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Research Article

The role of P-glycoprotein/cellular prion protein interaction in multidrug-resistant breast cancer cells treated with paclitaxel

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Abstract. We previously reported that treatment with P-glycoprotein (P-gp) substrates promotes *in vitro* invasion in multidrug-resistant (MDR) breast cancer cells. This effect is initiated by the P-gp pump function and mediated by interaction of P-gp with some unknown component(s). However, the underlying mechanism(s) remains poorly understood. Here we confirm a novel physical interaction between P-gp and cellular prion protein (PrP^c). Blocking P-gp activity or depletion of PrP^c inhibited paclitaxel (P-gp substrate)-induced invasion. Paclitaxel further facilitated the formation of P-gp/PrP^c clusters residing in caveolar domains and promoted the association of P-gp with caveolin-1. Both caveolin-1 and the integrity of caveolae were required for the drug-induced invasion. In addition, the P-gp/PrP^c complex also played an important role in anti-apoptotic activity of MCF7/Adr cells. These data provide new insights into the mode by which MDR breast cancers evade cytotoxic attacks from P-gp substrates and also suggest a role for P-gp/PrP^c interaction in this process.

Keywords. P-glycoprotein, PrP^c, CD147, EGFR, caveolin-1, *in vitro* invasion, apoptosis, paclitaxel.

Introduction

Emerging evidence has suggested a functional linkage between multidrug resistance (MDR) and tumor metastasis, which are the two main causes of treatment failure and increased mortality in cancer patients [1, 2]. MDR cell lines exhibit an increased *in vitro* invasive ability as compared with the sensitive line and this metastatic phenotype is attributed to the differential expression profiling [3]. MDR cell lines produce more matrix metalloproteinases (MMPs), as well as CD147 (extracellular matrix metalloproteinase inducer), which is a glycoprotein enriched on the surface of tumor cells that can stimulate the production of MMPs. In our previous research, we confirmed that MCF7/Adr cells treated with P-glycoprotein (P-gp) substrates show greater metastatic potential, which is ascribed to up-regulation of MMPs and CD147 mediated by epidermal growth factor receptor (EGFR) [4]. However, P-gp alone cannot explain this phenomenon. The mechanisms for P-gp substrate-elicited augmentation of metastatic potential in MDR cancer cells are not yet fully elucidated. Yang et al. [5] have reported that interaction of drugs with P-gp induces membrane ruffling, an early indicator of cellular motility and metastatic potential, in MDR MCF7 cells, and that this effect may be mediated through activation of phosphatidylinositol-3-kinase (PI3K). Our prior studies also indicate that some

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component(s) may exist that structurally and functionally couples with P-gp. Pumping chemotherapeutic drugs out of cancer cells appears to be a triggering event that initiates interaction of P-gp with this unknown component and sequentially, activates the downstream events.

P-gp, encoded by the *MDR1* gene, is a broad-spectrum multidrug efflux pump that recognizes a variety of structurally diverse chemotherapeutic agents, which are called P-gp substrates [6–8]. In MDR cancer cells overexpressing P-gp, a portion of P-gp has been found to be localized in caveolae and associated with caveolin-1 [9]. PrP^c, the normal cellular prion protein, has also been reported to be enriched in caveolae or caveolae-like domains [10, 11], at least partially consistent with P-gp localization. Until now, no studies have suggested a possible physical connection between these proteins. However, some evidence suggests that they may be functionally linked. Most cancers derived from tissues that express P-gp are thought to be associated with a poor prognosis, presumably because they impart resistance to cancer treatment and acquire a propensity towards invasion and metastasis as suggested in colon cancer cells [12, 13]. An emerging role of PrP^c is its ability to promote cell invasion in metastatic tumors. PrP^c is up-regulated in an MDR gastric carcinoma cell line. The N-terminal region of PrP^c promotes the invasive and metastatic abilities of gastric cancer cells through activation of EGFR and consequent transactivation of MMPs [14]. The original work in the MDR gastric carcinoma cell line suggested that PrP^c confers resistance of both P-gp-related and P-gp-nonrelated drugs on cancer cells [15]. All the above evidence combined with our preliminary experiments imply that PrP^c may act as the P-gp-coupled factor mediating the drug-induced effects lying downstream of P-gp.

