co., Ltd.; cat. no. G3335). The PCR cycling program was as follows: 50°C for 30 min, 98°C for 2 min, followed by 39 cycles of 98°C for 15 sec, 55°C for 20 sec and 72°C for 20 sec. A final extension step was carried out at 72°C for 5 min. The primer sequence (5'-3') information

is as follows: SBEM forward, TCTGCCCAGAATCCGACA AC, and reverse GGGCTTCATCATCAGCAGGA; GAPDH forward, CTGGGCTACACTGAGCACC, and reverse AAG TGGTCGTTGAGGGCAATG. GAPDH was used as the internal reference gene and the 2^{- $\Delta\Delta$ Cq} was used as the relative expression level of the target gene mRNA (28).

Western blotting. Breast cancer cell suspensions were collected and lysed using RIPA lysis buffer (Wuhan Servicebio Technology Co., Ltd.; cat. no. G2002). Lysates were incubated on ice for 30 min and then centrifuged at 12,000 x g for 15 min at 25°C. The supernatant was collected and proteins were denatured in a 95°C water bath for 15 min. Protein concentrations were determined using a BCA protein quantification kit (Wuhan Servicebio Technology Co., Ltd.; cat. no. G2026). Cell lysis and protein-loading buffers (Beyotime Institute of Biotechnology; cat. no. P0015) were added to normalize the protein concentrations across samples. Then, 10 μ l (30 μ g) of each protein solution was loaded into the wells of the gel (10%). Electrophoresis was performed at 80 V for 30 min, followed by 120 V for 60 min. After electrophoresis, the proteins were transferred to PVDF membranes at 260 mA for 2 h. PVDF membranes were blocked with 5% skim milk (Wuhan Servicebio Technology Co., Ltd.; cat. no. GC310001) at room temperature for 2 h, then washed three times with TBST (0.1% Tween 20) (Wuhan Servicebio Technology Co., Ltd.; cat. no. G0004) for 5 min each. Primary antibody solutions were added and incubated overnight at 4°C. After incubation, secondary antibody solutions were applied and incubated at room temperature for 1 h. Details of the antibodies, including manufacturer cat. no. and dilution ratios, are provided in Table SIV. After washing with TBST, chemiluminescent detection was performed using a luminescent substrate (Wuhan Servicebio Technology Co., Ltd.; cat. no. G2161) and images were captured using a gel imaging system (Mona Biotechnology Co., Ltd.; cat. no. GD50202). Images were analyzed with ImageJ 1.5.2a software (National Institutes of Health) for subsequent statistical analyses.

Cell apoptosis. Exponentially growing breast cancer cells were treated as aforementioned in the *Cell experimental grouping* subsection, then centrifuged and resuspended as previously described. According to the instructions of the flow cytometry apoptosis detection kit (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1511), 5 μ l FITC solution was added to each cell suspension, followed by incubation in the dark at room temperature for 30 min. Subsequently, PI solution was added and incubated in the dark for an additional 5 min. Cells were analyzed using a Attune™ NxT flow cytometer (Thermo Fisher Scientific, Inc.; cat. no. A24858) and Attune™ NxT software (version 6; Thermo Fisher Scientific, Inc.) within 2 h of staining.

Co-immunoprecipitation (Co-IP). Exponentially growing breast cancer cells were treated as aforementioned in the *Cell experimental grouping* subsection, then centrifuged and resuspended as previously described. Cell lysis buffer was added according to the Co-IP kit protocol (Beyotime Institute of Biotechnology; cat. no. P2179M) and the samples were incubated at room temperature for 30 min. The lysates

