

SB225002 inhibits prostate cancer invasion and attenuates the expression of BSP, OPN and MMP-2

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Abstract. The mechanisms of malignant cell metastasis to secondary sites are complex and multifactorial. Studies have demonstrated that small integrin-binding ligand N-linked glycoproteins (SIBLINGs), particularly bone sialoprotein (BSP) and osteopontin (OPN), are involved in neoplastic growth and metastasis. SIBLINGs promote malignant cell invasion and metastasis by enhancing matrix metalloproteinase 2 (MMP-2) and MMP-9 expression. Moreover, BSP and OPN can combine with integrin, which is located on the tumor cell surface, to further promote the malignant behavior of tumor cells. In the present study, we investigated whether SB225002, a specific CXCR2 receptor antagonist, can inhibit prostate cancer cell expression of BSP and OPN and reduce cancer cell invasion ability. A series of experiments showed that after SB225002 treatment, the proliferation, invasion and migration of two androgen-independent prostate cancer cell lines were inhibited, but this inhibitory effect was not observed on androgen-dependent prostate cancer cells. Western blotting showed that the PI3K signaling pathway could regulate the expression of SIBLING and MMP family proteins, and SB22055 could reduce the expression of BSP, OPN and MMP-2 in prostate cancer cells by inhibiting AKT/mTOR phosphorylation. Finally, *in vivo* experiments confirmed that SB225002 inhibited the proliferation of prostate cancer cells *in vivo*, and the expression levels of BSP, OPN and MMP-2 were also inhibited.

Introduction

Metastasis is a major obstacle in cancer therapy; despite constant standardization of chemotherapy or surgical procedures (1,2), some patients die due to distant metastasis of tumor cells. The purpose of this experiment was to explore a new method to inhibit the invasion of tumor cells more efficiently.

Tumor metastasis is a multistage process during which malignant cells spread from the primary tumor to distant organs (3). With the participation of multiple proteins and cytokines, tumor cells infiltrate the circulatory system and avoid immune system attacks, eventually reaching target organs for implantation and growth (4). The SIBLING (small integrin-binding ligand N-linked glycoprotein) family of proteins regulates malignant tumor behaviors such as malignant cell proliferation, detachment, invasion, and metastasis by combining with integrin protein (5). SIBLINGs are over-expressed in many cancers (2,6-8). The level of SIBLING protein in serum is often used to predict the prognosis of many cancer patients, especially in patients with prostate and breast cancer (9-11). Two proteins in this family, namely, OPN (osteopontin) and BSP (bone sialoprotein), have garnered the most attention, and their reported levels of expression are closely correlated with tumor aggressiveness. For invasion, BSP and OPN can activate specific metalloproteinases (BSP activates MMP-2, OPN activates MMP-3) to enhance the ability of cancer cells to hydrolyze the extracellular matrix (ECM) (6,12). The binding of BSP and integrins contributes to metastasis formation of breast cancer cells, particularly bone metastasis (13). Moreover, BSP-transfected breast cancer cells showed increased primary tumor growth following injection into the mammary fat pad of nude mice (14), and OPN stimulated human prostate cancer (PCa) cell proliferation when transferred to a mouse xenograft model system (15). These effects mainly occurred through BSP and OPN activation of the epidermal growth factor receptor (EGFR) and integrin-mediated intracellular Ca²⁺ signaling (16). Therefore, it is important to identify a method that can inhibit the expression of BSP and OPN in tumor cells to prevent tumor cell metastasis.

Studies have suggested that interleukin-8 (IL-8) and its cognate receptors, namely, C-X-C chemokine receptor-1 (CXCR1) and CX-C chemokine receptor-2 (CXCR2), mediate the initiation and development of various types of cancers, including breast cancer, PCa, lung cancer, colorectal carcinoma and melanoma (17-21). IL-8 also integrates with multiple intracellular signaling pathways to produce coordinated effects. In terms of invasion, IL-8 promotes prostate and breast cancer expression of matrix metalloproteinases (MMPs) (especially MMP-2 and MMP-9) to enhance their cell aggressiveness (22,23). The ectopic expression of IL-8

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stimulated by IL-1 β and TNF- α can enhance the metastatic potential of breast cancer since a high level of IL-8 can promote angiogenesis and attract neutrophils to release enzymes involved in tissue remodeling and tumor establishment (24). Increased IL-8 secretion by PCa cells is similarly associated with the malignant biological behaviors of cancer cells. IL-8/CXCR2 promotes castration-resistant growth and proliferation of AIPC cells (androgen-independent PCa cells) by activating cyclin D1 expression in a PI3K/Akt/mTOR and MAPK pathway-dependent manner (25,26).

SB22502 is a specific CXCR2 receptor antagonist, and studies have shown that SB225002 induces apoptosis in ovarian cancer cells and cell death and cell cycle arrest in acute lymphoblastic leukemia cells (27,28). However, few studies have described the inhibition of cancer cell invasion or metastasis by SB225002. This study shows for the first time that SB225002-treated human PCa DU-145, LNCAP and PC-3 cells exhibited reduced invasion ability. At the same time, we detected the expression of BSP, OPN, MMP-2, MMP-9 and $\alpha\text{v}\beta\text{3}$ after treatment with SB225002 and different signaling pathway inhibitors to further clarify the underlying molecular mechanism of SB225002 function in PCa cells.

Materials and methods

Cells and culture. Human androgen-independent prostate cancer DU-145 cells were obtained from Biotechnology Company (Shenyang, China) and were cultured in MEM medium (Corning Inc., Corning, NY, USA), supplemented with 10% FBS and 1% penicillin/streptomycin and cultured in 5% CO₂ at 37°C. Androgen-independent prostate cancer PC-3 cells and androgen-dependent prostate cancer LNCAP cells were provided by the Brain and Spinal Injury Laboratory of Liaoning Province (Liaoning, China). PC-3 cells were cultured in F-12, and LNCAP cells were cultured in RPMI-1640; the other culture conditions were the same as those of DU-145 cells.

Reagents and treatment. LY294002 (Akt inhibitor), U0126 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK1/2 inhibitor) and SB225002 (CXCR2 receptor antagonist) were purchased from Selleck Chemicals (Houston, TX, USA). The primary antibodies for PI3K (cat. no. sc-71891), AKT (cat. no. sc-5270)/p-AKT (cat. no. sc-271966) and mTOR (cat. no. sc-293089PE)/ p-mTOR (cat. no. sc-293133) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for MMP-2 (WL1579), MMP-9 (WL01580), and OPN (WL02848) were obtained from Wanleibio (Shenyang, China). BSP (BA2329) was purchased from Boster Biological Technology (Wuhan, China). $\alpha\text{v}\beta\text{3}$ (bs-1310R) and p-PI3K (bs-6417R) were purchased from Bioss Biological Technology (Beijing, China). MTT and DAPI were provided by Solarbio (Beijing, China), and Transwell Matrigel was purchased from Corning Inc. The anti-human BSP Elisa kit was purchased from AMEKO (Shanghai Lianshuo Biological Technology Co., Ltd., Shanghai, China).