The purpose of the present study was to investigate whether or not a physical and functional interaction exists between P-gp and PrP^c. We found co-expression and co-localization of P-gp and PrP^c on the cell membrane of MDR breast cancer cells. Furthermore, we demonstrated that in response to treatment with P-gp substrate, MCF7/Adr cells displayed increased *in vitro* invasion and anti-apoptotic activity *via* P-gp/PrP^c interaction. Disruption of this interaction had profound effects on tumor survival, aggressive behavior, and clinical effects of chemotherapy.

Materials and methods

Cell culture. The human breast carcinoma cell line MCF7, its adriamycin-resistant counterpart MCF-7/Adr, and MCF-7/MDR1(a human *MDR1* gene stable transfectant clone) were kindly supplied by Dr. R. David (Washington University, St. Louis, MO) and maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). MCF-7/Adr cells were cultured in the presence of adriamycin (Sigma, St. Louis, MO). For drug treatment, paclitaxel (Sigma), a P-gp substrate routinely used in the first-line treatment of metastatic breast cancers, was adopted in our study. The final concentration of paclitaxel was 45 μg/ml and the duration of treatment was 48 h.

Reverse transcription and quantitative real-time PCR. Quantitative RT-PCR was carried out using the basic procedure described previously [4]. The primers used in the real-time PCR reactions were designed based on information from the human genomic database. The following primers were used for the specific amplification of *GAPDH, MDR1* and *PRNP: GAPDH* forward primer: 5'-catcaagaaggtggtgaagc-3', and reverse primer: 5'-ggaaattgtgagggagatgc-3'; *MDR1* forward primer: 5'-cccatcattgcaatagcagg-3', and reverse primer: 5'-gttcaaacttctgctcctga-3'; *PRNP* forward primer: 5'-ctgctggatgctggttct-3', and reverse primer: 5'-tggcttactcggcttgtt-3'.

Western blotting and co-immunoprecipitation. Total protein was extracted from cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blots, 50 μg protein extract/lane were electrophoresed, transferred to PVDF membranes, and incubated overnight with antibodies against PrP^c (clone 3F4, Sigma), CD147 (Santa Cruz Biotechnology), P-gp (Chemicon International, Temecula, CA), MMP2 (Neomarkers, Fremont, CA), MMP9 (Santa Cruz), MMP11 (Santa Cruz), EGFR (Santa Cruz), p-EGFR (Santa Cruz), caveolin-1 (Santa Cruz) and p-caveolin-1 (Santa Cruz), respectively. Membranes were treated with the appropriate AlexaFluor700/800 nm-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). and analyzed using the Odyssey Infra-Red Imaging System (Li-Cor BioSciences, Lincoln, NE). For co-immunoprecipitation, 1 mg protein lysate from each sample was incubated with 2 μg anti-P-gp, caveolin-1 or PrP^c overnight at 4°C. Immunoblotting detections were performed as described above.

Immunofluorescence confocal microscopy analysis. Cells were grown on glass slides and treated as indicated. The slides were quickly washed with PBS followed by fixing in 100% methanol at –20°C for 10 min. The samples were probed with the appropriate primary and secondary antibodies (Cy3-conjugated anti-rabbit antibody, red fluorescence; FITC-conjugated anti-goat antibody, green fluorescence). The

fluorescence was visualized under Leica SP5 confocal microscope (Leica).

Plasmid construction and PrP^c transfection of MCF7. The *PRNP* cDNA, 750 bp in full length, were amplified from total cDNA of MCF7/Adr cells. Primers for *PRNP* cDNA were: forward: 5'-gggttctagaggagtgcaggtggagactatctcc-3'; reverse: 5'-gggcgtagtctggtacgtcgtacggta ttg-3'. The resulting PCR products were then introduced in the *Hin*dIII and *Bam*HI sites of pCEP4 vector. This recombinant plasmid (named pCEP4-PrP^c) was confirmed by restriction digestion and full-length sequencing, respectively. Transfection of MCF7 with pCEP4-PrP^c was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions.

***In vitro* invasion assays.** A bioassay for *in vitro* cell invasion using Matrigel Invasion Chambers (Corning Costar, Corning, NY) was performed as described previously [4].