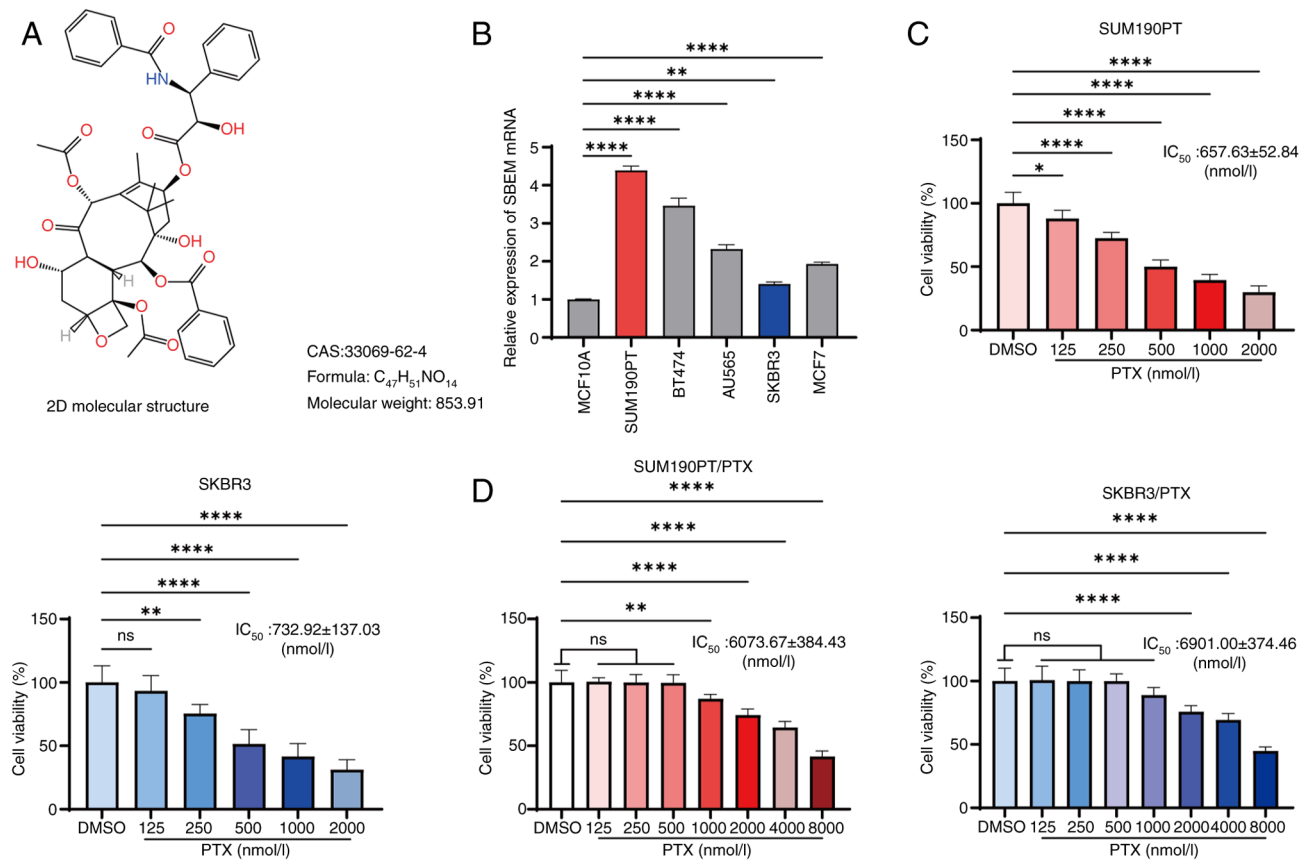


Figure 1. Inhibition of breast cancer cell viability by PTX and the construction of PTX-resistant strains. (A) 2D molecular structure of PTX. (B) Relative expression levels of SBEM mRNA in MCF10A, SUM190PT, BT474, AU565, SKBR3 and MCF7 cells were measured using reverse transcription-quantitative polymerase chain reaction. (C) CCK-8 assay showing changes in the viability of SUM190PT and SKBR3 cells treated with varying PTX concentrations. (D) CCK-8 assay showing changes in the viability of SUM190PT/PTX and SKBR3/PTX cells treated with varying PTX concentrations. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. ns, not significant; PTX, paclitaxel; SBEM, small breast epithelial mucin; CCK-8, Cell Counting Kit-8.

were then centrifuged at $1,200 \times g$ for 3 min at $25^{\circ}C$ and the supernatant was collected. Protein A/G magnetic beads pre-conjugated with the primary antibody were added ($20 \mu l$ of magnetic bead suspension to every $500 \mu l$ of protein sample) and the mixtures were incubated overnight at room temperature. After incubation, the beads were washed with washing buffer and the immunocomplexes bound to the beads were eluted using the elution buffer. The eluted target proteins were analyzed via western blotting. Antibody details are provided in Table SIV.

Statistical analysis. Data were analyzed using SPSS version 22.0 (IBM Corp.). All experiments were independently repeated at least three times. Results are expressed as mean $\pm$ standard deviation. Comparisons between two groups were performed using unpaired t-tests. Differences among multiple groups were assessed using one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SBEM induces PTX drug resistance. The 2D structural formula of PTX is shown in Fig. 1A. The SBEM mRNA expression levels in various breast cancer cell and normal breast epithelial cell lines were assessed using RT-qPCR. Compared with

MCF10A cells, SBEM mRNA expression was highest in SUM190PT cells and lowest in SKBR3 cells (Fig. 1B). Since SBEM was highly expressed in the SUM190PT cell line, a knockdown cell line using this cell line was initially planned. However, as the present study focuses on drug-resistant strains, a SBEM-knockdown cell line using the SUM190PT/PTX drug-resistant strain was ultimately constructed. Additionally, SKBR3 cells, which have low SBEM expression, were selected to create a SBEM-overexpressing cell line. Knockdown and overexpression experiments in cell lines representing the two extremes of SBEM expression were conducted to more clearly observe the impact of SBEM modulation on cellular behaviors, such as viability and PTX resistance. This maximized the differences in experimental outcomes and enhanced the persuasiveness of the findings.

The establishment of PTX-resistant cell lines was validated using the CCK-8 assay. After 48 h of PTX treatment, the SUM190PT and SKBR3 cell viabilities were significantly inhibited, with IC_{50} values of 657.63 ± 52.84 nmol/l and 732.92 ± 137.03 nmol/l, respectively (Fig. 1C). By contrast, the PTX-resistant SUM190PT/PTX and SKBR3/PTX cells exhibited respective IC_{50} values of 6073.67 ± 384.43 nmol/l and 6901.00 ± 374.46 nmol/l, with corresponding resistance coefficients of 8.95 ± 1.33 and 9.04 ± 1.46 (Fig. 1D). These data confirmed the successful generation of the PTX-resistant cell lines.