Cell viability analysis. Three PCa cell lines were seeded into 96-well plates (at a cell density of 2×10^3 cells/well) cultured in normal growth medium and treated with different

concentrations of SB225002 (0, 1, 3, 5, 10 and 15 μM) for 72 h. After incubation, MTT solution (0.5 mg/ml) was added to each well, and the plates were incubated in a humidified incubator at 37°C for 4 h. At the end of the incubation period, the medium was removed, formazan was dissolved in DMSO, and the optical densities were determined at 490 nm using a microplate reader. The cell growth inhibition rate was calculated using the following equation: Cell growth inhibition rate = 1-(absorbance of experimental value/absorbance of control).

Migration and invasion assays. *In vitro* invasion was determined in 24-well Transwell inserts with 8- μm pore-size filters. The basement membrane Matrigel was diluted to 200 $\mu\text{g/ml}$ with serum-free RPMI-1640 medium, and the filters were coated with 100 μl of basement membrane Matrigel, air-dried and hydrated with 100 μl of serum-free RPMI-1640 per well for 30 min prior to cell addition. Cells were added to the upper chamber inserts at a concentration of 5×10^4 cells in 0.2 ml of serum-free medium (at least 3 replicates for each sample). Three media (500 ml) containing 20% FBS were added to the lower chamber. In the SB225002 group, 5 μM of the drug was added. After incubation for 48 h at 37°C, cells in the upper part of the Transwells were removed with a cotton swab, and the chambers were washed with PBS. Cells that had migrated were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 10 min (13). At the same time, the experimental group and the control group without Matrigel were set up to calculate the invasion rate of the cancer cells. For migration, cells were plated in 6-well plates at a density of 5×10^5 cells per well until they reached 90% confluence. A single wound was scraped with a pipette tip (200 μl was used) in the center of the cell monolayer, and the wells were washed with PBS to remove cell debris. After an additional 48 h of culture, wound healing was visualized with an inverted microscope (Olympus IX51; Olympus Corp., Tokyo, Japan).

Western blot analysis. Cells were harvested and lysed with lysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Each group of protein samples was quantified using the BSA Protein Quantification kit. Equal amounts of protein (20 $\mu\text{g/lane}$) from the sample were electrophoresed on 10% SDS-PAGE gels and were transferred to PVDF membranes. The membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 for 1 h at room temperature. After washing with TBST three times, the membranes were co-incubated with the primary antibody (1:500 for anti-BSP, 1:500 for anti-MMP2, 1:500 for GAPDH) overnight at 4°C in TBST. After incubation with horseradish peroxidase (HRP) goat anti-rabbit IgG (1:10,000) in TBST for 60 min, the proteins were visualized using the ECL detection kit (Wanleibio). Imaging system used ImageQuant™ LAS 4000 (GE Healthcare, Chicago, IL, USA).

Tumor xenografts. Fifteen BALB/c mice (as SIBLING proteins help tumor cells escape immune system attack, nude mice were not selected) weighing 18-22 g were obtained from the Brain and Spinal Injury Laboratory of Liaoning (Liaoning, China) and reared at a temperature 18-29°C; relative humidity 50-60%; ventilation 8-12 times/h; light 10-12 h/day, and were randomly divided into three groups: untreated

Table I. MTT assay was used to detect the inhibitory effect of SB225002 on prostate cancer cell proliferation.

A, DU-145						
SB225002 ($\mu\text{mol/l}$)	OD (n=3, $\bar{x} \pm \text{SD}$)			Inhibition rate of cell growth (%)		
	24 h	48 h	72 h	24 h	48 h	2 h
0	0.211 \pm 0.023	0.274 \pm 0.197	0.281 \pm 0.026	0.00	0.00	0.00
5	0.109 \pm 0.017	0.137 \pm 0.013	0.110 \pm 0.028	48.34	50.00	60.85
10	0.059 \pm 0.015	0.042 \pm 0.010	0.003 \pm 0.001	72.22	84.85	98.91
15	0.026 \pm 0.012	0.021 \pm 0.009	0.002 \pm 0.001	87.81	92.34	99.01
B, PC-3						
SB225002 ($\mu\text{mol/l}$)	OD (n=3, $\bar{x} \pm \text{SD}$)			Inhibition rate of cell growth (%)		
	24 h	48 h	72 h	24 h	48 h	2 h
0	0.203 \pm 0.019	0.272 \pm 0.109	0.293 \pm 0.016	0.00	0.00	0.00
5	0.119 \pm 0.007	0.138 \pm 0.013	0.108 \pm 0.034	41.37	49.29	63.13
10	0.077 \pm 0.026	0.041 \pm 0.031	0.003 \pm 0.001	62.11	84.79	99.01
15	0.038 \pm 0.022	0.019 \pm 0.009	0.004 \pm 0.001	81.27	93.12	98.70
C, LNCAP						
SB225002 ($\mu\text{mol/l}$)	OD (n=3, $\bar{x} \pm \text{SD}$)			Inhibition rate of cell growth (%)		
	24 h	48 h	72 h	24 h	48 h	2 h
0	0.224 \pm 0.011	0.247 \pm 0.034	0.291 \pm 0.020	0.00	0.00	0.00
5	0.140 \pm 0.003	0.136 \pm 0.060	0.152 \pm 0.024	37.70	45.02	47.77
10	0.120 \pm 0.036	0.125 \pm 0.031	0.123 \pm 0.071	47.27	49.44	57.89
15	0.108 \pm 0.023	0.092 \pm 0.019	0.042 \pm 0.017	51.71	62.70	85.27

The MTT assay was used to detect SB225002 inhibition of cell proliferation in three PCa cell lines. For DU-145 and PC-3 cells, the inhibition rate with 5 μM SB225002 at 48 h reached 50%; however, the inhibitory effect of SB225002 on LNCAP cells was not obvious, 85% at 72 h with 15 μM SB225002. The values represent means \pm SD, and MTT assay data are representative of at least 3 independent experiments.

group, DU-145 implantation group and DU-145 implantation + SB225002 injection group. The number of cells implanted was 1×10^6 /mouse. All the procedures were performed in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of China. The tumor volume (V) was measured at 1 week (W) and 2 W after DU-145 implantation and SB225002 injection, and the measurements were calculated as $V = (\text{length} \times \text{width}^2)/2$.