Inhibition of PrP^c, caveolin-1 expression by RNA interference. Cells (2×10^5) were seeded in 6-well plates in triplicates and, after an overnight incubation, were transfected with various concentrations of small interference RNA (siRNA) in serum-free Opti-MEM medium (Gibco) using HiPerfect Reagent (Qiagen, Valencia, CA) as suggested by the manufacturer's instructions. At 48 h after transfection, total protein was extracted and gene expression was determined by Western blotting. Anti-β-actin was used as protein loading control. The siRNA used to target *PRNP* and *caveolin* mRNA sequence were synthesized by Qiagen.

PrP^c siRNA–transfected cells invasion assay. Cells were trypsinized and resuspended in serum-free Opti-MEM. From this single-cell suspension, 3×10^4 cells were seeded in the upper compartments that were then placed in the lower companion 24-well plate without cell culture medium. After 12 h of incubation, the cells were transfected with PRNP siRNA and the plate wells were filled with RPMI 1640 plus 10 % FBS without removing the upper compartments. After another 36-h incubation with chemotherapeutic agents, a modified cell migration assay was performed using Matrigel Invasion Chambers as described above.

Isolation of caveolae-enriched membrane domains. Low-density caveolae-enriched domains were isolated by a carbonate-based fractionation method as described previously [9]. Briefly, confluent MCF7/Adr or MCF7 cells cultured in 100-mm dishes (containing about 10^7 cells) were scraped into 2 ml 0.5 M sodium carbonate (pH 11) and homogenized extensively using a Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts at medium speed) and a sonicator (three 20-s bursts at 50 % maximal power). The resulting homogenate was brought to 45 % sucrose by the addition of 2 ml 90 % sucrose in Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 150 mM NaCl) and overlaid with two layers of 35 % and 5 % sucrose in MBS containing 0.25 M carbonate (4 ml each). The gradient was then centrifuged at 200 000 *g* for 18 h using a Beckman SW41Ti rotor. For analysis of the resulting gradient, 1-ml fractions were collected from the top to the bottom of the gradient. From each fraction, 10 or 20 µl was subjected to SDS-PAGE for the detection of P-gp, PrP^c or caveolin.

Flow cytometry assay. Flow cytometry (FCM) was used to quantitatively detect the apoptotic rate. Cells (1×10^6) were plated into 10-cm tissue culture dishes 1 day before the treatment, and were then treated with paclitoxel. After the treatment, floating and attached cells were harvested, washed with PBS, fixed in 70 % ethanol overnight at 4°C and stained with 50 mg/ml propidium iodide (Sigma). The sub-G1 peak (DNA content less than 2N) was measured with a FACScan (Becton Dickinson Labware, Franklin Lakes, NJ) and analyzed by CellQuest software.

Caspase activity assay. Cells were collected and resuspended in ice-cold lysis buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 % 3-[(3-cholamidopropyl)–dimethylammonio]–2- hydroxy-1-propanesulfonic acid (CHAPS), 2 mM dithiothreitol (DTT), 0.1 mM EDTA for 15 min on ice and centrifuged at 12 000 *g* for 15 min at 4°C. The supernatants (cytosolic extract) were collected as the samples for caspase activity detection. The samples were incubated with 500 mM caspase substrates in 100 ml caspase activity assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1 % CHAPS, 10 mM DTT, 0.1 mM EDTA and 10 % glycerol) for 4 h at 37°C. Optical density was measured at 405 nm.

Statistical analysis. Statistics were conducted by SPSS software. The results are presented as mean ± SEM. ANOVA, Student's *t*-test analysis and Dunnett's multiple comparison tests were used to compare mean values. A p value of less than 0.05 was defined as statistical significance.