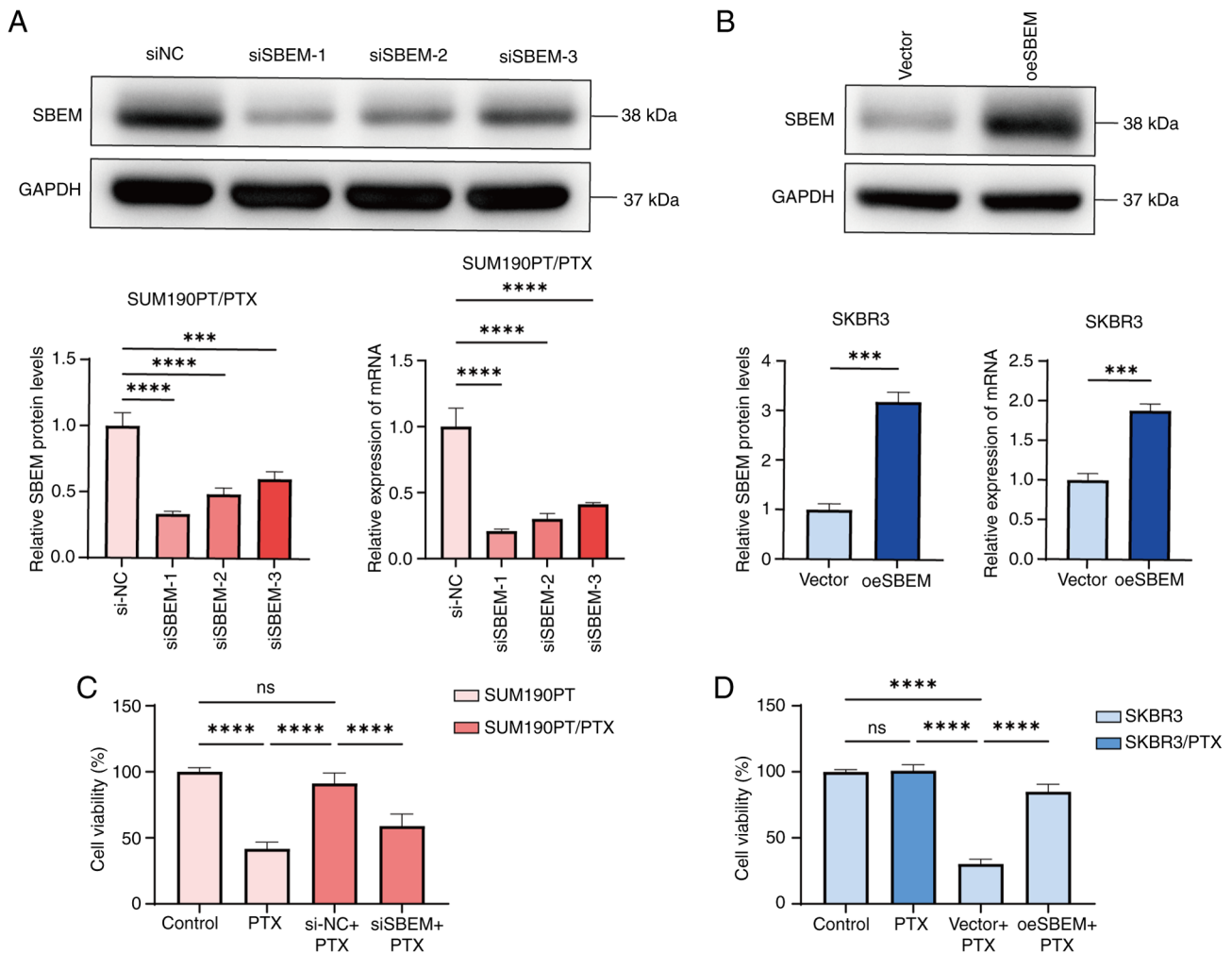


Figure 2. Changes in SBEM expression and the effect on PTX resistance. (A) Western blotting and RT-qPCR analyses of SBEM protein and mRNA levels in SUM190PT/PTX cells 48 h post-transfection with siSBEM. (B) Western blotting and RT-qPCR analyses of SBEM protein and mRNA levels in SKBR3 cells 48 h post-transfection with the SBEM overexpression plasmid. (C) SUM190PT cells were treated with PTX (500 nmol/l) for 48 h. In the case of SUM190PT/PTX cells, the PTX concentration in the drug-resistant medium was maintained at 500 nmol/l. Subsequently, siRNA (SBEM) was transfected for 48 h. The CCK-8 assay was used to detect the changes in cell viability of SUM190PT and SUM190PT/PTX cells after treatment. (D) SKBR3 cells were treated with PTX (500 nmol/l) for 48 h. Subsequently, the cells were transfected with the SBEM-overexpressing plasmid and incubated for another 48 h. For SKBR3/PTX cells, the PTX concentration in the drug-resistant medium was maintained at 500 nmol/l. The CCK-8 assay was used to detect the changes in cell viability of SKBR3 and SKBR3/PTX cells after treatment. *** $P < 0.001$, **** $P < 0.0001$. ns, not significant; PTX, paclitaxel; SBEM, small breast epithelial mucin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8; si, small interfering (RNA); NC, negative control; oe, overexpression.

To further explore the association and potential mechanism of SBEM in PTX resistance, cell lines with upregulated or downregulated SBEM expression were established (Fig. 2A and B). Among the siRNAs tested, siSBEM-1 most effectively inhibited both SBEM mRNA and protein expression and was therefore selected for subsequent experiments. The effects of SBEM knockdown and overexpression on the proliferation of SUM190PT/PTX and SKBR3 cells were assessed using the CCK-8 assay. The results demonstrated that SBEM knockdown restored PTX sensitivity in SUM190PT/PTX cells, whereas SBEM overexpression reduced PTX sensitivity (Fig. 2C and D). These findings suggest that SBEM expression may be associated with PTX sensitivity in breast cancer cells.

SBEM has binding sites for PTX and interacts with DUSP16. While it was demonstrated that SBEM may be associated with PTX sensitivity, its precise regulatory mechanism remains unclear. The following analyses were conducted to

screen the rational domains of the SBEM protein. The prediction results showed that the protein ipTM=-pTM=0.23, with a relatively low overall confidence level (Fig. S1A). In addition, amino acids 1-20 were predicted to form the signal peptide region (Fig. S1B). Next, PrankWeb was used, which is based on machine learning; it learns the characteristics of a large number of known binding sites through a Random Forest algorithm to predict the protein structure (28). The results are shown in Fig. S1C and the red area represents the active site. The score of this active site (residue site: 1) is >1 (score: 1.45) and the average AlphaFold score is >70 (score: 83.21), both of which indicate a high level of confidence in this site (27). Additionally, it was noted that SBEM is also known as MUCL1. MUCL has two subtypes: MUCL1 and MUCL3. To detect the conserved sequences, a sequence alignment between MUCL1 (ID: Q96DR8) and MUCL3 (ID: Q3MIW9) was performed (Fig. S1D). The alignment results were consistent with the