Immunohistochemistry. After xenograft implantation was completed at 1 W and 2 W, the tumors were harvested, paraffin embedded, deparaffinized and rehydrated through a gradient alcohol series using standard protocols. Next, endogenous peroxidase was inactivated with 3% hydrogen peroxide for 6 min, antigen unmasking was performed by heat retrieval performed using citrate buffer (pH 6.1), and the slides were washed in PBS three times. Each group was incubated with the respective primary antibody overnight at 4°C in a humid

chamber after blocking in goat serum at room temperature for 15 min. Thereafter, anti-rabbit biotinylated secondary antibody was added, followed by incubation at 37°C for 1 h, the addition of HRP labeled streptavidin, and incubation at room temperature for 30 min with DAB color 3 min. Finally, hematoxylin was used to dye the nuclei. The results were evaluated by two pathologists who performed a double-blind reading as follows: Colorless, 0 points; light yellow, 1 point; dark yellow, 2 points; brown, 3 points. The percentage of positive cells was counted in each field. The percentage of positive cells per 100 cells was counted under a upright microscope (Olympus BX53; Olympus Corp.) (magnification, $\times 200$): 0 indicates negative, 1 indicates the percentage of positive cells <10%, 2 indicates the percentage of positive cells 10-50%, 3 indicates the percentage of positive cells >50-75%, and 4 indicates the percentage of positive cells >75%. The average score for each group was calculated as the average color depth multiplied by the percentage of the positive cell score. For the final score, <1 indicates negative (-), 1-2

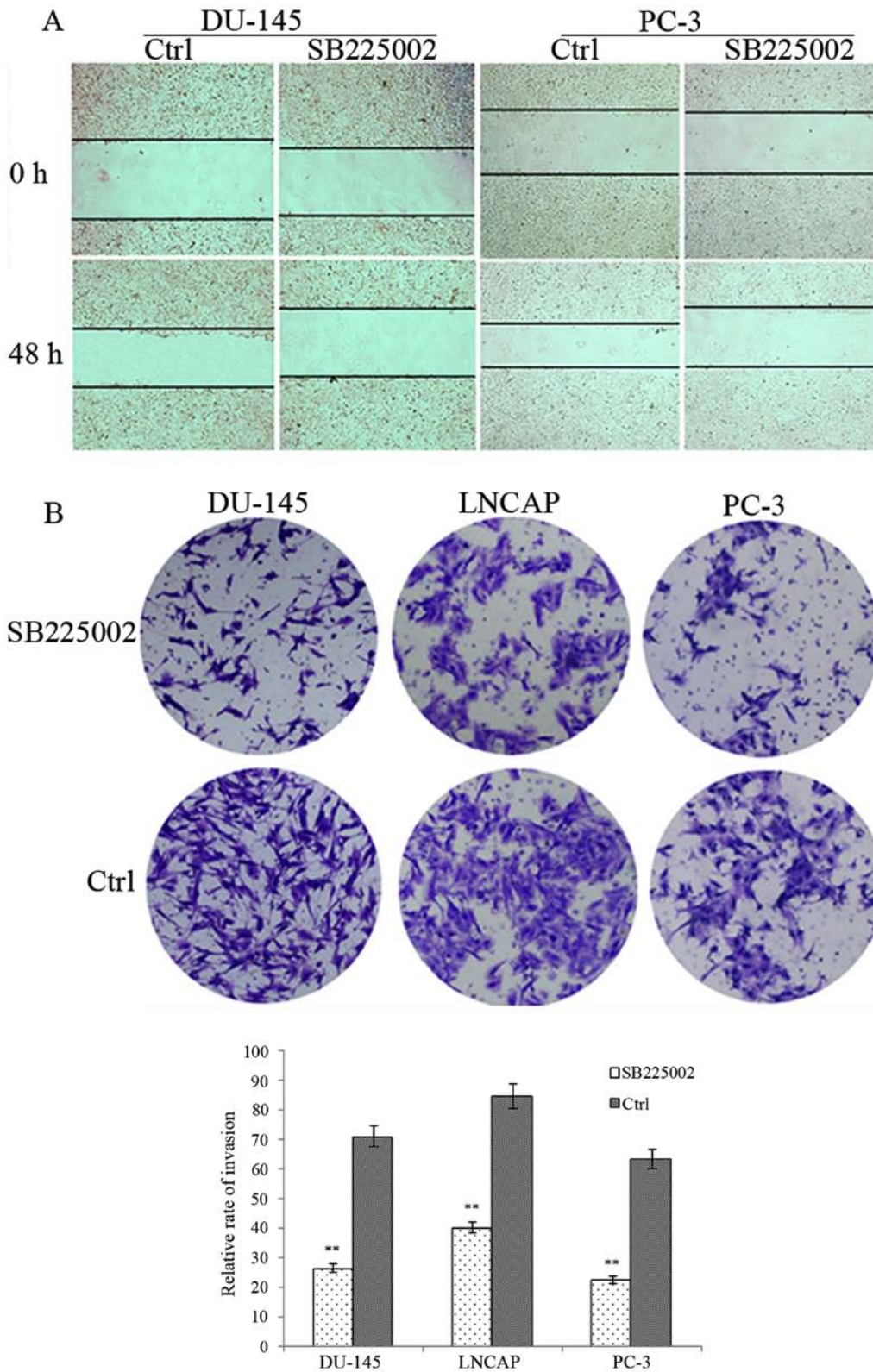


Figure 1. Transwell and migration assays were performed before and after SB225002 treatment. (A) The migration assay showed that although two prostate cancer cell lines showed a slower speed of healing, the wound healing in the SB225002 group was still slower than that of the control group. (B) The Transwell assay indicated that SB225002 suppressed the invasion ability of the three PCa cell lines through the Matrigel barrier. For DU-145 cells, the relative rate of invasion was 26.54% in the SB225002 group compared with 71.21% in the control (P=0.005). For LNCAP cells, the relative rate of invasion was 40.31% in the SB225002 group compared with 84.64% in the control (P=0.005). Finally, for PC-3 cells, the relative rate of invasion was 22.65% in the SB225002 group and 63.44% in the control. **P<0.01. Results of the Transwell assays represent at least 3 independent experiments. PCa, prostate cancer; SB225002, a specific CXCR2 receptor antagonist.

indicates weakly positive (+), 3-4 indicates positive (++) , and >4 indicates strongly positive (+++).