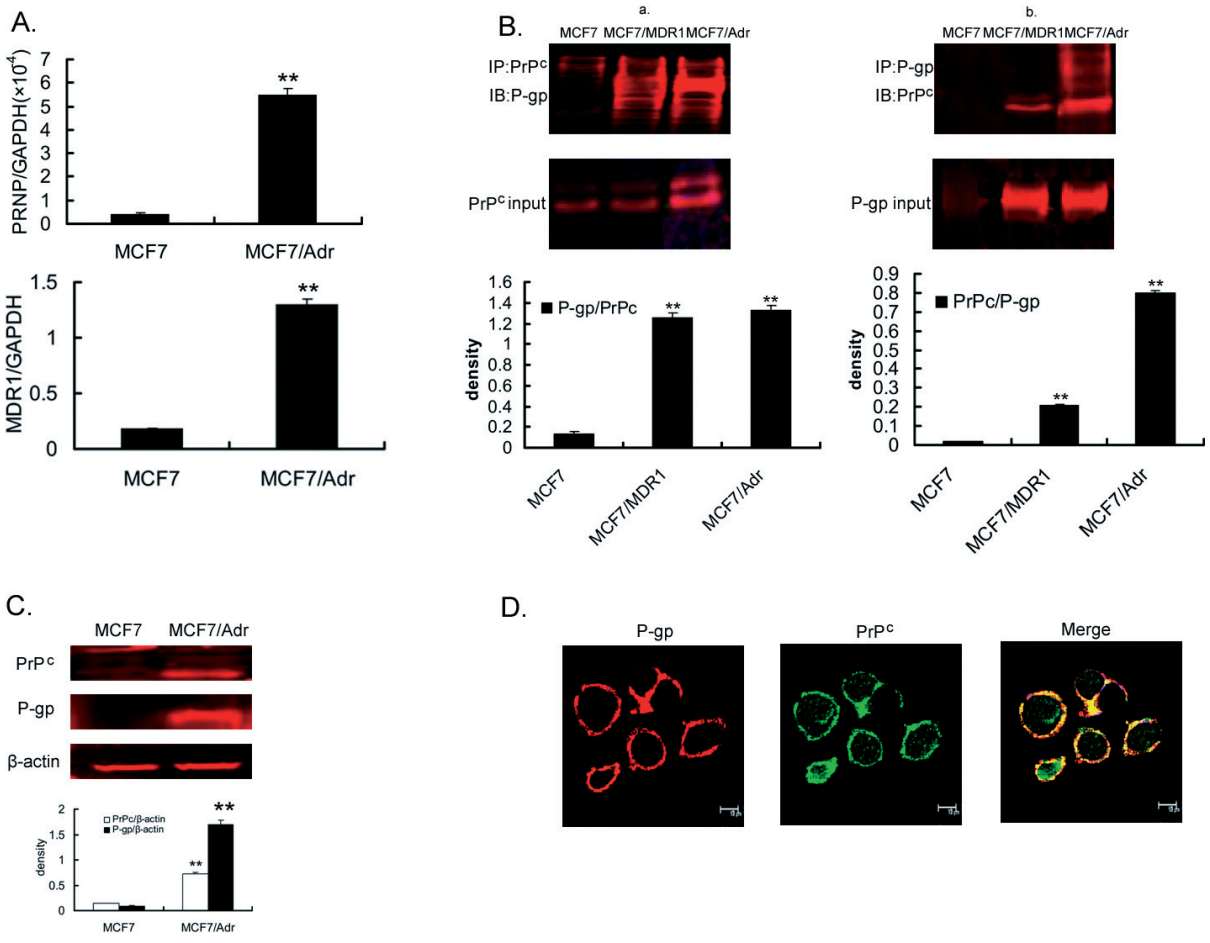

Figure 1. Cellular prion protein (PrP^c) is overexpressed and physically linked with P-glycoprotein (P-gp) in MCF7/Adr cells. P-gp and PrP^c expression at mRNA (*A*) and protein (*B*) levels were assessed by real-time PCR and immunoblotting, respectively. (*C*) Lysates from MCF7, MCF7/MDR1 and MCF7/Adr cells were immunoprecipitated with the indicated antibodies and immunoblotted with corresponding antibodies. Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ *versus* corresponding control cells (Student's *t*-test). (*D*) MCF7/Adr cells were double-stained for P-gp (red) and PrP^c (green). Bar 10 μm.

Results

PrP^c is highly expressed and physically interacted with P-gp in MCF7/Adr cells. In MCF7/Adr cells, the expressions of PRNP, MDR1 mRNA were 13- and 6.9-fold, while the expressions of PrP^c, P-gp protein were 5.6- and 18.2-fold higher than those in sensitive cells (Fig. 1A, C). After pre-treating the total extracted protein with proteinase K, prion protein became undetectable (data not shown), which demonstrated that it is normal PrP^c and not proteinase K-resistant isoform of PrP^{sc} that exists and functions in breast cancer cells.