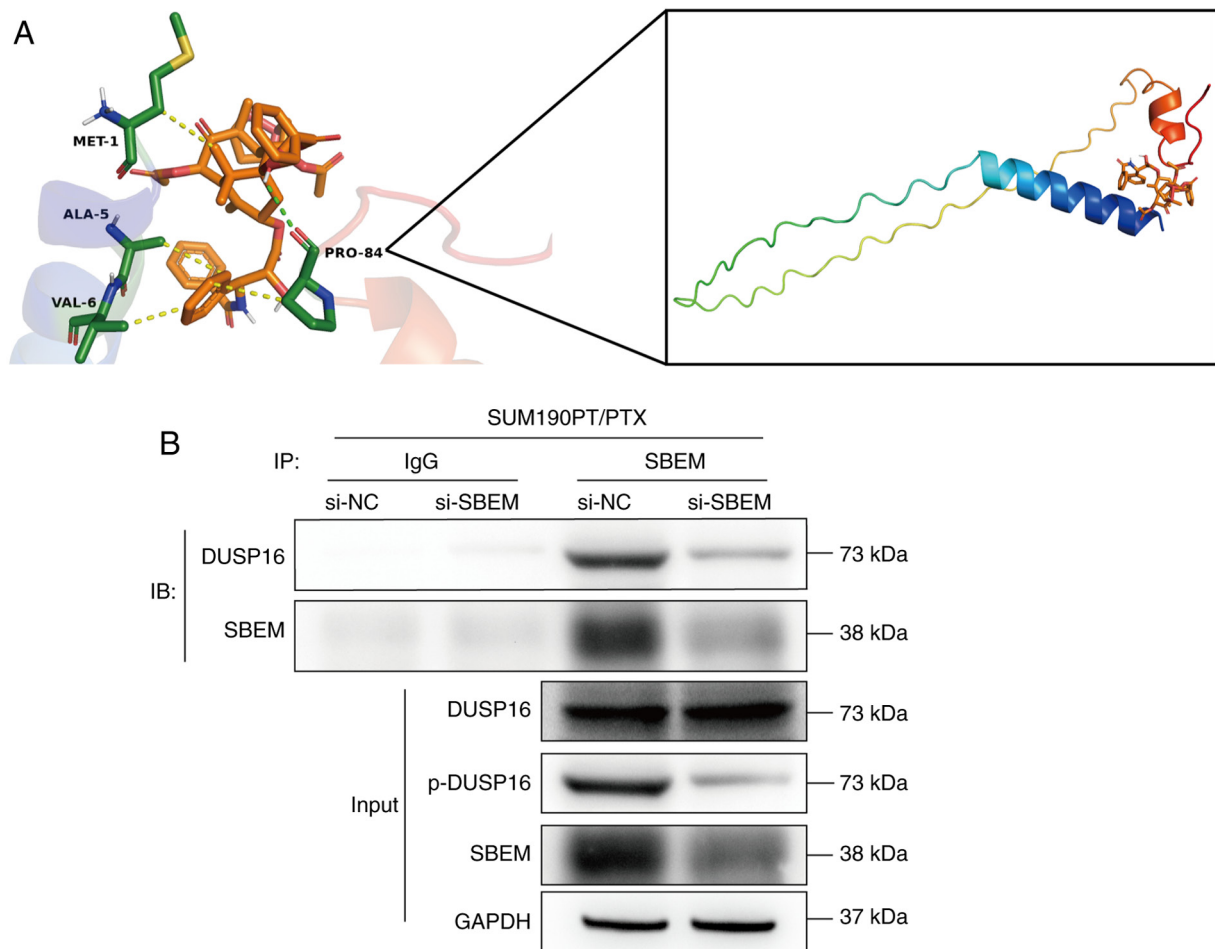


Figure 3. SBEM interacts with DUSP16. (A) Molecular docking analysis showing the interaction between PTX and SBEM. (B) Western blot analysis of the SBEM and p-DUSP16 protein levels in the SBEM protein complex, extracted by immunoprecipitation following siSBEM transfection for 48 h of SUM190PT/PTX cells. PTX, paclitaxel; SBEM, small breast epithelial mucin; DUSP16, dual specificity phosphatase 16; p-, phosphorylated; si, small interfering (RNA); NC, negative control.

prediction of the active site, both of which are located in the N-terminal region. The molecular docking results showed that PTX can bind tightly to SBEM (Fig. 3A). The strongest calculated binding free energy was -6.4 kcal/mol. In addition, each docking free energy was < -5.0 kcal/mol, demonstrating a good binding between the two. PTX was predicted to not only bind to the Pro84 amino acid of SBEM through a hydrogen bond but also form hydrophobic interactions with Met1, Ala5, Val6 and Pro84. Most notably, the distance of each interaction was < 4 Å, indicating a relatively strong bond energy (Table SV).

Additionally, to verify the interaction between SBEM and DUSP16, a Co-IP assay was performed. DUSP16 was detected in the SBEM IP, supporting a physical interaction between the two proteins. Furthermore, SBEM knockdown led to a reduction in the phosphorylated (p-)DUSP16 levels in the input group. However, no significant change in total DUSP16 protein expression was observed compared with the siNC group, suggesting that SBEM may regulate DUSP16 phosphorylation rather than its expression (Fig. 3B).

Downregulation of SBEM restores PTX sensitivity in breast cancer cells. Following SBEM knockdown, the apoptosis rate of SUM190PT/PTX cells (in culture medium containing 500 nmol/l PTX) significantly increased. The apoptosis rate

of the SBEM-knockdown group increased approximately three-fold, rising from 16.53% in the control group (siNC + PTX) to 44.25% in the knockdown group (siSBEM + PTX) (Fig. 4A). By contrast, SBEM overexpression in SKBR3 cells (in culture medium containing 500 nmol/l PTX) significantly decreased apoptosis (Fig. 4B). To further investigate this observation, the expression of apoptosis-related proteins was analyzed.

In SUM190PT/PTX cells treated with PTX, compared with the siNC + PTX group, the siSBEM + PTX group had increased expression levels of Bax and cleaved-Caspase-3/Caspase-3, while the Bcl2 expression was decreased (Fig. 4C). Conversely, in SKBR3 cells treated with PTX, compared with the Vector + PTX group, overexpression of SBEM in the oeSBEM + PTX group decreased the expression of Bax and cleaved-Caspase-3/Caspase-3 and increased the expression of Bcl2 (Fig. 4D).