ELISA. Enzyme-linked immunosorbent assay was performed to determine whether SB225002 reduced BSP and OPN

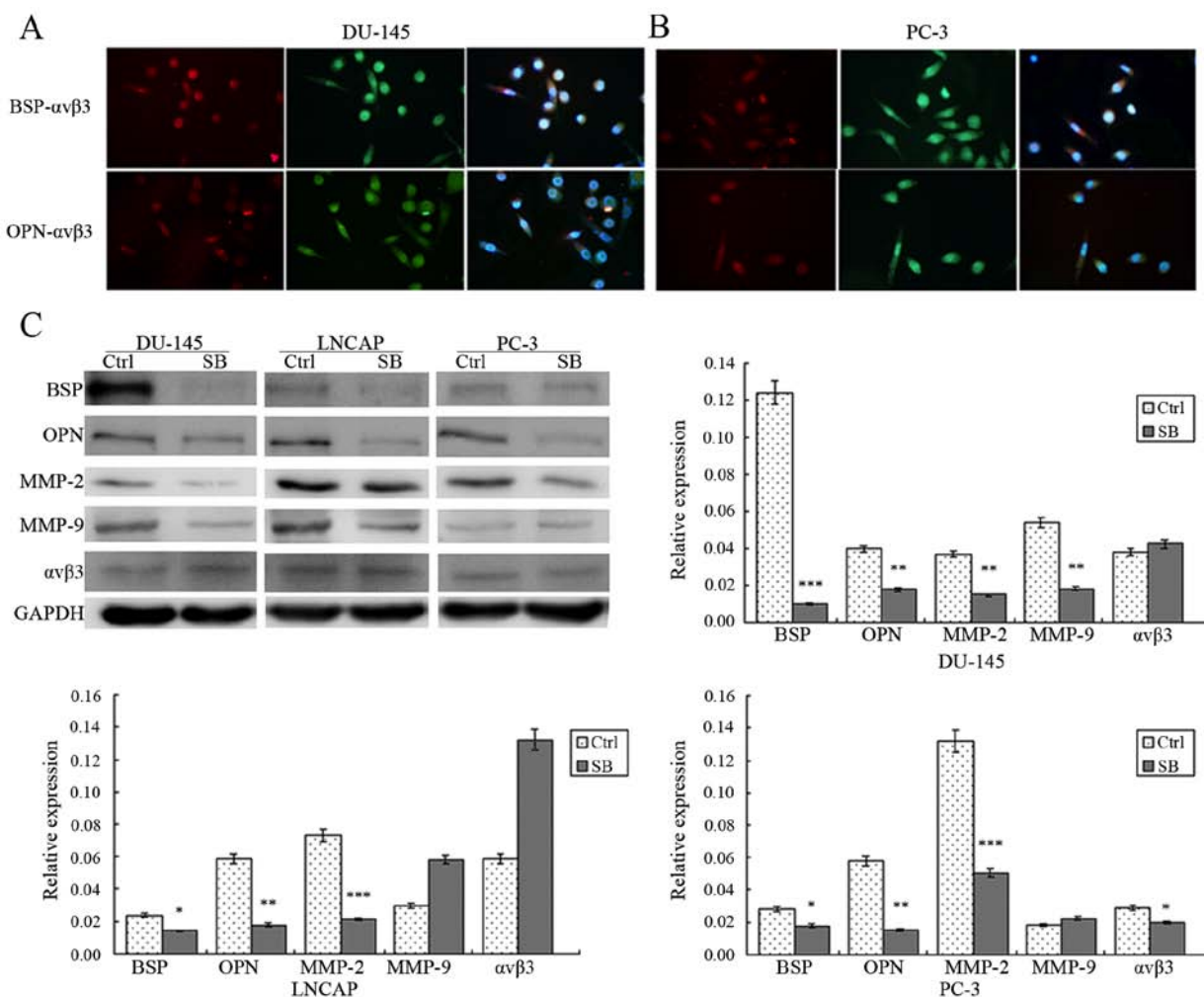


Figure 2. (A and B) Immunofluorescence detected that BSP/OPN and $\alpha v \beta 3$ were co-expressed in DU-145 and PC-3 cells. (C) Western blotting showed that SB225002 reduced expression of BSP, OPN and MMP-2 in the PCa cells ($P < 0.05$), but not that of MMP-9 and $\alpha v \beta 3$. Each histogram was compared with the semi-quantitative result of western blot analysis; each experiment was repeated at least 3 times, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PCa, prostate cancer; BSP, bone sialoprotein; OPN, osteopontin; MMP, metalloproteinase; $\alpha v \beta 3$, integrin alpha v and integrin beta 3; SB, SB225002 (a specific CXCR2 receptor antagonist); Ctrl, control; LY, LY294002 (Akt inhibitor); U0, U0126 (ERK1/2 inhibitor); S, SB203580 (p38 MAPK inhibitor); SP, SP600125 (JNK1/2 inhibitor).

secretion *in vivo*. At the end of 7 days of SB225002 injection, mouse blood was obtained through eye arteries. After centrifugation at 1,000 rpm for 10 min, the serum was separated from whole-blood samples, and BSP and OPN were detected using the Human BSP/OPN ELISA kit.

Immunofluorescence. DU-145 and PC-3 cells were seeded into 96-well plates. When the cell density reached 60%, the medium was removed, and the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. The cells were then permeabilized using Triton X-100, and each well was treated with blocking buffer (1X TBST, 3% goat serum) for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies (BSP, 1:300; OPN, 1:300; $\alpha v \beta 3$, 1:100) diluted in blocking buffer. The samples were washed 3 times with 1X PBS and were incubated with secondary antibodies for 1 h before mounting with Prolong Gold antifade reagent (Solarbio, Beijing, China) with DAPI.

Statistical analysis. All the statistical analyses were evaluated using SPSS 21.0 software (IBM Corp., Armonk,

NY, USA). Data are presented as the means \pm SD (standard deviation). Statistical analysis was performed using one-way ANOVA followed by the Bonferroni or Dunnett (2-sided) test for comparisons. The level of significance was set at $P < 0.05$.