Figure 1Ba shows that immunoprecipitation with anti-PrP^c coprecipitated P-gp in cell lysates from MCF7/Adr cells. Immunoprecipitation with anti-P-gp also coprecipitated PrP^c in the cell lysates (Fig. 1Bb). Lysates from the parental sensitive cell lines that did not express P-gp were used as negative controls. Another control was MCF-7/MDR1 cells, a MCF7 cell line transfected with *MDR1* expression vector. In this PrP^c negligibly expressed control, anti-P-gp antibodies immunoprecipitated relatively less PrP^c and *vice versa.* We then analyzed the cellular localization of the two proteins by confocal microscopy. The results showed the expression of PrP^c (green) in MCF7/Adr cells, which merged with P-gp (red), suggesting their physical association within the cell membrane (Fig. 1D). As a negative control, PBS was used instead of the first antibody (data not shown).

Paclitaxel induces *in vitro* invasion of MCF7/Adr cells *via* P-gp/PrP^c interaction. To examine the role of P-gp/PrP^c interaction in paclitaxel-induced invasion, we first introduced PrP^c siRNA into MCF7/Adr cells, and found that induction of either EGFR activity (p-EGFR) or CD147 (Fig. 2A, lane 5) was markedly decreased in response to paclitaxel treatment, while