Downregulation of SBEM inhibits p-DUSP16 expression and the MAPK pathway. To elucidate the mechanism by which SBEM affects PTX sensitivity, the expression levels of DUSP16 and MAPK-related proteins were analyzed. In SUM190PT/PTX cells, compared with the siNC + PTX group, knockdown of SBEM in the siSBEM + PTX

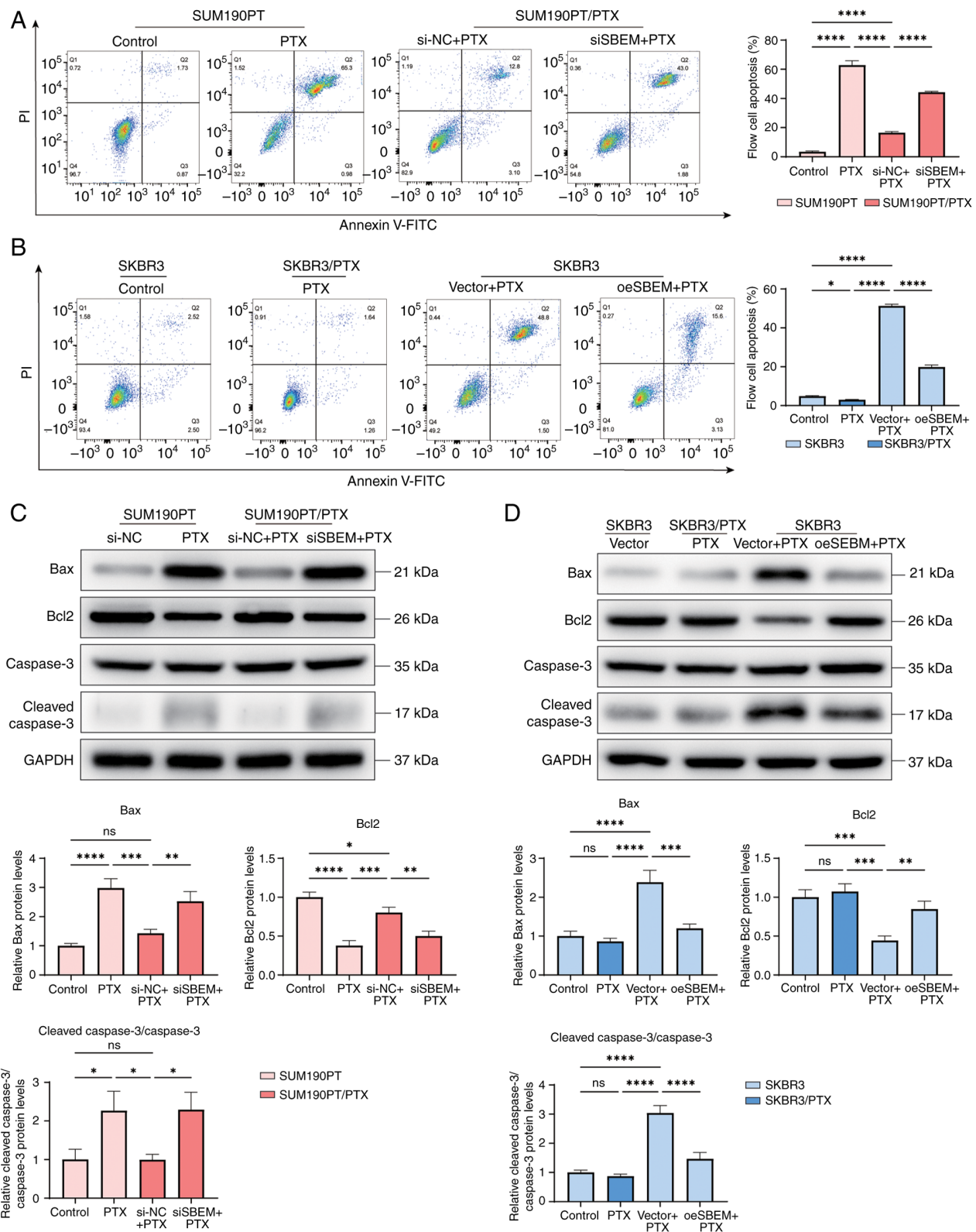


Figure 4. SBEM modulates PTX-induced apoptosis in breast cancer cells. (A) Flow cytometry analysis of apoptosis in SUM190PT/PTX and SUM190PT cells following various treatments. (B) Flow cytometry analysis of apoptosis in SKBR3/PTX and SKBR3 cells following various treatments. (C) Western blot analysis of Bax, Bcl2, Caspase-3 and cleaved-Caspase-3 in SUM190PT/PTX and SUM190PT cells. (D) Western blot analysis of Bax, Bcl2, Caspase-3 and cleaved-Caspase-3 in SKBR3/PTX and SKBR3 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant; PTX, paclitaxel; SBEM, small breast epithelial mucin; si, small interfering (RNA); NC, negative control; oe, overexpression.

group decreased the ratios of p-DUSP16/DUSP16 and p-MAPK/MAPK (Fig. 5A). By contrast, in SKBR3 cells treated with PTX, compared with the Vector + PTX group,

upregulation of SBEM in the oeSBEM + PTX group increased these ratios (Fig. 5B). These findings suggest that SBEM enhances DUSP16 phosphorylation and activates the