Results

SB225002 inhibits PCa cell proliferation and invasion. The MTT results showed that, after 72 h of culture, DU-145 and PC-3 proliferation was inhibited in a concentration- and time-dependent manner upon treatment with SB225002. The growth inhibition rate of the DU-145 and PC-3 cells reached 50% after treatment with 5 μ M SB225002 for 48 h, and it reached almost 100% after treatment with 10 μ M SB225002 for 72 h. For LNCAP cells, 15 μ M SB225002 for 72 h showed an inhibition rate of 85% (Table I). To further explore the effect of SB225002 on invasion ability, the Transwell assay was used to validate the effect of SB225002 on the invasive ability of PCa cells. After 48 h of treatment, the number of SB225002-treated cancer cells from the three cell lines

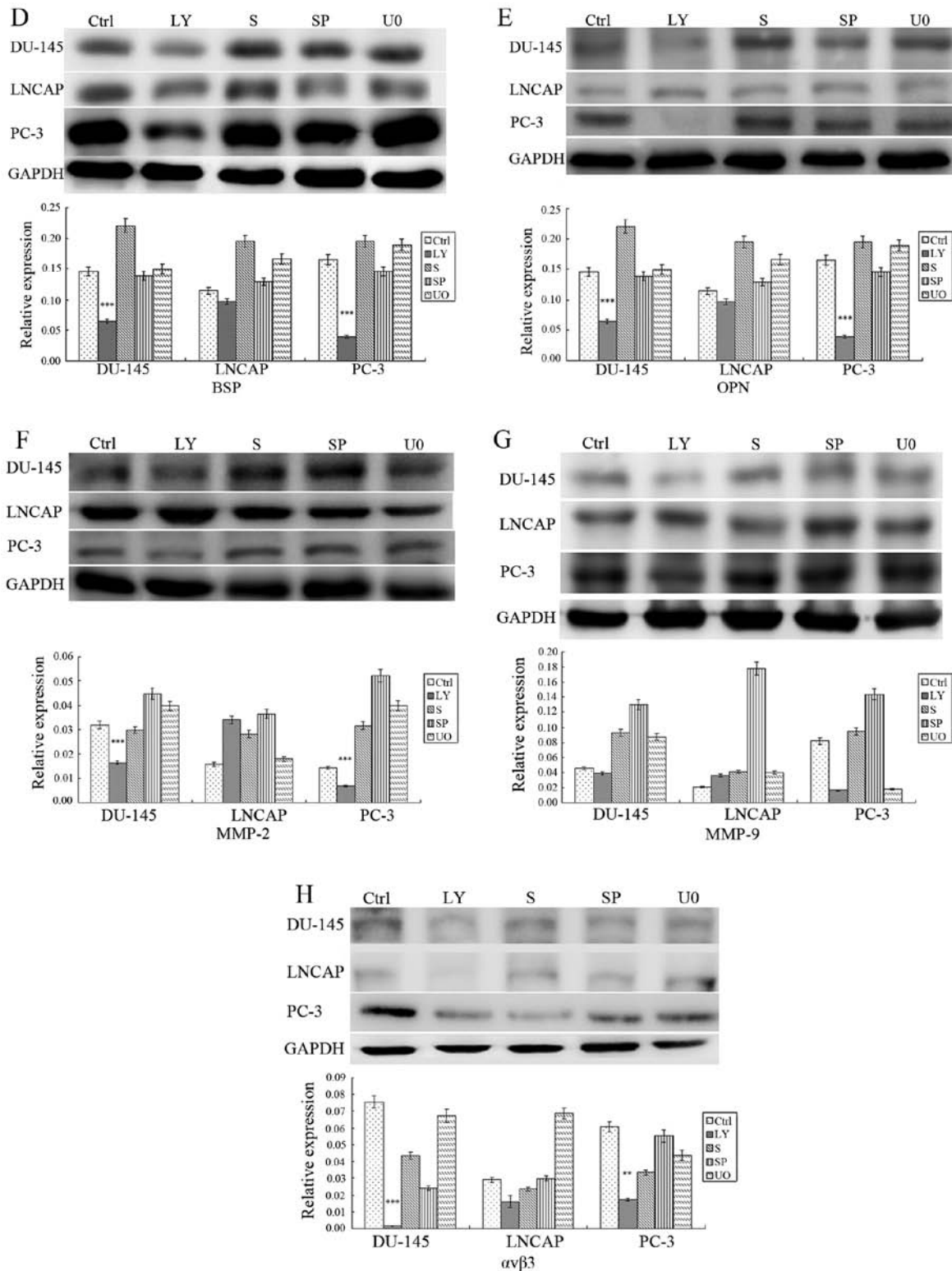


Figure 2. Continued. (D-H) Effect of different signaling pathway inhibitors on BSP, OPN, MMP-2, MMP-9 and $\alpha v \beta 3$ expression. The expression levels of BSP, OPN, MMP-2 and $\alpha v \beta 3$ were decreased significantly in the LY294002 group of the DU-145 and PC-3 cells; however, levels of these five proteins were not obviously changed in the LNCAP cells. Each histogram was compared with the semi-quantitative result of western blot analysis; each experiment was repeated at least 3 times, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PCa, prostate cancer; BSP, bone sialoprotein; OPN, osteopontin; MMP, metalloproteinase; $\alpha v \beta 3$, integrin alpha v and integrin beta 3; SB, SB22502 (a specific CXCR2 receptor antagonist); Ctrl, control; LY, LY294002 (Akt inhibitor); UO, U0126 (ERK1/2 inhibitor); S, SB203580 (p38 MAPK inhibitor); SP, SP600125 (JNK1/2 inhibitor).

invading through the Matrigel barrier was less than that of the control groups (Fig. 1B). After the cells were counted under random high magnification, the DU-145 penetration rate of the control group was 71.21% and that of the SB225002 group was

26.54% ($P = 0.005$). The LNCAP penetration rate of the control group was 84.67% for the control and 40.31% after SB225002 treatment ($P = 0.005$). Finally, the PC-3 penetration rate was 63.44% for the control and 22.65% after SB225002 treatment