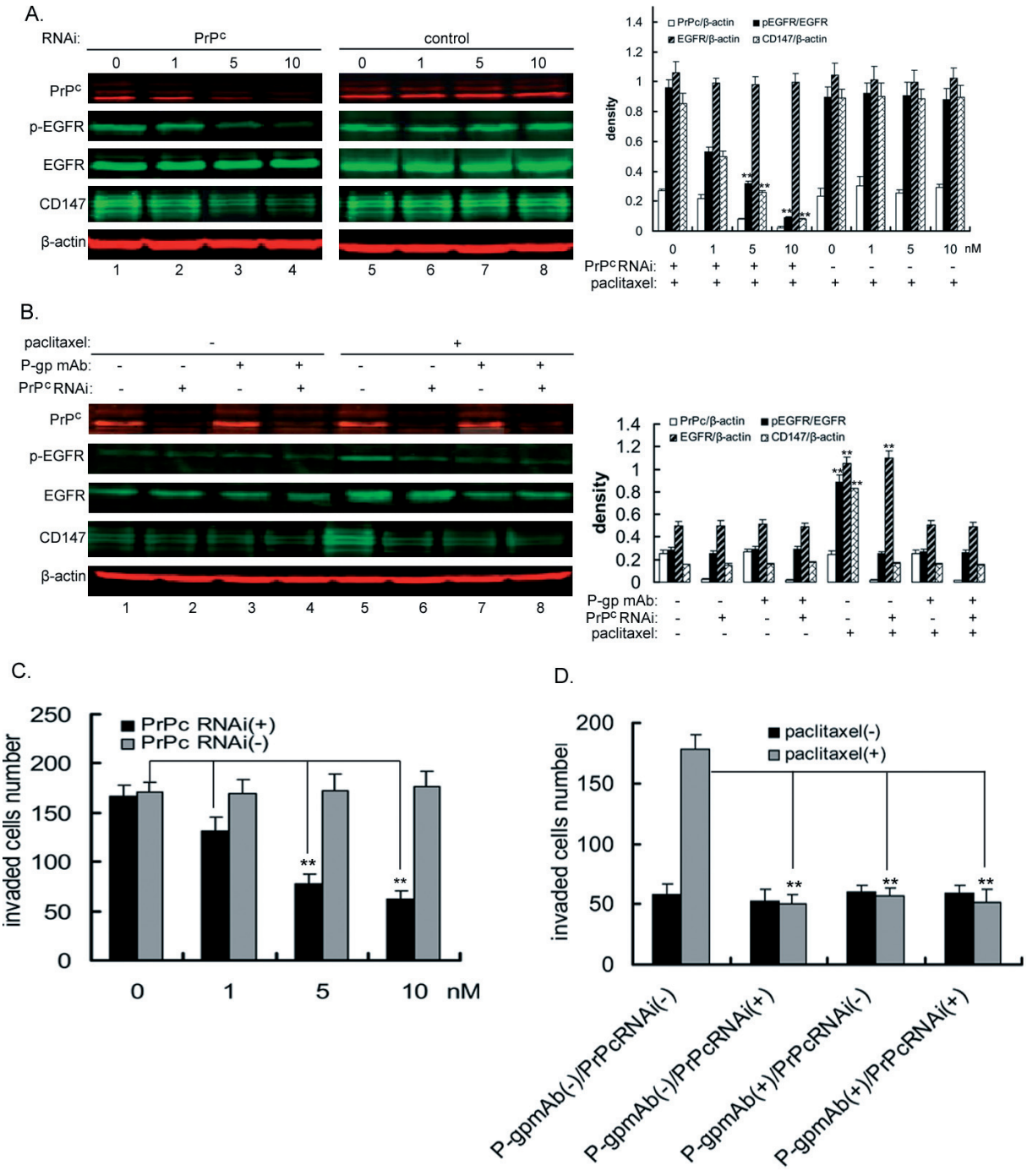

Figure 2. The paclitaxel-induced invasion is initiated by P-gp pump function and mediated through P-gp/PrP^c interaction. Expressions of pEGFR, EGFR, CD147 (*A* and *C*) and invasive potentials (*B* and *D*) in siPrP^c-transfected MCF7/Adr cells incubated without or with paclitaxel or/and P-gp mAb were determined by immunoblotting and *in vitro* invasion assay, respectively. Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ *versus* corresponding control cells (Student's *t*-test).

EGFR expression remained unchanged under the same conditions (Fig. 2A, lanes 2–4). Consequently, paclitaxel could no longer facilitate invasiveness after PrP^c siRNA treatment (Fig. 2C).

We then assessed p-EGFR and CD147 levels after blocking P-gp function. Upon P-gp block (P-gp mAb, Hyb-241 [16], 20 μg/ml), p-EGFR and CD147 induction were largely abolished in PrP^c-reduced cells as well as in control cells (Fig. 2B, lanes 7 and 8). These results were in accordance with the data of *in vitro* invasion assays (Fig. 2D), suggesting that the positive effects of paclitaxel treatment on tumor invasion are

initiated by P-gp pumping function, and that P-gp/PrP^c interaction is indispensable to this process.

P-gp or PrP^c alone is not involved in paclitaxel-induced invasion. To further elucidate whether paclitaxel-induced invasion was elicited by P-gp or PrP^c alone, we knocked down PrP^c in MCF7 cells that harbor P-gp (MCF7/MDR1) to generate P-gp(+)PrP^c(-) cells. As expected, PrP^c depletion decreased p-EGFR and CD147 induction as compared to the control group (Fig. 3A, lanes 3 and 4). In P-gp-deficient derivatives [P-gp(**-/-**)] (MCF7), paclitaxel did not change p-EGFR or CD147 expression regardless of whether PrP^c expression was reduced (Fig. 3A, lanes 7 and 8). Correspondingly, only stable P-gp expressing MCF7/MDR1 cells without PrP^c depletion [P-gp(+/+)PrP^{c}siRNA(-)] displayed increased invasion rate in response to paclitaxel treatment (Fig. 3C). We also transfected PrP^c into MCF7 cells and determined P-gp and PrP^c expression by immunoblot. In these PrP^c(+)P-gp(-) clones (MCF7/PRNP), overexpression of PrP^c did not alter the expression levels of p-EGFR, EGFR and CD147 (Fig. 3B, lanes 1 and 3), nor did they change when the transfectants were treated with paclitaxel (Fig. 3B, lanes 1 and 2). However, PrP^c promoted *in vitro* invasion of MCF7 cells by 2.4-fold (Fig. 3Da) and unregulated MMP11 expression in the transfectants (Fig. 3B). To demonstrate the possible role of MMP11 in PrP^c-related invasion, the transfectants were pretreated with MMP11 monoclonal antibody (mAb) (1 μg/ml) or control IgG before performing invasion assay. Treatment with MMP11 mAb blocked the invasive activities of MCF7/PRNP cells by 66% and the effect of MMP11 mAb was specific since the control IgG had no effect on invasion of the transfectants (Fig. 3D b).When MCF7/PRNP cells were treated with paclitaxel, MMP11 expression remained unchanged (Fig. 3B), in accordance with *in vitro* invasion assays (Fig. 3Da). These findings indicate that P-gp or PrP^c alone is not involved in paclitaxel-induced invasion.