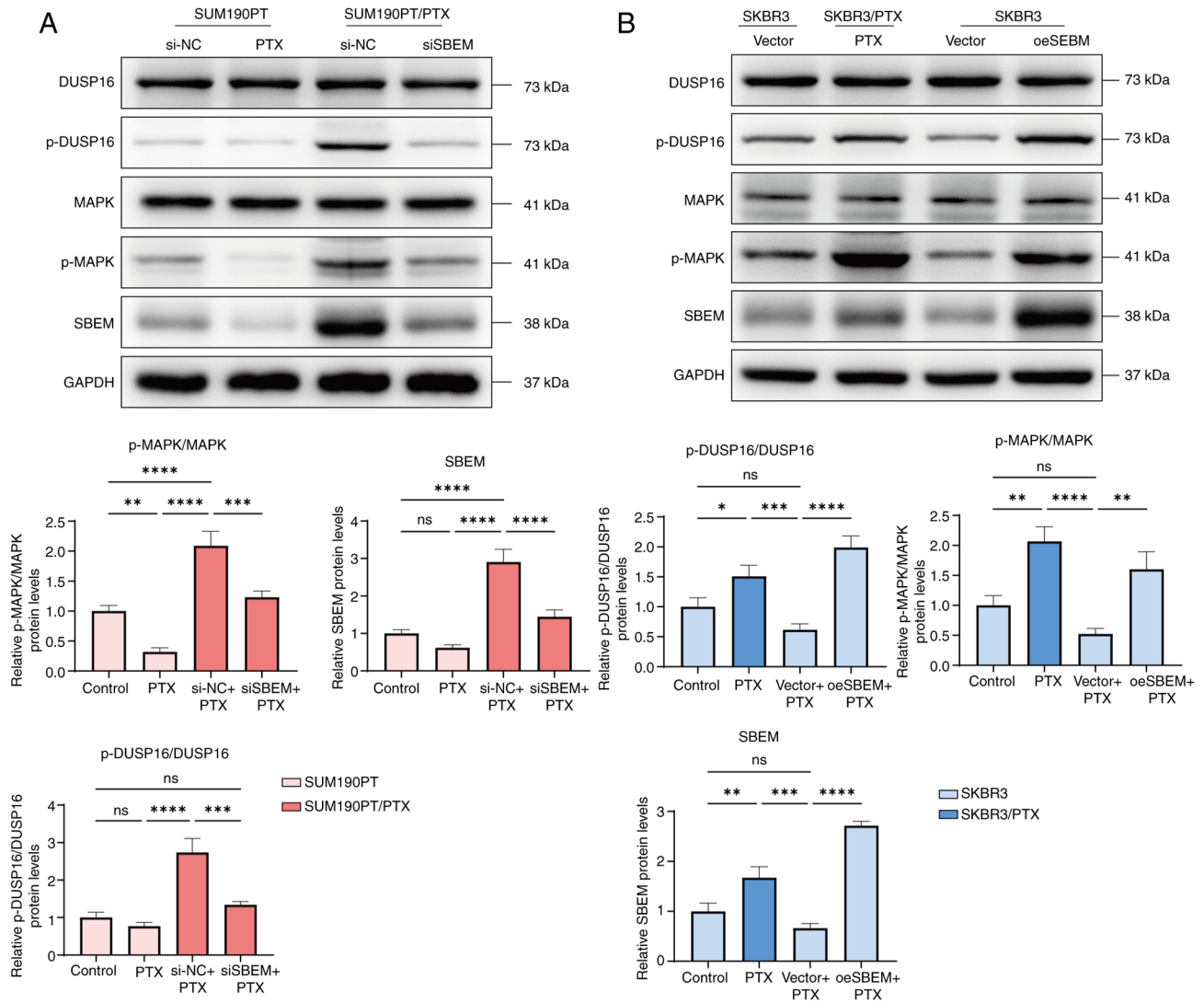


Figure 5. SBEM regulates p-DUSP16 and the MAPK signaling pathway. (A) Western blotting of DUSP16, p-DUSP16, MAPK, p-MAPK and SBEM in SUM190PT and SUM190PT/PTX cells. (B) Western blotting of DUSP16, p-DUSP16, MAPK, p-MAPK and SBEM in SKBR3 and SKBR3/PTX cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant. PTX, paclitaxel; SBEM, small breast epithelial mucin; DUSP16, dual specificity phosphatase 16; p-, phosphorylated; si, small interfering (RNA); NC, negative control; oe, overexpression.

MAPK signaling pathway, thereby potentially contributing to PTX resistance.

SBEM induces PTX resistance in breast cancer cells by activating the MAPK pathway. To further investigate the regulatory effect of SBEM on the MAPK pathway, SUM190PT/PTX cells were treated with the MAPK pathway activator, AMPK activator 13. The results showed that in SUM190PT/PTX cells treated with PTX, compared with the siSBEM group, the level of apoptosis in the siSBEM + AMPK activator 13 group decreased significantly while the cell viability increased significantly. The results indicated that AMPK activator 13 counteracted the inhibitory effects of SBEM on the viability and induction of apoptosis in SUM190PT/PTX cells (Fig. 6A and B). Furthermore, compared with the siSBEM group, the addition of AMPK activator 13 (siSBEM + AMPK activator 13 group) significantly reduced the levels of Bax and cleaved-Caspase-3 and increased the expression of Bcl2 (Fig. 6C). The effect of

AMPK activator 13 on apoptosis-related proteins reversed the impact of siSBEM.

In addition, in SUM190PT/PTX cells treated with PTX, compared with the siSBEM group, the p-DUSP16/DUSP16 level decreased and the p-MAPK/MAPK level increased in the siSBEM + AMPK activator 13 group (Fig. 7). These findings suggest that SBEM may contribute to drug resistance in breast cancer cells by activating the MAPK pathway.

Discussion

PTX, a key chemotherapeutic agent for breast cancer, is frequently associated with the development of drug resistance, making it crucial to investigate the underlying mechanisms of this resistance (29). In the present study, a predicted binding interaction between SBEM and PTX was identified and it was demonstrated that downregulation of SBEM can restore PTX sensitivity. However, the specific binding sites (single or multiple) between PTX and SBEM remain unclear. In future,