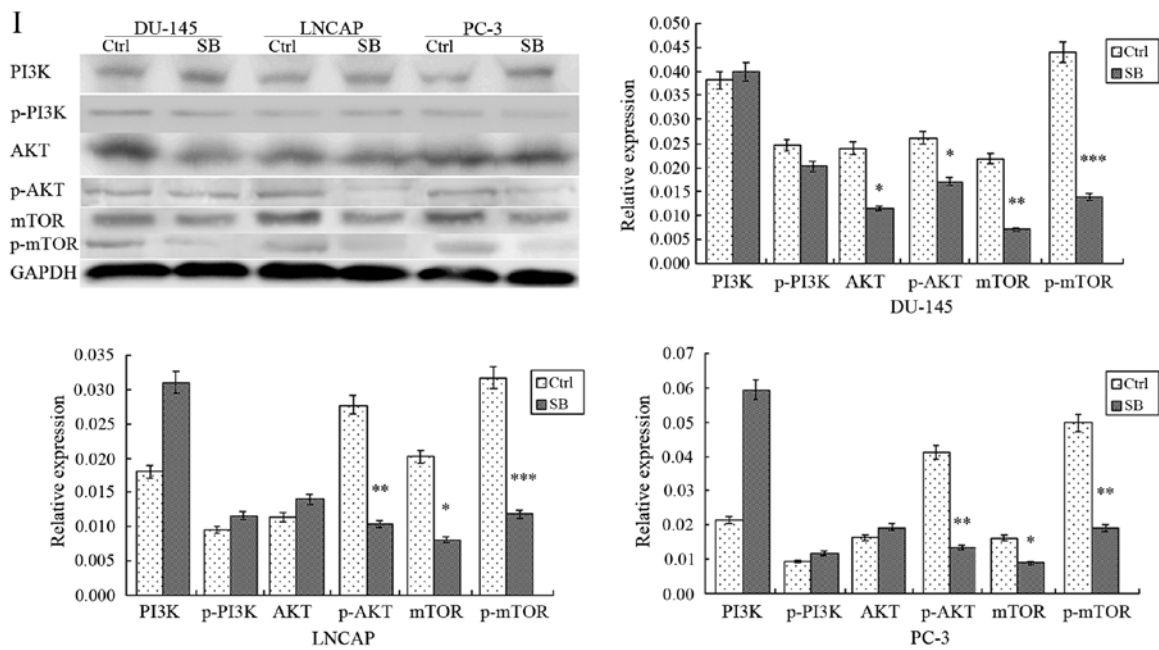


Figure 2. Continued. (I) Expression of p-AKT and p-mTOR was decreased obviously in the DU-145 and PC-3 cells after SB225002 treatment. Each histogram was compared with the semi-quantitative result of western blot analysis; each experiment was repeated at least 3 times, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PCa, prostate cancer; BSP, bone sialoprotein; OPN, osteopontin; MMP, metalloproteinase; $\alpha\beta 3$, integrin alpha v and integrin beta 3; SB, SB225002 (a specific CXCR2 receptor antagonist); Ctrl, control; LY, LY294002 (Akt inhibitor); UO, U0126 (ERK1/2 inhibitor); S, SB203580 (p38 MAPK inhibitor); SP, SP600125 (JNK1/2 inhibitor).

($P = 0.001$). For the migration assay (Fig. 1A), after 48 h of incubation, the wounds in the control groups of the DU-145 and PC3 cells were significantly reduced by 25% for DU-145 and 30% for PC-3; however, in the SB225002 group, the two cell lines migrated poorly, showing a decrease of 10 and 5%, respectively, in wound healing.

Co-expression of BSP/OPN and $\alpha\beta 3$ in DU-145 and PC-3 cells. It was reported that BSP/OPN combine with integrin proteins, especially $\alpha\beta 3$, on the cell surface to promote cell migration and invasion. We used immunofluorescence to determine whether BSP/OPN and $\alpha\beta 3$ were co-expressed in the PCa cells (Fig. 2A and B). After labeling with different colors of the fluorescent secondary antibody, BSP and OPN were shown in the images as red fluorescence and $\alpha\beta 3$ showed green fluorescence. Additionally, nuclei stained with DAPI exhibited blue fluorescence. Co-expression of BSP- $\alpha\beta 3$ and OPN- $\alpha\beta 3$ was evident in the DU-145 and PC-3 cells.

SB225002 inhibits the expression of BSP, OPN, MMP2, MMP9 and $\alpha\beta 3$ in PCa cells. To explore the molecular mechanism of the suppression of PCa invasion by SB225002, western blotting was performed after treatment of the three PCa cell lines with SB225002 (5×10^{-6} M in 72 h). The results showed that the expression levels of BSP, MMP-2 and OPN were reduced significantly following SB225002 treatment compared with levels noted in the control group: BSP was reduced by 9.04-fold in DU-145 cells, by 1.7-fold in LNCAP cells and by 5.4-fold in PC-3 cells; OPN was reduced by 3.0-fold in DU-145 and PC-3 cells and by 2.8-fold in LNCAP cells; MMP-2 was reduced by 3.2-fold in DU-145 cells, by 5.2-fold in PC-3 cells and by 4.5-fold in LNCAP cells, $P < 0.05$ (Fig. 2C). Regarding MMP-9 and $\alpha\beta 3$, the expression levels of both proteins were

not reduced significantly in the LNCAP cells ($P = 0.08$), while MMP-9 expression was decreased by 3.7-fold in the DU145 cells, and $\alpha\beta 3$ expression was reduced by 1.3-fold in the PC-3 cells. These results indicate that SB225002 may suppress PCa invasion through restraining the expression of BSP, MMP-2 and OPN.

ERK, JNK, P38 and PI-3K signaling pathways mediate the expression of BSP, OPN, MMP-2, MMP-9 and $\alpha\beta 3$. To identify which signal transduction pathway(s) regulate several of the abovementioned invasion-related protein expression levels, we applied the inhibitors LY294002 (Akt inhibitor), U0126 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), and SP600125 (JNK1/2 inhibitor) and detected the expression of these proteins by western blotting. The three cancer cell lines were treated with LY294002 (10^{-6} M), U0126 (10^{-6} M), SB203580 (10^{-6} M), and SP600125 (10^{-6} M) for 72 h. The findings revealed that BSP, OPN, MMP-2 and $\alpha\beta 3$ protein levels were significantly lower in the DU-145 and PC-3 cells after LY294002 treatment than after the other inhibitor treatments (Fig. 2D-F and H). This result is consistent with previous reports that PI3K can regulate tumor cell invasion. Although the expression level of MMP-9 in the LY294002 treatment group was lower than that in the control group (DU-145 and PC-3), the difference was not statistically significant (Fig. 2G). Regarding LNCAP cells, although the BSP and MMP-9 expression levels were reduced, no statistically significant difference was noted, and no obvious expression changes were found in the remaining three proteins (OPN, MMP-2 and $\alpha\beta 3$) after LY294002 treatment in LNCAP cells. Moreover, in the SP600125 treatment group, we found that the expression levels of BSP and $\alpha\beta 3$ showed little reduction, especially in DU-145 and PC-3 cells, suggesting that the JNK signaling pathway can also regulate tumor cell invasion.

