WNT pathway inhibitor pyrvinium pamoate inhibits the self-renewal and metastasis of breast cancer stem cells

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Abstract. Acquisition of chemoresistance and metastatic phenotype are the major causes of breast cancer treatment failure and cancer-related mortality. Recently, a plethora of experimental and clinical studies points toward a central role of cancer stem cells (CSCs) in the chemoresistance and metastasis. In the present study, we demonstrated that pyrvinium pamoate (PP), an anthelmintic drug, inhibited proliferation of different subtypes of breast cancer cells (luminal: MCF-7, claudin-low: MDA-MB-231, basal-like: MDA-MB-468 and Her-2 enriched: SkBr-3) as a novel WNT pathway inhibitor. Additionally, PP was also shown to inhibit self-renewal of breast cancer stem cells (BCSCs) and decrease both $CD44^{+}CD24^{-/low}$ and ALDH-positive BCSCs content in a panel of breast cancer cell lines. Besides, the metastatic potential and expression of EMT markers (such as N-cadherin, vimentin, Snail) were also found suppressed by PP. By using a xenograft model, we next tested the efficacy of PP on tumorigenicity of MDA-MB-231, one of the most aggressive breast cancer cell lines, and we observed PP significantly delayed tumor growth *in vivo*. Moreover, in-depth analysis revealed that PP caused inhibition of WNT pathway activity and stemness regulator expression including NANOG, SOX2 and OCT4, which were inherently upregulated in the BCSCs as compared with the bulk of cells within the tumor. Collectively, our findings provide direct evidence for PP serving as a promising high-yield agent targeting BCSCs and cancer heterogeneity. Therefore, strategies combining PP with standard chemotherapy drugs which fail to eliminate the BCSCs hold promise to overcome BCSCs associated treatment resistance and achieve a better therapeutic outcome.

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Introduction

Currently, a rapidly growing body of research demonstrates that breast cancer arises from a small population of cancer cells termed as 'cancer stem cells' (CSCs) or 'tumor-initiating cells' (TICs) (1). CSCs are endowed with self-renewing and unlimited proliferation potential (2,3), and it is conceivable that CSCs also share with normal stem cells several properties such as the relative quiescence, resistance to drugs or toxins through expression of drug transporters, a better ability to repair DNA and resistance to apoptosis and hypoxia, which is critical to enable them to survive for extended periods (4-7). As a result, typical chemo-radiotherapies could only eliminate the bulk of the tumor, but CSCs would survive and develop into a new tumor over time. Therefore, the discovery and development of specific therapies that target CSCs has the potential to revolutionize the treatment of malignant tumors (4,8).

Signaling pathways that support stem cell self-renewal appear to be promising cancer treatment candidates for personalized therapy. Several developmental pathways, such as Notch, Sonic Hedgehog (Shh), WNT are involved in regulation of self-renewal of normal stem cells. However, dysregulation of these pathways also contributes to the maintenance of CSCs (9-13). In fact, numerous 'stemness' related genes are also oncogenes, and many genes that inhibit self-renewal are also tumor suppressor genes. These observations suggest the CSCs originate from normal stem cells with somatic mutants accumulation during aging, and cancer is essentially a disease of 'stemness' gone awry (14). Compounds that converge on these cell-intrinsic pathways may overcome the dynamic nature of CSCs and thereby prevent the evolution of CSC clones that drive tumor initiation, maintenance, and relapse (15,16).

Notably, some researchers have reported pyrvinium pamoate (PP), a well-known anthelmintic drug, exhibited a potent antitumor activity against several cancers including myeloma (17), glioblastoma (15), colon cancer (18) and lung cancer (19) as a selective WNT pathway inhibitor. Although the WNT signaling pathway is important for cell proliferation and differentiation, cell movement and polarity, and maintenance of self-renewal in CSCs (20), whether pharmacologic blocking of the WNT signaling pathway with PP in breast cancer could provide therapeutic possibility by inhibiting breast cancer stem cells (BCSCs) remains to be elucidated.

Importantly, breast tumors are comprised of phenotypically diverse populations of breast cancer cells (21). In the past decade, the genomic studies have established at least five different breast cancer subtypes with difference in incidence, drug response and survival: the luminal A and B, Her-2 overexpressing (OE), basal-like, and normal breast-like tumors (22). Moreover, BCSCs are also heterogeneous and the existence of various BCSC subpopulations which would lead to a rapid relapse after primary treatments might pose a problem for cancer therapy (23,24). Virtually, therapeutic failure is in part due to the heterogeneity imparting phenotypic diversity within the CSCs (25,26). Each cancer subtype contains distinct CSC subpopulations expressing different CSC markers, and the molecular difference of the CSCs also imply different outcome in response to the current treatment (24,27).

In the present study, PP was tested for its ability to suppress the self-renewal and mammosphere-formation ability of BCSCs derived from distinct molecular subgroups (luminal: MCF7, Her-2 OE: SK-BR3, claudin-low: MDA-MB-231, basal-like: MDA-MB-468). Moreover, we also evaluated the efficacy of PP suppressing breast cancer motility and EMT process *in vitro*. Additionally, the ability of PP to suppress BCSC self-renewal *in vivo* and the potential mechanisms involved were also examined.

Materials and methods

Antibodies and reagents. Rabbit monoclonal anti-NANOG, anti-SOX2, anti-GAPDH, anti-E-cadherin, anti-Ki67 and anti-vimentin were from Cell Signaling Technology. Rabbit polyclonal anti-OCT4, monoclonal anti-N-cadherin were from Abcam. Goat anti-rabbit IgG-HRP was from Santa Cruz Biotechnology. Antibodies to FITC-conjugated CD44, and PE-conjugated CD24 were from Miltenyi Biotec. Pyrvinium pamoate was purchased from Sigma-Aldrich, and it was dissolved in DMSO at a concentration of 1 μmol/l and was stocked in aliquots at -20˚C.

Cell culture. Human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468 and SkBr-3 were obtained from American Type Culture Collection (www.atcc.org). These cells above were routinely cultured in their recommended media containing 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37˚C in a humidified chamber with 5% CO_2.

Mammosphere formation assay. For mammosphere assay, a single cell suspension was prepared at a density of 10^4/ml in culture medium and were plated in the ultra-low attachment 6-well plates (Corning). The mammosphere culture medium was serum-free DMEM/F-12 (1:1) (Hyclone) supplemented with 20 ng/ml human basic fibroblast growth factor (Gibco), 20 ng/ml human epidermal growth factor (Gibco), 1xB27 (Invitrogen), and 5 μg/ml insulin (Sigma-Aldrich). Culture medium was replenished every 3 days and images were taken at day 7.

Colony formation assay. Cells were resuspended in culture medium containing 10% FBS with or without PP and seeded at a density of $1x10^3$/dish into a 6-cm dish. Cells were kept for two weeks and monitored for colony formation. To evaluate the colony formation, cells were fixed and then stained with crystal violet (Beyotime) for 15 min after the culture period. The clones consisting of a minimum of 50 cells were counted.