Paclitaxel induces clustering of P-gp/PrP^c complexes in caveolar domains. PrP^c clustering leads to the recruitment of downstream signaling molecules, which are associated with cell motility [17, 18]. To make clear whether P-gp substrates induce cell invasion by affecting the co-localization of P-gp and PrP^c on the cell membrane, MCF-7/Adr cells were treated with paclitaxel, labeled with fluorescent anti-PrP^c and anti-P-gp antibodies, and analyzed by confocal microscopy. Immunofluorescence revealed the formation of small clusters of P-gp/PrP^c complexes at the surface of the cells (Fig. 4Ab), while no similar phenomenon was observed on the control cells (Fig. 4Aa), MCF7/PRNP cells (data not shown), or MCF7/Adr cells sequentially treated with P-gp mAb and paclitaxel (Fig. 4Ac). These phenomena suggested that pumping paclitaxel out of cells by P-gp can be the initial event triggering the clustering of P-gp/PrP^c complexes.

Both P-gp and PrP^c are physically associated with caveolin-1 [9, 10], the marker of caveolae. Therefore, we investigated the effects of paclitaxel on P-gp/PrP^c localization in caveolae-like microdomains. First, paclitaxel did not affect P-gp or PrP^c expression in MCF7/Adr cells (Fig. 4B). Subsequently, the same number of cells for each treatment were fractionated by the carbonate method for isolation of caveolar-like microdomains (Fig. 4C). When MCF7/Adr cells were grown in the absence of paclitaxel, two populations of P-gp, PrP^c and caveolin-1 were immunodetected. One population was associated with fractions 4–6 and the other was detected in fractions 9–12. Paxillin and FAK, which are negative markers of caveolae domains in endothelial cells, were only detected in fractions 9–12. The profile of P-gp, PrP^c and caveolin-1 distribution was modified when cells were grown in the presence of paclitaxel. In accordance with caveolin-1 redistribution, P-gp and PrP^c became more strongly detected in fractions 4–6, suggesting a strong enrichment of these proteins in the caveolae fractions caused by paclitaxel treatment (Fig. 4C). In MCF7/Adr cells transfected with PrP^c siRNA, P-gp clustering (Fig. 5A) as well as P-gp, caveolin-1 distribution in sucrose fractions (Fig. 5B) were unaffected by paclitaxel treatment, implying that P-gp/PrP^c interaction is essential to the enrichment of P-gp/PrP^c clusters in caveolar domains.

PrP^c dictates increased association of P-gp with caveolin-1 induced by paclitaxel, and caveolin-1 is required for the drug-induced invasion. We next examined whether paclitaxel affects the physical interaction between P-gp, PrP^c and caveolin-1. As shown in Figure 6, paclitaxel treatment increased the physical association of P-gp with caveolin-1 (Fig. 6A), while the P-gp/PrP^c interaction remained unchanged under the same conditions (Fig. 6B). Moreover, in view of decreased formation of P-gp/caveolin-1 complex by PrP^c depletion (Fig. 6C), we inferred that PrP^c promotes the interaction between P-gp and caveolin-1.

We also assessed the role of caveolin-1 and caveolae in drug-induced invasion. Figure 7A shows that paclitaxel increased p-caveolin-1 expression, which could be inhibited by PrP^c RNA interference (RNAi). The induction of both p-EGFR and CD147 was markedly decreased in paclitaxel-treated cells after caveolin-1 depletion (Fig. 7B). Thus, it can be deduced that caveolin-1 lies downstream of the P-gp/PrP^c complex in a pathway that mediates the enhanced invasiveness.