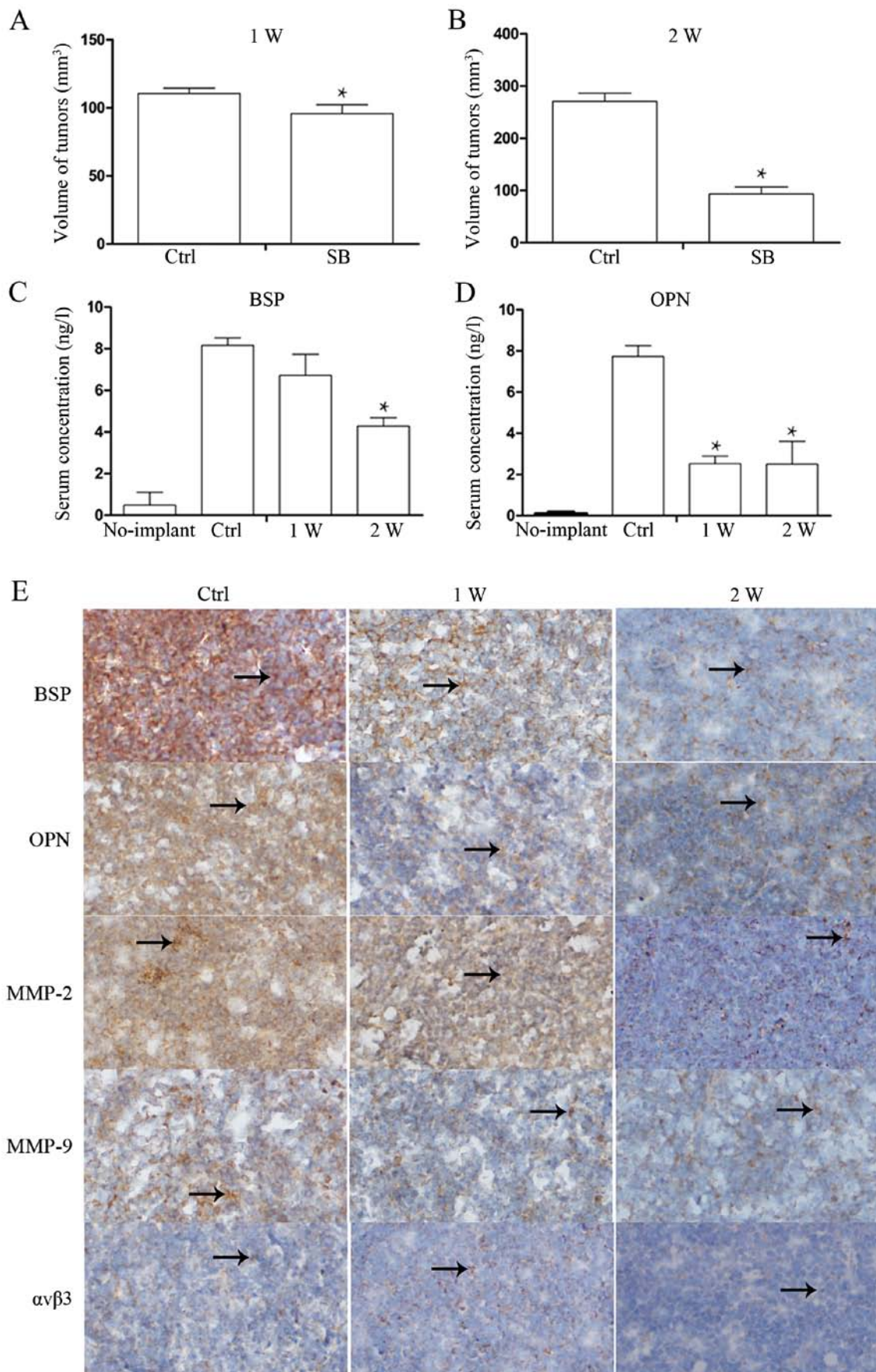


Figure 3. Subcutaneous tumorigenesis in mice was used to examine the effect of SB225002 on DU-145 cells *in vivo*. (A and B) At the end of the intraperitoneal injection at 1 week (W) and 2 W, the tumor volumes were measured, and SB225002 (SB) was found to inhibit xenograft tumor growth obviously after 2 weeks. (C and D) The serum levels of BSP and OPN were detected by ELISA, and the results showed that BSP and OPN secreted by xenograft tumors were significantly decreased after 2 W of SB225002 injection ($P < 0.05$). (E) The expression levels of the five invasion-related proteins in the xenograft tumor tissues were detected by immunohistochemistry at 1 W and 2 W. Arrows indicate protein-positive expression. Ctrl, control; BSP, bone sialoprotein; OPN, osteopontin.

Table II. Expression intensity assessments of five proteins.

	BSP	OPN	MMP-2	MMP-9	$\alpha v\beta 3$
Ctrl	+++	+++	+++	++	+
1 week	++	+	++	+	+
2 weeks	+	+	+	+	-

BSP, bone sialoprotein; OPN, osteopontin; MMP-2, metalloproteinase 2; $\alpha v\beta 3$, integrin alpha V and integrin beta 3; Ctrl, control.

SB225002 inhibits the phosphorylation/activation of AKT and mTOR. To identify the effect of SB225002 on the PI3K signaling pathway, we treated the three PCa cell lines with SB225002 (10^{-6} M for 72 h). The primary proteins of the PI3K signaling pathway (PI3K/p-PI3K, AKT/p-AKT and mTOR/p-mTOR) were detected by western blotting. The result shows that SB225002 did not significantly promote the phosphorylation of PI3K, and PI3K expression was not significantly changed. When we further detected the PI3K downstream protein AKT, only DU-145 cells demonstrated a decrease in the expression of AKT, and AKT in LNCAP and PC-3 cells was not changed; however, phosphorylated Akt in all three cancer cell lines showed a significant downward trend. Finally, the expression levels of mTOR and p-mTOR in the SB225002 group were lower than those of the control group (Fig. 2I). The above results illustrate that SB225002 has a certain blocking effect on the PI3K signaling pathway, and this block effect may begin with the inhibition of phosphorylation of AKT.

SB225002 suppresses PCa cell growth and the secretion of BSP and OPN in vivo. To examine whether SB225002 inhibits the secretion of BSP and OPN from PCa cells *in vivo*, after the xenografts were harvested at 1 week (W) and 2 W, whole blood from the treated and control group mice was obtained from the eye artery, and enzyme-linked immunosorbent assay was performed after centrifuging the blood at 1,000 rpm for 10 min. The results revealed that the serum BSP and OPN levels in the no implant mice were low BSP, 0.724 ± 0.3 ng/l; OPN, 0.132 ± 0.01 ng/l). However, after two weeks of SB225002 administration, the serum levels of BSP (9.201 ± 0.4 ng/l in the control group and 4.821 ± 0.6 ng/l in the SB225002 group; $P=0.01$) and OPN (8.431 ± 0.5 ng/l in the control group and 3.812 ± 1.4 ng/l in the SB225002 group; $P=0.04$) were decreased by 2-fold compared with the levels in the control group (Fig. 3C and D), indicating that SB225002 can inhibit PCa cell secretion of BSP and OPN *in vivo*. We also measured the volume of the xenografts at 1 W and 2 W after SB225002 injection. At the end of the first week, the tumor volume was 110.5709 ± 3.98 mm³ in the control group and 95.8498 ± 6.49 mm³ in the SB225002 group. At the end of the second week, the tumor volume of the control group had reached 270.7950 ± 15.59 mm³, and the tumor volume of the SB225002 group was 93.3554 ± 13.34 mm³ ($P=0.006$) (Fig. 3A and B). The BSP, OPN and MMP-2 expression levels in xenografts were similarly determined by immunohistochemistry. In xenografts, positively expressed proteins were stained to yellow or brown yellow and appears in the shape