In vitro proliferation assay. Cells ($1x10^4$) were suspended in 200 μl culture medium and then seeded into 96-well plates (Corning) in quintuplicate overnight. Cells were treated with indicated concentrations of PP (0-8,000 μM). After incubating for 3 days, Cell Counting kit-8 (CCK8) assay was conducted according to the manufacturer's protocol (28). CCK8 (10 μl) (Dojindo) was added into each well and incubated at 37˚C for 1 h. The absorbance was measured using a microplate reader at 450 nm (Tecan). The measured optical density (OD) values were directly proportional to the number of viable cells. Then, dose-response curves were fitted to the data and the half-maximal inhibitory concentrations (IC_{50}s) were calculated using SPSS software package (v19.0; IBM Corp., Armonk, NY, USA). All experiments were repeated at least three times. The cell proliferation rate was calculated as follows: Cell proliferation rate (%) = Experimental group OD value/Control group OD value x 100% (1).

$CD44^+/CD24^{-/low}$ cell population. Cells were resuspended as single cell in PBS with 5% FBS and incubated with FITC mouse anti-human CD44 (#130-095-195, 1:100, Miltenyi Biotec) and PE mouse anti-human CD24 (#130-095-953, 1:100, Miltenyi Biotec) for 15 min at 4˚C in the dark. Analysis was performed using a FACS Aria II cell sorter (BD Biosciences).

ALDEFLUOR assay. The ALDEFLUOR kit (Stem Cell Technologies) was used to detect the cell populations with high aldehyde dehydrogenase (ALDH) enzyme activity according to the manufacturer's instructions. After incubating with or without PP for 72 h, cells were resuspended at a density of 10^6/ml in ALDH assay buffer containing the ALDH substrate BAAA (1 mM) and incubated for 30 min at 37˚C. As a negative control, a sample of cells was incubated with 50 mM of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. A FACS Aria II cell sorter was used to analyse the ALDH-positive cell population.

Migration/invasion assay. Migration assays were performed in 24-well Falcon tissue culture plate with non-coated membrane transwells (pore size, 8.0 μm, Merck Millipore). PP-pretreated ($1x10^5$; MDA-MB-231: 500 nM, 72 h; MDA-MB-468: 100 nM, 72 h) or untreated breast cancer cells were seeded on the top chamber and starved overnight, and then incubated for 24 h

using 10% FBS DMEM as chemoattractant. Then the cells on the top of the insert were removed with a cotton swab. The invasion assay was performed as described for migration assay by using $1.5x10^5$ cells and Matrigel-coated membrane. Migrated cells on the lower surface were fixed with ice-cold 4% paraformaldehyde, stained with 0.1% crystal violet and then photographed and counted.

In vivo xenograft assay. NOD/SCID mice were housed under aseptic conditions in individually ventilated cages. For xenografting, $5x10^6$ PP-pretreated or untreated breast cancer cells (MDA-MB-231) were resuspended in a 1:1 mixture of culture medium and Matrigel (BD Biosciences) and then transplanted into the fourth pair of mammary fat pads of mice (4-6-week-old) as previously described (29). After injection, tumor size was measured by calipers each day and tumor growth was plotted. Upon reaching the endpoint, mice were sacrificed and tumors were harvested. All the tumors were formalin-fixed, and paraffin-embedded for hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining. All staining was performed with standard protocols and analyzed by a pathologist (Xiaochun Fei) who specializes in breast cancer. The rabbit anti-Ki67 monoclonal antibody (#9027S, 1:200) used for IHC was purchased from Cell Signaling Technology. All experiments were performed in accordance with guidelines of Shanghai Jiaotong University (SJTU) animal care and use committee.

qPCR assay. Total RNA was extracted using TRIzol reagent (Invitrogen) according the manufacturer's instructions. RNA integrity was verified using the Experion automated electrophoresis station (Bio-Rad), and the RNA concentration was measured at 260 nm. The qPCR assays were conducted with the aid of a FastStart Universal SYBR Green Master kit (Roche) and an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The cycler protocol was 5 min at 95˚C, 40 cycles with 15 sec at 95˚C, 60 sec at 60˚C, and 5 min at 72˚C. Gene of interest expression was normalized to the reference genes GAPDH, and fold expression was calculated with the $2^{-\Delta\Delta Ct}$ method (30). The primers used in the present study are listed in Table I.

Western blotting. Cultured cells were washed with ice-cold PBS three times, harvested, and lysed in RIPA buffer (Pierce) for immunoblot analysis. In brief, the supernatants containing 10 μg total protein were electrophoresed on 10-12% gradient sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in 5% (w/v) skim milk for 1 h at room temperature and then incubated at 4˚C overnight with the primary antibodies. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature and detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare). Images were obtained using a LAS-3000 Imager (Fuji film). The primary antibodies used were NANOG (#4903S, 1:1,000, Cell Signaling Technology), SOX2 (#3579S, 1:1,000, Cell Signaling Technology), GAPDH (#5174, 1:2,000, Cell Signaling Technology), OCT4 (#ab109183, 1:1,000, Abcam), E-cadherin (#3195, 1:1,000, Cell Signaling Technology), N-cadherin (#ab18203, 1:1,000, Abcam) and vimentin (#5741, 1:1,000, Cell Signaling Technology).

Statistical analysis. All graphs and statistical analyses were made using Prism 5 statistical software (GraphPad Software, Inc.), unless otherwise stated. Student's t-test was employed for two-group comparisons. The results are expressed as mean ± standard deviation (SD). All experimental data were obtained from at least three experimental repeats and P-values <0.05 were considered statistically significant. Bar graphs show mean values with 95% confidence intervals.

Results

PP inhibits proliferation of different breast cancer cells. In order to evaluate the effects of PP on proliferation in breast cancer cells, cell viabilities were examined after exposing four breast cancer cell lines to varying concentrations of PP for 3 days. We found PP efficiently decreased the viabilities on MCF-7 (luminal), MDA-MB-231 (claudin-low), MDA-MB-468 (basal-like) and SkBr3 (HER2-OE) cells in a dose-dependent manner (Fig. 1A). The half-maximal inhibitory concentrations (IC_{50}s) of PP were used at a nanomole concentration and they vary considerably among diverse subtypes. Of interest, MDA-MB-231, a claudin-low breast cancer cell line, was relatively insensitive to the PP treatment with a IC_{50} value of 1170±105.0 nM (Fig. 1B).

PP inhibits self-renewal capacity of BCSCs in vitro. As the cardinal property of stemness, self-renewal is defined by the ability of a cell, at each cell division, to generate an identical copy of itself and a cell of the same or different phenotype (31). Moreover, because the mammosphere culture mirrors *in vitro* tumorigenic capacity and it can also retrospectively identify CSCs that develop from single stem cell-like clones (32,33), mammosphere formation assay was utilized in our study. As shown in Fig. 2A, mammospheres were successfully generated from MCF-7, MDA-MB-468, SkBr3, and MDA-MB-231 cells. Furthermore, we evaluated whether PP could exert influence on self-renewal capacity of BCSCs. In the mammosphere formation assay, PP was shown to significantly reduce both the number and size of mammospheres *in vitro* (Fig. 2B). As a consequence of these findings, we next tested its effect on colony formation. As expected, our findings also directly illustrated that PP was effective against colony formation across all four cell lines tested (Fig. 2C and D). Taken together, our results demonstrated that PP significantly inhibits self-renewal and proliferation of BCSCs.