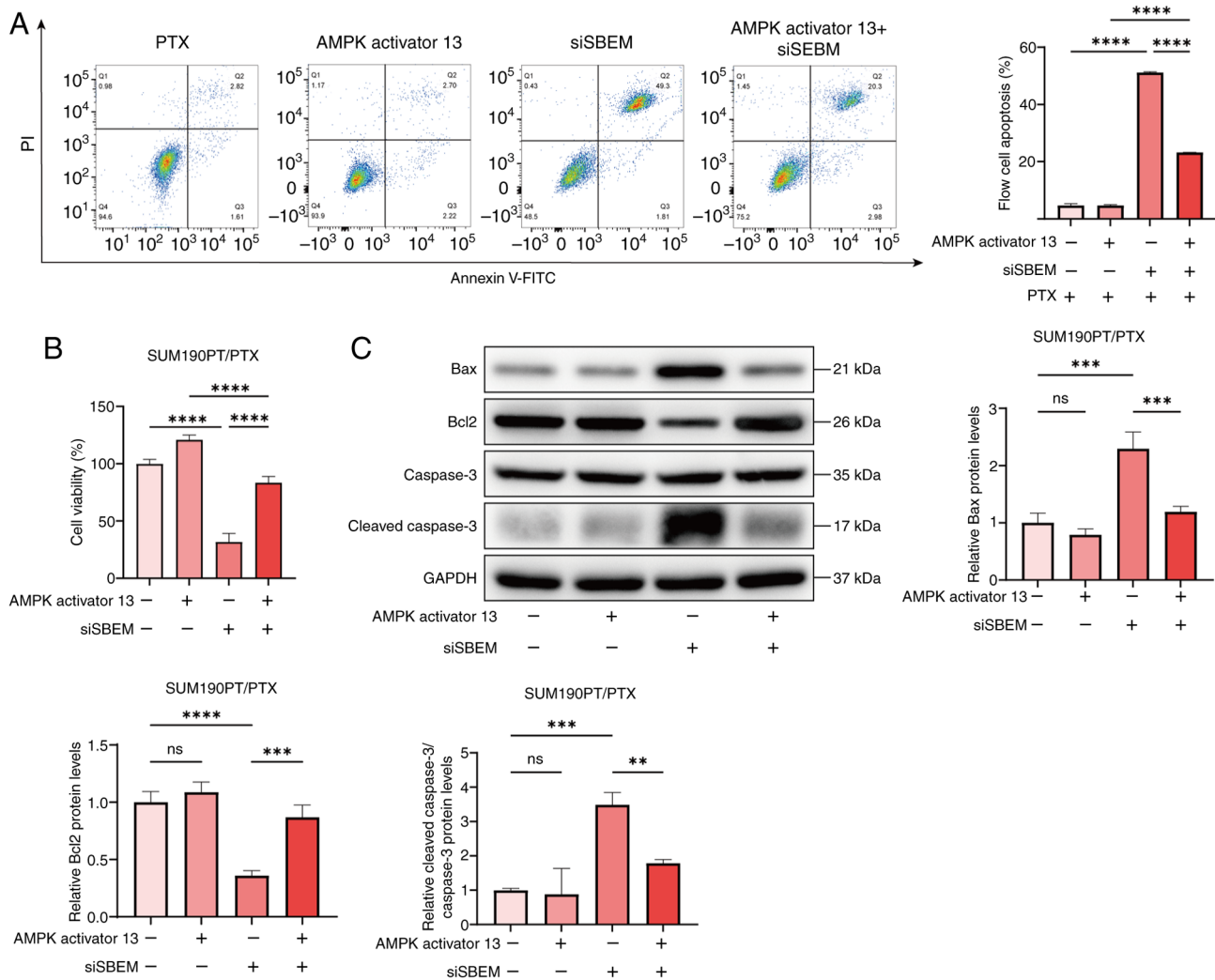


Figure 6. SBEM induces PTX resistance in breast cancer cells by activating the AMPK pathway. (A) Flow cytometry analysis of apoptosis in SUM190PT/PTX cells transfected with siRNA for 48 h and treated with AMPK activator 13 (5 μ mol/l) for 24 h. (B) The treatment of SUM190PT/PTX cells was the same as aforementioned. After the treatment, the change in cell viability was determined by the CCK-8 assay. (C) The treatment of SUM190PT/PTX cells was the same as aforementioned. After the treatment, the relative expression levels of Bax, Bcl2, Caspase-3 and cleaved-Caspase-3 proteins were detected by western blotting. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant; PTX, paclitaxel; SBEM, small breast epithelial mucin; CCK-8, Cell Counting Kit-8; si, small interfering (RNA).

we plan to utilize protein structure-based approaches such as site-directed mutagenesis and crystallographic analysis to systematically elucidate the interaction between PTX and SBEM. This will further validate and refine the findings of the present study. Apoptosis, a form of programmed cell death (30), is one of the primary mechanisms by which PTX exerts its anticancer effects. However, tumor cells often develop resistance to PTX, diminishing its therapeutic efficacy (31,32). The present study demonstrated that over-expression of SBEM in SKBR3 cells conferred resistance to PTX and reduced its cytotoxic effect. Conversely, SBEM knockdown in SUM190PT/PTX cells restored apoptosis upon PTX treatment, indicating that SBEM inhibits PTX-induced apoptosis. Bax, a member of the Bcl2 protein family, plays a crucial role in PTX-induced apoptosis by promoting mitochondrial membrane permeabilization (33). During apoptosis, Bax translocates from the cytoplasm to the mitochondrial membrane and forms homodimers, increasing mitochondrial outer membrane permeability and leading to the release of

cytochrome c and other pro-apoptotic factors, which activate the Caspase cascade and ultimately induce apoptosis (34,35). In line with this mechanism, the findings of the present study revealed that SBEM downregulation significantly increased the expression of Bax and cleaved-Caspase-3 proteins while reducing Bcl2 expression, thereby promoting apoptosis in SUM190PT/PTX cells.

PTX has been shown to activate the AMPK signaling pathway, which plays an important role in its anticancer effects (36,37). To further investigate the mechanism of SBEM-induced PTX resistance, the present study focused on the involvement of DUSP16 and the AMPK signaling pathways. AMPK, a cellular energy sensor, is activated under conditions of energy deficiency; it promotes catabolic pathways and inhibits anabolic processes. The MAPK family, which includes ERK, JNK and p38, responds to growth factors, stress and inflammatory signals to regulate cell proliferation, apoptosis, and differentiation (38). DUSP16 is mainly located downstream of the MAPK signaling pathway, and its function