of dot or sheet. The arrows indicate protein positive expression (Fig. 3E). The expression levels of BSP, OPN and MMP-9 were significantly decreased after SB225002 injection for one week, while MMP-2 expression level remained strongly. But the MMP-2 expression was significantly reduced after two weeks of injection. As for $\alpha v\beta 3$, there was no significant change before and after SB225002 injection. After 2 weeks, the positive staining scores of the five proteins were calculated and are shown in Table II.

Discussion

Invasion and metastasis are two major obstacles to the treatment of malignant tumors (1,2). Many patients lose the chance of surgical treatment due to the transfer of primary tumors to distant organs. For PCa, although most patients respond initially to androgen removal, most patients eventually develop castration resistance and have a high risk of bone metastases (21). Thus, there is an urgent need to explore effective strategies to prevent distant tumor metastasis to improve the prognosis of patients.

At the molecular level, malignant cells must be able to detach from their primary tissues, evade the host immune system, cross the walls of the vasculature, penetrate through the extracellular matrix in tissue, and finally take up residence and survive in tissues quite different from their origins (3). Studies over recent years have suggested that, besides MMP family proteins, small integrin binding ligand N-linked glycoproteins (SIBLINGs) also regulate many of the activities required for the distant metastasis of tumor cells, especially for malignant bone metastases. Additionally, the serum levels of BSP and OPN (two primary proteins of the SIBLING family and the most frequently investigated) are often used to predict the occurrence of bone metastases (2,7,9-11). As mentioned previously, BSP and OPN contain an integrin-binding RGD (Arg-Gly-Asp) sequence that can bind to integrins to enhance the invasion and adhesion of tumor cells (29). However, OPN peptides must be cleaved by MMP-9 first, followed by the enhancement of matrix degradation by activating MMP-3 (38). In terms of immune escape, after tumor cells enter the vasculature, these two SIBLINGs can bind complement factor H and prevent tumor cells from complement attack (30). In addition, some studies have indicated that SIBLING proteins can also be combined with the CD44 regulation of tumor cell proliferation and apoptosis (3). Because SIBLINGs are important factors in the regulation of tumor metastasis, to decrease tumor cell expression, SIBLINGs may be an efficiency strategy by which to overcome the distant metastasis of PCa. Many studies have reported that phosphoinositide-3-kinase (PI3K) is an important signaling pathway responsible for malignant neoplasm growth and transformation processes (31). For invasion, the PI3K signaling pathway mediates the expression of MMP-2 and MMP-9 (32,33). Zhang *et al* demonstrated that the PI3K/Akt pathway inhibitor LY294002 attenuated the migration, invasion, expression and activity of MMP and expression of p-PI3K and p-Akt in U87 and U251 cells (34). However, few studies have reported on the factors that regulate SIBLING expression. In this study, a series of *in vitro* and *in vivo* experiments confirmed that SB225002 could decrease PCa expression of BSP and OPN through the PI3K pathway.

As our results showed, following treatment of three prostate cell lines with different concentrations of SB225002, concentration- and time-dependent growth inhibition was demonstrated in DU145 and PC-3 cells but not in LNCAP cells. The lack of an effect in LNCAP cells is likely due to LNCAP belonging to the androgen-dependent cell group, and some reports have demonstrated that IL-8 and its receptors are not expressed or negligibly expressed in androgen-dependent PCa (18,35-37). Additionally, the Transwell assay exhibited that SB225002 could decrease the number of cancer cells that crossed the Matrigel barrier, indicating that SB225002 can reduce the invasion of PCa cells. Many studies have demonstrated SIBLING and integrin expression in breast cancer, but few have been reported in PCa. Considering that SIBLINGs enhance invasion through combining with integrin receptors, we evaluated the co-expression of BSP, OPN and $\alpha\beta3$ in DU-145 and PC-3 cells, and immunofluorescence analysis indicated all three proteins were expressed in PCa. Simultaneously, western blotting was performed to detect the influence of SB225002 on these invasion-related proteins, and SB225002 treatment was found to decrease the expression of BSP, OPN and MMP-2 in the three cell lines. However, MMP-9 expression was only reduced in DU-145 cells, and SB225002 did not inhibit the expression of $\alpha\beta3$. By contrast, following treatment with SB225002 treatment, the $\alpha\beta3$ expression levels showed an increasing trend in the three cell lines. Next, we treated cells with different signaling pathway inhibitors to detect which pathways control tumor cell invasion primarily. After U0126, SP600125, SB230580 and LY294002 treatment, the expression of the five proteins in the LY294002 group was obviously inhibited, consistent with previous reports describing that PI3K regulates the invasion of malignant neoplasms (32-34). Next, we tested the signaling protein in the PI3K pathway in the SB225002 and control groups to determine whether SB225002 suppresses PCa cell invasion through the PI3K pathway. Western blotting showed that, in the SB225002 group, P-AKT expression was decreased obviously, the expression levels of downstream protein mTOR and P-mTOR were significantly reduced, and the expression levels of PI3K and P-PI3K did not change, suggesting that the function of SB225002 to restrain tumor cell invasion was achieved by inhibiting the phosphorylation of AKT. Finally, we implanted DU-145 cells into mice subcutaneously, through two weeks of continuous intraperitoneal administration and confirmed that SB225002 suppressed PCa cell expression and secretion of BSP and OPN *in vivo*, in addition to MMP-2.

In conclusion, many studies have confirmed that SB225002 is an IL-8 receptor antagonist (17,38,39). SB225002 exhibits many antitumor effects by blocking the binding of IL-8 to CXCR2 receptors. This experiment confirmed that SB225002 has an inhibitory effect on the expression of invasion-related proteins. These findings may provide new ideas and methods to prevent the distant metastasis of tumors in clinical practice.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

HJ and MX conceived and designed the study. MX, JL, HW, BL and ZG performed the experiments. MX wrote the paper. HJ and HW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal experiment was approved by the JinZhou University Laboratory Animal Ethics Review Committee (JinZhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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