PP decreases different BCSC subpopulations. Both $CD44^+/CD24^{-/low}$ and ALDH-positive have been widely used as specific markers to identify the BCSCs from breast cancer tissues, and the putative BCSCs are capable of self-renewal and generating tumors resembling breast cancer (34). To this end, we further evaluated whether PP was able to eliminate the BCSCs with $CD44^+CD24^{-/low}$ or ALDH-positive phenotype directly. Results of the flow cytometric assay depicted that after 3-day treatment, PP markedly reduced the $CD44^+CD24^{-/low}$ population in different breast cancer cell lines (MCF-7, MDA-MB-231: Fig. 3A and B; MDA-MB-468: data not shown), compared with

Table I. Primer sequences.

Gene		Primers	*Sapiens*
GAPDH	Forward:	5' GGA GCG AGA TCC CTC CAA AAT 3'	*Homo*
	Reverse:	5' GGC TGT TGT CAT ACT TCT CAT GG 3'	*Homo*
N-cadherin	Forward:	5' AGC CAA CCT TAA CTG AGG AGT 3'	*Homo*
	Reverse:	5' GGC AAG TTG ATT GGA GGG ATG 3'	
E-cadherin	Forward:	5' ATT TTT CCC TCG ACA CCC GAT 3'	*Homo*
	Reverse:	5' TCC CAG GCG TAG ACC AAG A 3'	
Slug	Forward:	5' TGT GAC AAG GAA TAT GTG AGC C 3'	*Homo*
	Reverse:	5' TGA GCC CTC AGA TTT GAC CTG 3'	
Snail	Forward:	5' ACT GCA ACA AGG AAT ACC TCA G 3'	*Homo*
	Reverse:	5' GCA CTG GTA CTT CTT GAC ATC TG 3'	
Twist1	Forward:	5' GTC CGC AGT CTT ACG AGG AG 3'	*Homo*
	Reverse:	5' GCT TGA GGG TCT GAA TCT TGC T 3'	
ZEB1	Forward:	5' TTA CAC CTT TGC ATA CAG AAC CC 3'	*Homo*
	Reverse:	5' TTT ACG ATT ACA CCC AGA CTG C 3'	
ZEB2	Forward:	5' GCG ATG GTC ATG CAG TCA G 3'	*Homo*
	Reverse:	5' CAG GTG GCA GGT CAT TTT CTT 3'	
NANOG	Forward:	5' TTT GTG GGC CTG AAG AAA ACT 3'	*Homo*
	Reverse:	5' AGG GCT GTC CTG AAT AAG CAG 3'	
OCT4	Forward:	5' CTT GAA TCC CGA ATG GAA AGG G 3'	*Homo*
	Reverse:	5' GTG TAT ATC CCA GGG TGA TCC TC 3'	
SOX2	Forward:	5' TAC AGC ATG TCC TAC TCG CAG 3'	*Homo*
	Reverse:	5' GAG GAA GAG GTA ACC ACA GGG 3'	
KLF4	Forward:	5' CAG CTT CAC CTA TCC GAT CCG 3'	*Homo*
	Reverse:	5' GAC TCC CTG CCA TAG AGG AGG 3'	
ABCG2	Forward:	5' ACG AAC GGA TTA ACA GGG TCA 3'	*Homo*
	Reverse:	5' CTC CAG ACA CAC CAC GGA T 3'	
ALDH1	Forward:	5' GCA CGC CAG ACT TAC CTG TC 3'	*Homo*
	Reverse:	5' CCT CCT CAG TTG CAG GAT TAA AG 3'	
CD44	Forward:	5' CTG CCG CTT TGC AGG TGT A 3'	*Homo*
	Reverse:	5' CAT TGT GGG CAA GGT GCT ATT 3'	
WNT1	Forward:	5' CGA TGG TGG GGT ATT GTG AAC 3'	*Homo*
	Reverse:	5' CCG GAT TTT GGC GTA TCA GAC 3'	
WNT7B	Forward:	5' GAA GCA GGG CTA CTA CAA CCA 3'	*Homo*
	Reverse:	5' CGG CCT CAT TGT TAT GCA GGT 3'	
MYC	Forward:	5' CAC CTT GTA GCA CGT CCT G 3'	*Homo*
	Reverse:	5' GAC TCC CCA AGA TGT GGT GG 3'	
LRP5	Forward:	5' TGG CCC GAA ACC TCT ACT G 3'	*Homo*
	Reverse:	5' GCA CAC TCG ATT TTA GGG TTC T 3'	
FZD1	Forward:	5' AGC CAT CCA GTT GCA CGA G 3'	*Homo*
	Reverse:	5' GAG TCG GGC CAC TTG AAG TT 3'	
FZD10	Forward:	5' GGC GGT GAA GAC CAT CCT G 3'	*Homo*
	Reverse:	5' GGC GGT GAA GAC CAT CCT G 3'	
CTNNB1	Forward:	5' CAT CTA CAC AGT TTG ATG CTG CT 3'	*Homo*
	Reverse:	5' GCA GTT TTG TCA GTT CAG GGA 3'	
Gli1	Forward:	5' GTG CAA GTC AAG CCA GAA CA 3'	*Homo*
	Reverse:	5' ATA GGG GCC TGA CTG GAG AT 3'	
Gli2	Forward:	5' CAT GGA GCA CTA CCT CCG TTC 3'	*Homo*
	Reverse:	5' CGA GGG TCA TCT GGT GGT AAT 3'	
HES1	Forward:	5' TCA ACA CGA CAC CGG ATA AAC 3'	*Homo*
	Reverse:	5' GCC GCG AGC TAT CTT TCT TCA 3'	