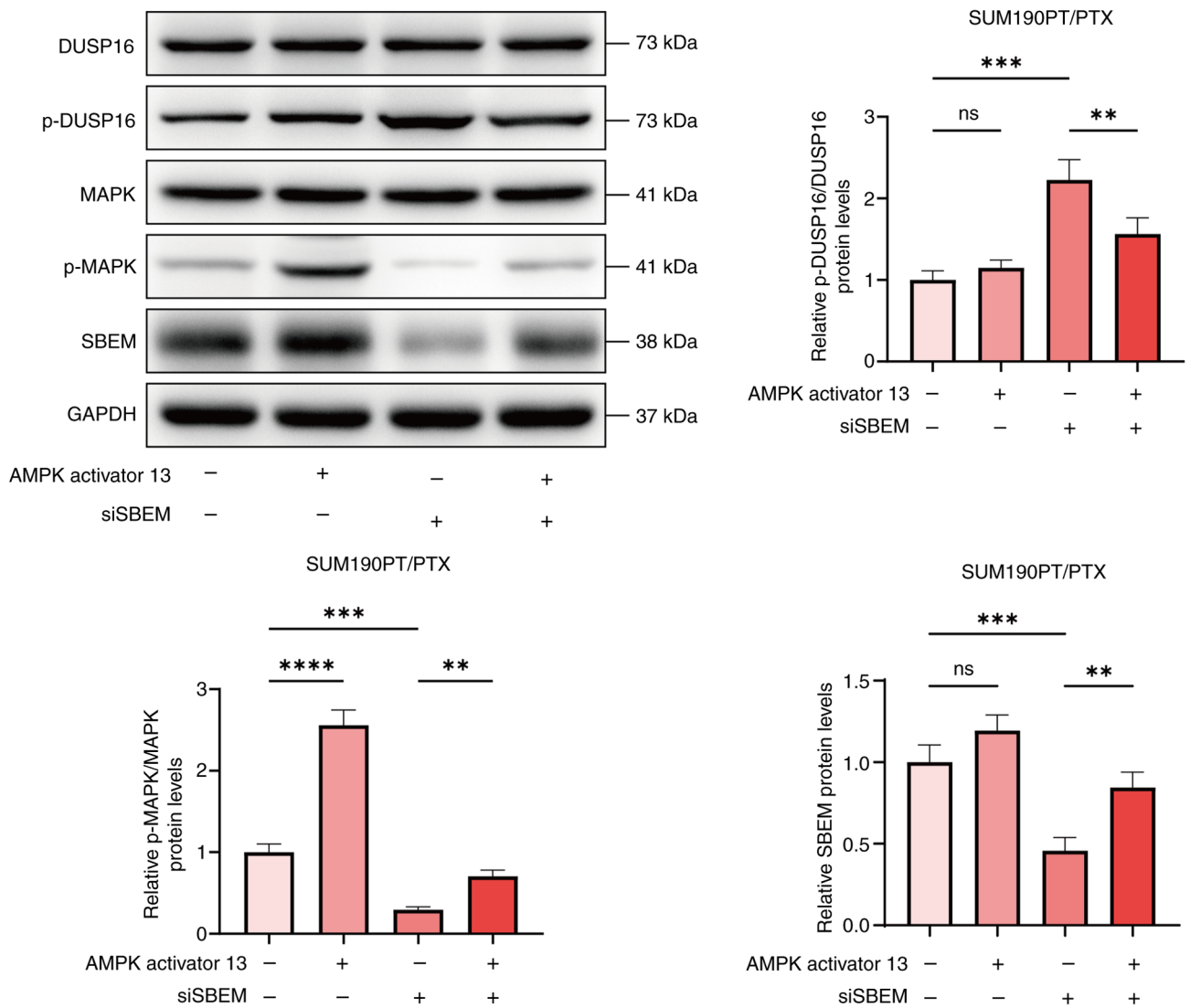


Figure 7. AMPK activator 13 restores drug sensitivity to PTX. Western blotting of DUSP16, p-DUSP16, MAPK, p-MAPK and SBEM protein levels in SUM190PT/PTX cells transfected with siRNA for 48 h and treated with AMPK activator 13 for 24 h. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant; PTX, paclitaxel; SBEM, small breast epithelial mucin; DUSP16, dual specificity phosphatase 16; p-, phosphorylated; si, small interfering (RNA).

is to inhibit the activities of p38 and JNK MAPKs (19,20). The results of the present study revealed an association between SBEM and DUSP16 expression. Overexpression of SBEM significantly increased the levels of p-DUSP16 and p-AMPK. Moreover, treatment with an AMPK activator reversed the effects of siSBEM on breast cancer apoptosis and associated protein expression. Therefore, we hypothesize that SBEM may regulate PTX resistance by activating the AMPK signaling pathway and inducing DUSP16 phosphorylation. The results of the Co-IP experiment further confirmed that SBEM and DUSP16 may interact with each other. However, based on the current results, it cannot be determined whether SBEM and DUSP16 affect protein expression through direct binding or indirect binding. Therefore, further experimental verification is needed.

The major contribution of the present study is the identification of SBEM as a regulator of DUSP16 phosphorylation and the MAPK signaling pathway, ultimately promoting PTX resistance in breast cancer cells. However, several limitations should be acknowledged. *In vivo* validation using xenograft

tumor mouse models is necessary. Furthermore, SBEM expression should be compared with established drug resistance markers, such as P-glycoprotein and β III-tubulin (39,40). Although a positive association was observed between SBEM and phosphorylated DUSP16 and MAPK proteins, the direct binding relationship between SBEM and phosphorylated DUSP16 remains to be elucidated and will be a key focus of future research. Additionally, PTX resistance involves complex signaling networks, necessitating further extensive investigation. At present, to the best of our knowledge, the relationship between SBEM expression and PTX resistance has not been examined in clinical breast cancer samples. Future work will focus on clinical collaboration to collect and analyze samples from patients with PTX-resistant breast cancer, to further validate the clinical relevance and therapeutic potential of targeting SBEM.

In conclusion, the results of the present study demonstrated that downregulation of SBEM enhanced the sensitivity of breast cancer cells to PTX by targeting DUSP16 and inhibiting the MAPK signaling pathway. These findings suggest that

SBEM could serve as a therapeutic target to overcome PTX resistance. Thus, the present study provides a theoretical basis for the use of SBEM inhibitors in combination with PTX to improve treatment efficacy in breast cancer.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LL conceived, designed and supervised the study. NL and XL performed the data analysis and drafted the manuscript. QY, SL and JW collected the data. HS and YD were responsible for conceptualization and data curation. XL revised the manuscript. NL and XL confirm the authenticity of all the raw data. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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