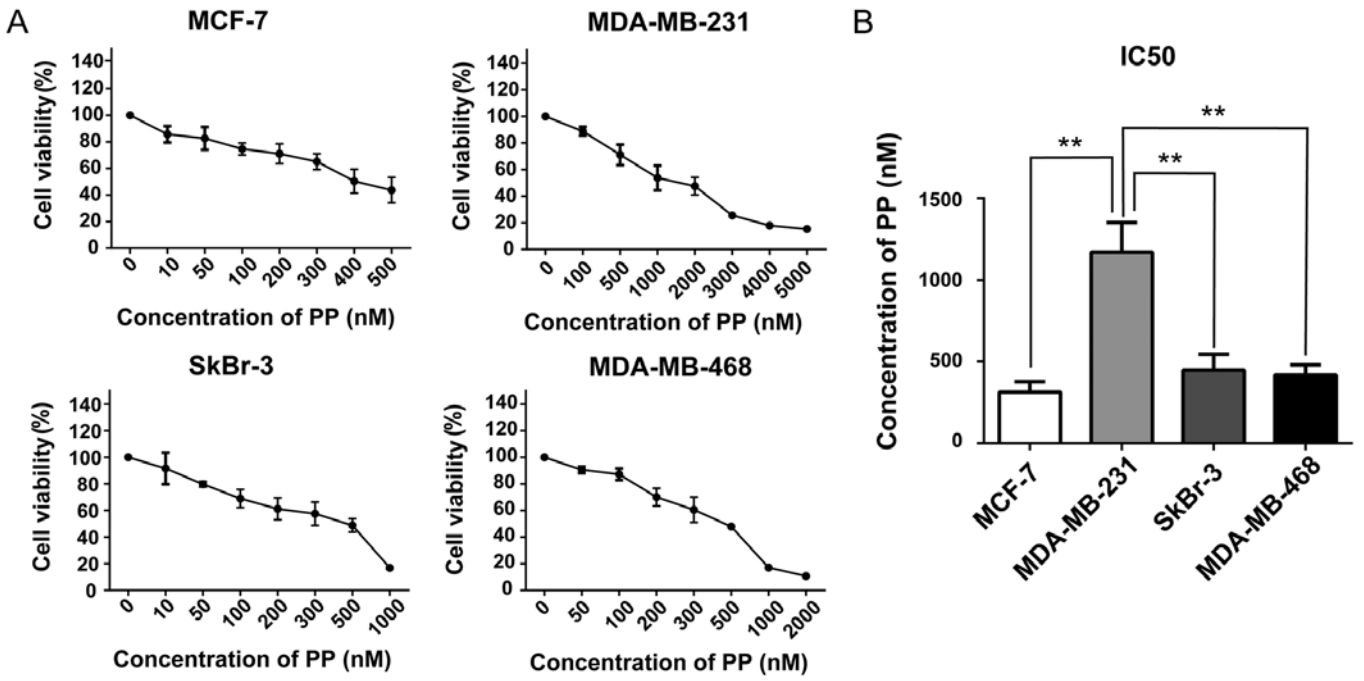

Figure 1. PP inhibits proliferation of breast cancer cells. (A) Different breast cancer cells growing in the log phase were treated with increasing concentrations of PP for 96 h. The anti-proliferation effect of PP was evaluated by Cell Counting kit-8 (CCK8) assay. (B) IC_{50}s of the four breast cancer cell lines. $^{*}P<0.05$ and $^{**}P<0.01$. All the experiments were repeated three times and error bars represent standard deviations (SD). PP, pyrvinium pamoate; IC_{50}, the half-maximal inhibitory concentration.

the control (P<0.05). Similarly, a decline of ALDH-positive cell population was also observed in PP-treated cells (MCF-7, MDA-MB-231: Fig. 3C and D; SkBr-3, MDA-MB-468: data not shown). Actually, recent studies have identified $CD44^{+}CD24^{-/low}$ and ALDH-positive phenotypes probably refer to different BCSC populations (2,35). Our results therefore indicated PP can suppress BCSC population with a distinct phenotype.

PP reduces tumorigenicity of BCSCs in vivo. In our xenograft model, $5x10^{6}$ PP-pretreated or untreated breast cancer cells (MDA-MB-231) were injected into the cleared mammary fat pads of NOD/SCID mice. All the tumor tissues were confirmed with hematoxylin and eosin (H&E) staining. We observed that PP-pretreatment strongly delayed tumor size and tumor weight (Fig. 4A and B). Furthermore, the tumor growth curves demonstrated that the tumor volume of PP-pretreated group was markedly decreased, compared with control group (P<0.05) (Fig. 4C). Immunohistochemical staining also found significantly lower Ki-67 expression in the PP-pretreated group (Fig. 4D), supporting our hypothesis of PP effectively targeting self-renewal and proliferation of BCSCs *in vitro* and *in vivo*.

PP inhibits breast cancer cell invasiveness and EMT process. To extend our analysis of the role of PP in cell motility, we applied Transwell assays to evaluate the breast cancer cell migratory and invasive potential of two of the most aggressive breast cancer cell lines (MDA-MB-231, MDA-MB-468) in the absence or presence of PP. As shown in Fig. 5A-D, PP significantly inhibited cell motility and reduced the number of cells that migrated through the membrane. Because EMT has a major role in cancer metastasis and maintenance of BCSCs, the expression levels of epithelial and mesenchymal markers were also examined. We found PP greatly increased the expression of the epithelial marker E-cadherin. For mesenchymal markers N-cadherin and vimentin, however, PP treatment effectively decreased their expression both at translational and transcriptional levels (Fig. 5E and F). Moreover, a high mRNA expression of well-known transcriptional repressors of E-cadherin (such as Snail, ZEB1 and Twist1) were also observed (Fig. 5E). Collectively, these results indicated PP attenuates the migratory and invasive properties of cancer cells and EMT process.

PP effectively attenuates WNT signaling and downregulates stemness regulators. We next investigated the mechanisms underlying the inhibitory effects of PP on BCSCs. Recently, WNT signaling pathway was reported to play a pivotal role in sustaining self-renewal potential and chemoresistance in CSCs (20). Aberrant activation of CTNNB1, MYC, and LRP5 is known as key process of the WNT signaling pathway. As shown in Fig. 6A, we observed that PP significantly decreased average expression levels of FZD1, FZD10, WNT1, WNT7B, CTNNB1, MYC, and LRP5 at transcriptional level compared with control. Additionally, a prior study has reported that pyrvinium is a potent inhibitor of Sonic Hedgehog (Shh) signaling, which acts by reducing the stability of the Gli family of transcription factors (36). Interestingly, a decrease of Gli1 and Gli2 was also confirmed with Q-PCR assay in the present study, whereas the mRNA level of HES1, the target gene of Notch pathway, remained unaltered (Fig. 6A).

Because several self-renewal genes including NANOG, OCT4 and SOX2 are key regulators of stemness in CSCs (37), we further explored whether PP downregulates these stemness

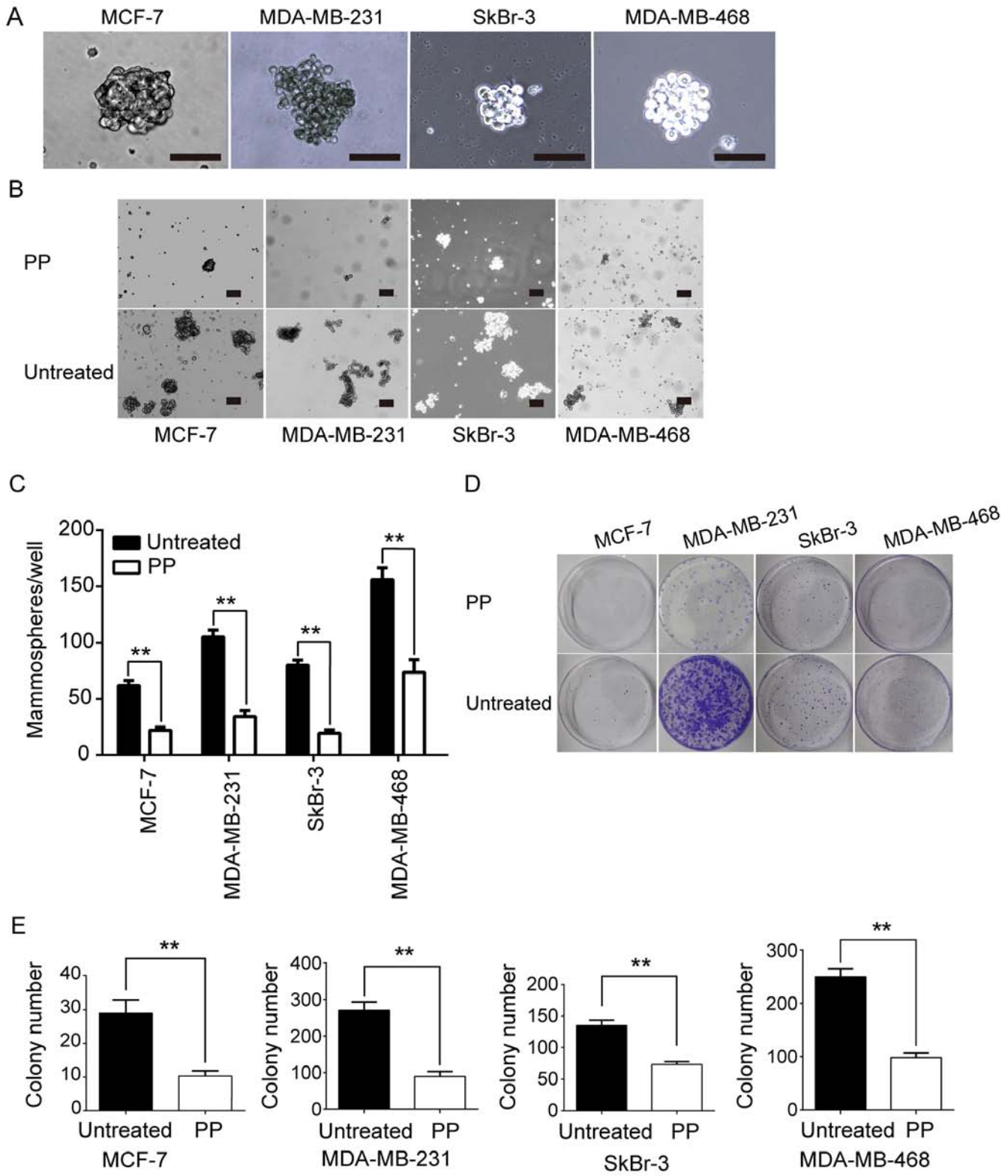

Figure 2. PP effectively inhibits self-renewal of BCSCs. (A) Morphology of mammospheres derived from different breast cancer cell lines. Cells were cultured in non-adherent culture conditions for 7 days, and images were captured by a microscope. (B) Representative images of mammosphere formation assay of four breast cancer cell lines in the absence or presence of PP. The dose of PP used for MCF-7, SkBr-3, MDA-MB-231, and MDA-MB-468 was 100, 200, 500 and 200 nM, respectively. (C) Cell counting results of mammosphere formation assay. (D) Colony formation assay of different breast cancer cell lines in the absence or presence of PP. The dose of PP used for MCF-7, MDA-MB-231, SkBr-3 and MDA-MB-468 was 100, 200, 100 and 100 nM, respectively. (E) Cell counting results of colony formation assay. Data are reported as means ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001. Scale bar, 100 μm. PP, pyrvinium pamoate; BCSC, breast cancer stem cell.

genes *in vitro*. Our data revealed significantly lower expression of these stem cell markers in the PP-treated breast cancer cells in comparison with untreated cells both at mRNA and protein levels (Fig. 6B and C). Moreover, PP also efficiently down-

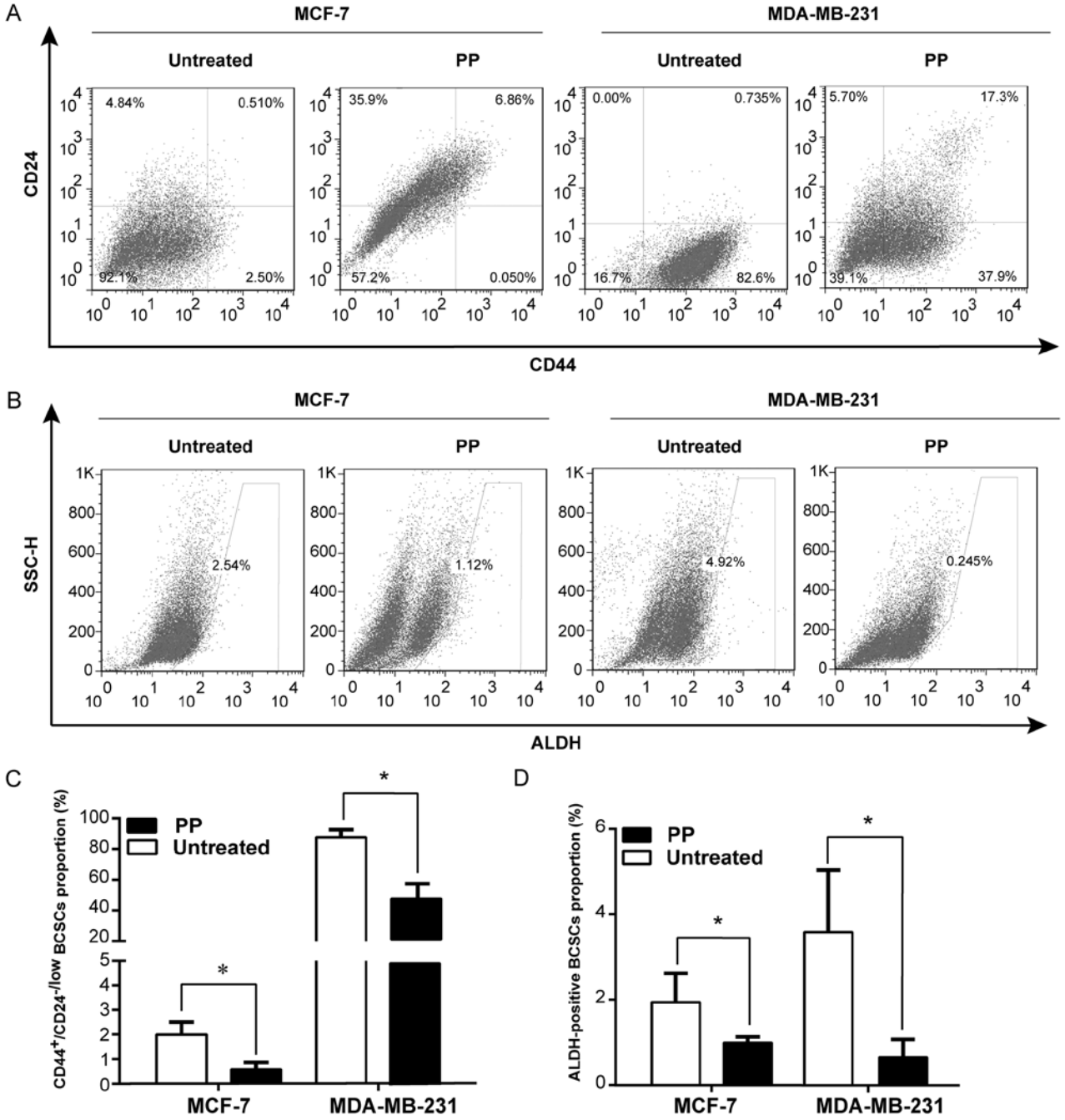

Figure 3. Effect of PP on the $CD44^+CD24^{-/low}$ and ALDH-positive putative BCSC content *in vitro*. (A and C) Proportions of $CD44^+CD24^{-/low}$ putative BCSCs in PP-treated MCF-7 and MDA-MB-231 cells. (B and D) Proportion of ALDH-positive population in PP-treated MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 breast cancer cells were treated with PP for 72 h at a concentration of 200 and 1,000 nM, respectively. The $CD44^+CD24^{-/low}$ and ALDH-positive cells were measured by flow cytometry. Similar results were obtained in three independent experiments, and the representative flow cytometry plots are shown. $^*P<0.05$. PP, pyrvinium pamoate; BCSC, breast cancer stem cell; ALDH, aldehyde dehydrogenase.

regulated the expression of other stemness genes including ALDH1, CD44 and ABCG2 (Fig. 6B). To sum up, all these results pointed towards the possibility that PP inhibits BCSC activity through attenuating WNT pathway activity and down-regulating stemness regulators.

Discussion

In the past decade, evidence has mounted for the role of the WNT signaling pathway during embryogenesis and physiological organogenesis (38). More recently, the WNT pathway also has been identified as an important regulator of self-renewal capacity in CSCs and a potential high-yield therapeutic target (20). Although no FDA-approved drugs that regulate WNT signaling are available to date (18), improved drug-screening platforms and new technologies have discovered agents that can alter WNT signaling in preclinical models (39,40). However, because the WNT pathway is shared with normal stem cells, most of the compounds may be proved difficult not to damage normal stem cells and thereby limit their use (8).

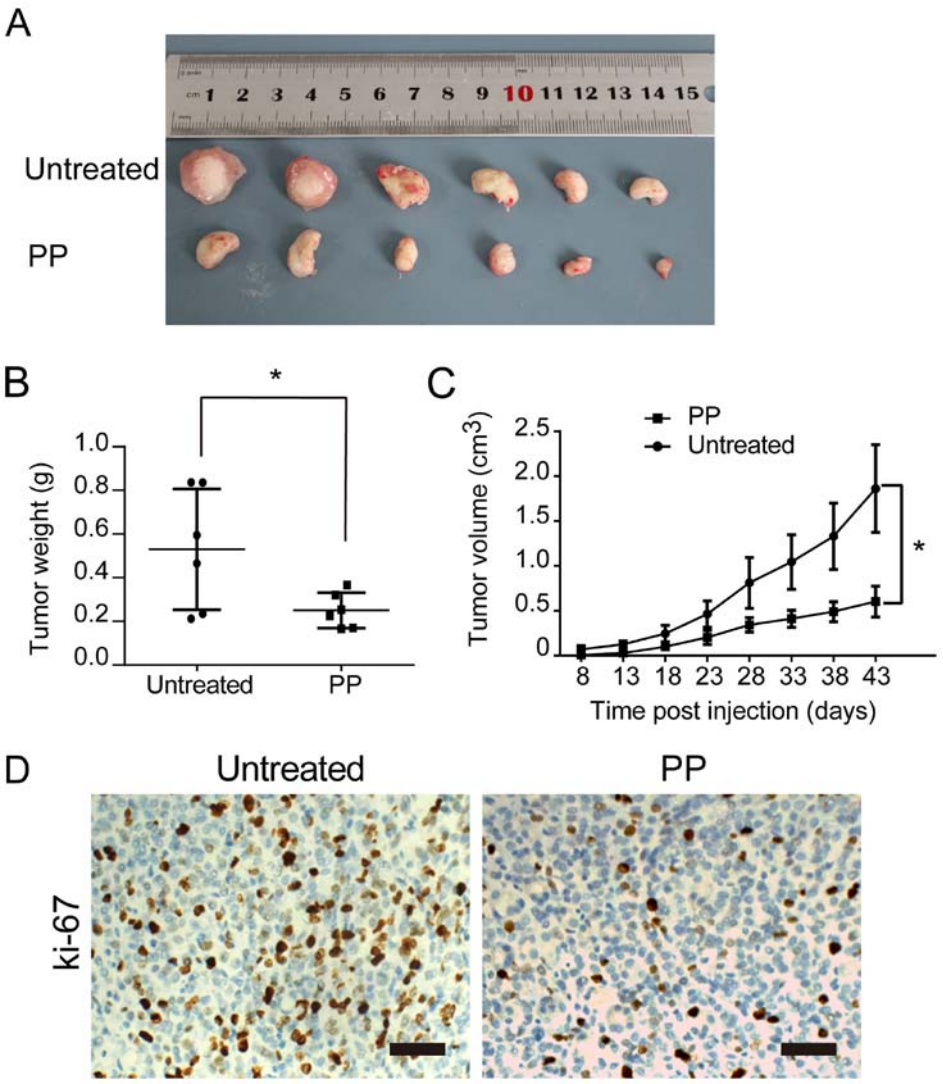

Figure 4. PP decreases tumorigenicity of BCSCs *in vivo*. (A) PP markedly reduced the tumor size in the xenograft model. A total of $5x10^6$ PP-pretreated (500 nM for 72 h) MDA-MB-231 cells or untreated MDA-MB-231 cells were injected into the mammary fat pad of the NOD/SCID mice. (B) Final weight of harvested tumors of each group (n=6). (C) Tumor growth curves of the PP-pretreated group and the control group (n=6). (D) Representative images of immunohistochemical analysis of Ki-67 from harvested formalin-fixed tumors of each group. Data are reported as mean ± SD (n=6). $^*P<0.05$. PP, pyrvinium pamoate; BCSC, breast cancer stem cell.

Hence, pyrvinium pamoate (PP), a classical anthelmintic drug, is attracting particular attention as a novel WNT pathway inhibitor. Recent studies have demonstrated that PP can exert a potent anticancer activity in several cancer types via inhibiting WNT pathway activity and autophagy process (15,17,19,41), but reports describing the effect of PP on breast cancer cells, especially on BCSCs, are scarce.

The CSC hypothesis has been proposed for years. Theoretically, if BCSCs were totally eliminated, the remaining non-stem tumor cells would be unable to re-grow or to promote new tumors. Given that conventional agents fail to eliminate the BCSCs that evade therapy to drive patient relapse, new potential targets and drugs that kill both BCSCs and non-tumorigenic cancer cells are now clinically warranted (42).

The present investigation examined the ability of PP to inhibit breast cancer cells proliferation. Our cell viability assays demonstrated that PP substantially suppressed proliferation of four genetically different breast cancer cell lines (Fig. 1A). Interestingly, the claudin-low breast cancer cell line MDA-MB-231 showed a relatively higher IC_{50}, compared with all other cell lines (Fig. 1B). These results may be partly explained by two phenomena: the claudin-low subtype most closely resembles the epithelial stem cells (43,44); and the $CD44^+/CD24^{-/low}$/claudin-low profile is increased in post-treatment samples after neoadjuvant chemotherapy or hormonal therapy (45). These findings together suggest different biological features (such as drug-resistance) associated with BCSCs converging in the claudin-low phenotype (43). PP has been described to function as a potent inhibitor of self-renewal via multiple mechanisms (15,19,46). Hence, we further explored the effect of PP on BCSC self-renewal capacity. Similarly, we also validated that PP has the capacity to inhibit mammosphere formation and colony formation of BCSCs (Fig. 2B-E). Moreover, our xenograft model also confirmed that the effect of PP on tumorigenicity decreased although the result was limited to a single breast cancer cell line (MDA-MB-231) (Fig. 4A and B). Indeed, MDA-MB-231 is one of the most aggressive breast cancer cell lines and has the highest

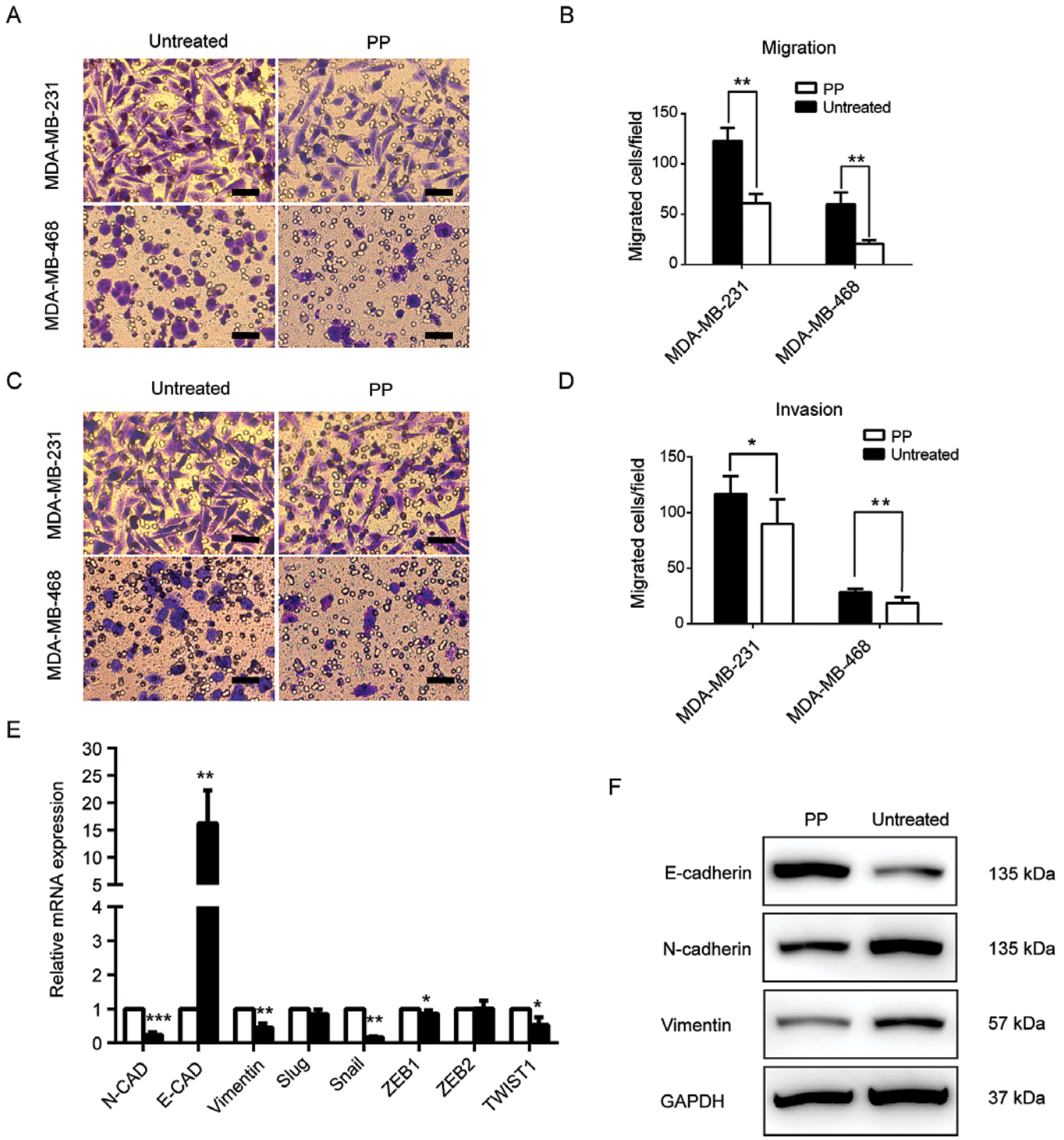

Figure 5. PP suppresses metastatic potential and EMT process in breast cancer. Transwell assay was used to evaluate the inhibitory effect of PP on migratory (A and C) and invasive potential (B and D) of MDA-MB-231 and MDA-MB-468 breast cancer cells. (E) Real-time PCR was used to analyse the gene expression of E-cadherin, N-cadherin, vimentin, Snail, Slug, ZEB1, ZEB2 and Twist1. (F) Western blot analysis of EMT-related markers. In Transwell assay, the concentration of PP used for MDA-MB-231 and MDA-MB-468 was 500 and 100 nM, respectively. The cell lysates for the immunoblot assay were obtained from the PP-treated MDA-MB-231 (1,000 nM, 72 h) and control cells. Data are reported as mean ± SD, *P<0.05, **P<0.01, ***P<0.001. Scale bar, 50 μm. PP, pyrvinium pamoate; EMT, epithelial-mesenchymal transition.

$CD44^{+}CD24^{-/low}$ BCSC frequency (~90%). Thus, our *in vivo* study result may be more broadly applicable including for less aggressive subtypes such as luminal breast cancer. Taken together, these findings clearly demonstrated the effectiveness of PP to overcome proliferation and self-renewal of both anchorage-dependent cells and BCSCs. At this juncture, it is

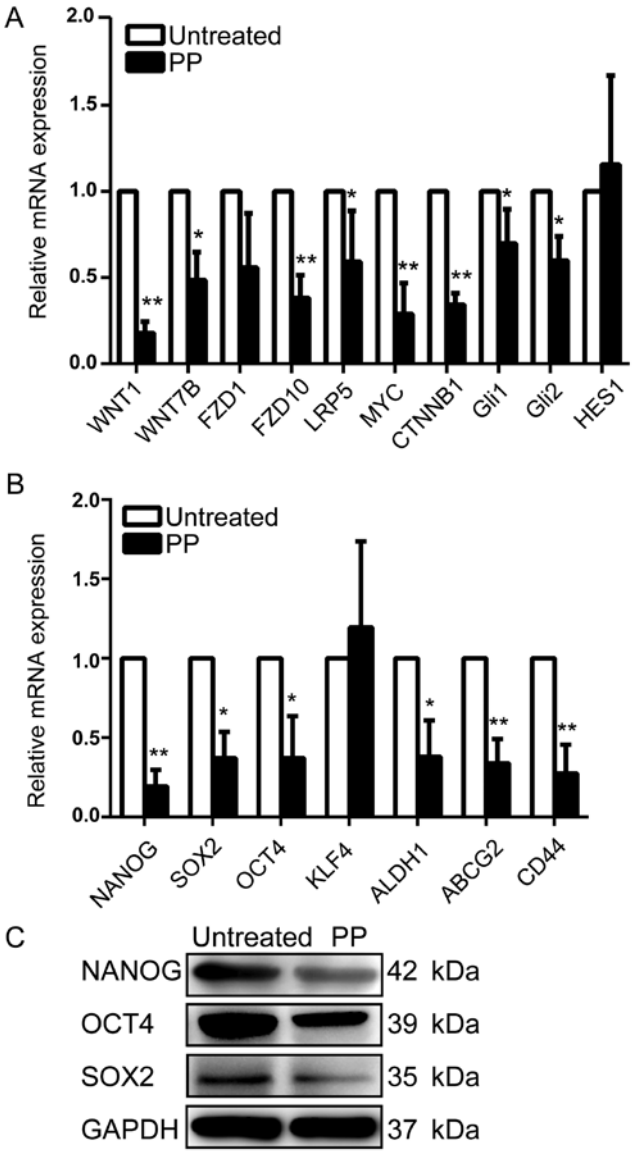

Figure 6. PP inhibits WNT pathway activity and stemness regulator expression. After 72 h PP treatment, real-time PCR was used to analyse (A) WNT, Shh, and Notch pathway activity and (B) stemness regulator expression. (C) Western blot analysis of stemness genes. The total RNA for the real-time PCR and the cell lysates for the immunoblotting assay were from the PP-treated MDA-MB-231 cells (1,000 nM, 72 h) and control. Data are reported as mean ± SD, *P<0.05, **P<0.01.

quite logical to postulate that PP might emerge as a promising drug for successful eradiation of breast cancer.

With continual refinement of massive parallel sequencing (MPS) technologies markedly shorten the path to fully personalized medicine, however, tumor heterogeneity will be one of the greatest challenges to manage in this endeavor (47). Notably, Venugopal *et al* revealed PP can selectively target TICs that drive tumor heterogeneity in human glioblastoma (15). In breast cancer, a previous study revealed that the overlap between $CD44^{+}CD24^{-/low}$ and ALDH-positive CSC phenotypes seems to be very small (<1%) (48). Additionally, it has been proven that their distributions among intrinsic breast cancer subtypes were different (2,49). Basal-like tumors contained a higher percentage of CSCs with $CD44^{+}CD24^{-/low}$ phenotype, whereas ALDH enzyme activity was mainly found in HER-OE and basal/epithelial breast cancer cells. The $CD44^{+}CD24^{-/low}$ and ALDH-positive subsets seem to identify CSCs with distinct levels of differentiation (2). Most importantly, previous studies reported variable therapeutic responses on different CSC populations (24,27). Due to these discrepancies, we next analysed whether PP was able to reduce $CD44^{+}/CD24^{-/low}$ and ALDH-positive subpopulation which are the most two consistently used methods to identify and isolate BCSCs. Interestingly, we noted that both $CD44^{+}CD24^{-/low}$ and ALDH-positive BCSCs population decreased after 3-day PP treatment (Fig. 3C and D). Thus, we provide direct evidence that PP is an inhibitor of BCSCs and a novel agent to overcome heterogeneity in both BCSCs and non-stem tumor bulk.

Unlike differentiated epithelial cells, when detaching from the extracellular matrix during the migratory process, CSCs can avoid apoptosis and survive in the blood stream. Then, as it travels all over the body, CSCs are able to select a suitable site distant from the primary tumor and thrive in its new environment (50). Thus, it is believed that CSCs are able to metastasize to distant organs where they serve as seeds of metastatic lesions (35). Of note, our results showed that PP significantly reduced metastatic capacity of two of the most aggressive breast cancer cell lines (MDA-MB-231, MDA-MB-468) (Fig. 5A-D). Consistent with the above findings, a prior study also observed that intraperitoneal injection of PP caused a trend toward decreased lung metastasis in mice (46). Moreover, EMT-related genes (N-cadherin, vimentin, Snail, Twist1) were also confirmed to be downregulated in the presence of PP (Fig. 5E and F). Indeed, one key link between CSCs and metastasis may be the EMT, the process by which epithelial cells shed their epithelial characteristics in order to become mesenchymal cells with enhanced motility and spindle cell shape (7). Therefore, although more studies are needed to provide more direct evidence for the conclusions, our results point towards the possibility that PP circumvents BCSC migratory and metastatic potential by suppressing the EMT process.

As a WNT pathway inhibitor, PP has two targets in the WNT signaling cascade (51). On the one hand, PP was able to inhibit WNT pathway by enhancing casein kinase 1α (CK-1α) activity (52). One the other hand, PP was also reported to inhibit *Pygopus* (PYG), a co-transcription factor of β-catenin, and thereby interfere with target gene transcription (51). However, other researchers also revealed PP is not a '*bona fide*' activator of CK-1α but promotes downregulation of Akt/PKB and GSK3 activation, thus, regulates WNT signaling (53). To delineate the mechanisms underlying the inhibitory effect of PP on BCSCs, we focused on the critical pathways that regulate the self-renewal of CSCs (WNT, Shh, and Notch signaling pathways). Similarly, we also found PP successfully attenuated WNT pathway activity and stemness regulator expression in BCSCs (Fig. 6A and B). Interestingly, the inhibitory function of PP was also identified in the Shh pathway, but not the Notch pathway (Fig. 6A). In line with our data, Li *et al* also showed that PP was a potent Shh pathway inhibitor, which acts by reducing the stability of the Gli family of transcription factors (36). Actually, crosstalk between the WNT and Shh pathways has been evidenced in cancer (54,55). On the basis of these findings, we speculate that PP targets the overlapping components of WNT pathway and Shh pathway,

and it warrants further studies (such as microarray assay and rescue experiment) to identify the target genes associated with the inhibition of BCSCs by PP.

Our current findings have direct implications with regard to evaluation of PP as a potent inhibitor of both BCSCs and non-tumorigenic cancer cells. However, it will take time for this information to be translated into clinic. Major concerns mainly focus on the absorption, distribution and systemic toxicity of PP. However, it is noteworthy that PP has been used as a classical anthelmintic drug for more than fifty years and the doses (0-1,000 nM) used in our study were relatively low and safe. Another challenge before us is that when taken orally, the absorption of PP from the gut is minimal (56). Thus, the development of pharmaceutical technology to improve the drug delivery is urgently needed. Because BCSCs are also highly associated with chemo-resistance behavior (57), PP combination therapy with current chemotherapeutic agents (such as anthracyclines and taxanes) should be evaluated in future studies both *in vitro* and *in vivo*.

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