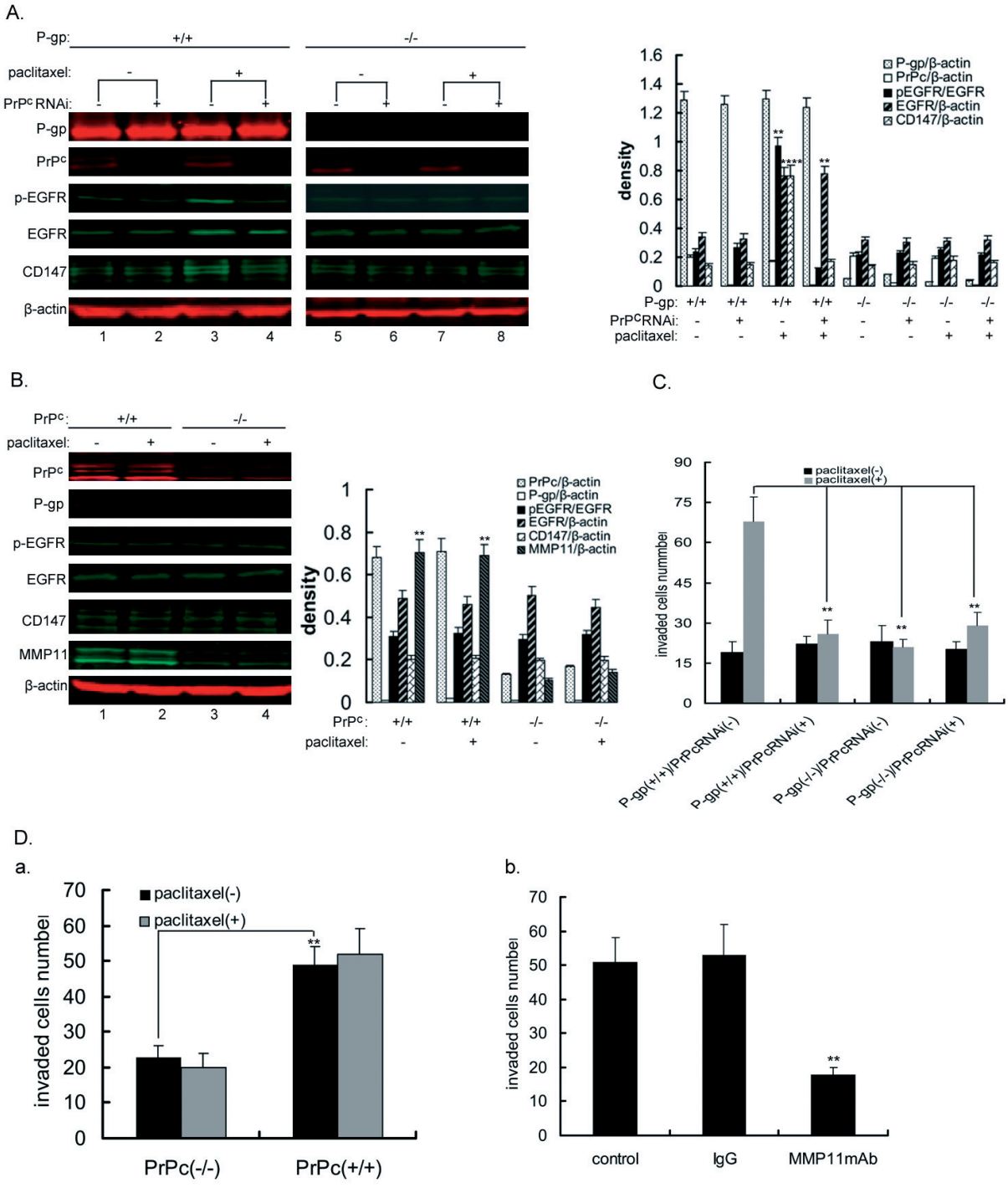

Figure 3. P-gp or PrP^c alone does not account for paclitaxel-induced invasion. Expressions of pEGFR, EGFR, CD147 (*A*) and invasive potentials (*C*) in siPrP^c-transfected MCF7/MDR1 cells, siPrP^c-transfected MCF7 cells incubated with paclitaxel were determined by immunoblotting and *in vitro* invasion assay, respectively. (*B*) Expressions of pEGFR, EGFR, CD147, MMP11 in MCF7/*PRNP* cells incubated with paclitaxel. (*D*) Invasive potentials in MCF7/*PRNP* cells incubated with paclitaxel (*a*) or MMP11 mAb (1 μg/ml) (*b*) Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ versus corresponding control cells (Student's *t*-test).

We then used the membrane-impermeable cholesterol-binding agent cyclodextrin, which depletes cell surface cholesterol to perturb the formation of caveolae. Cyclodextrin (5 mM) completely abrogated p-EGFR and CD147 induction in response to paclitaxel treatment. The effects of cyclodextrin could be reversed by coincubation with excess cholesterol (Fig. 7C), suggesting that paclitaxel-induced invasion

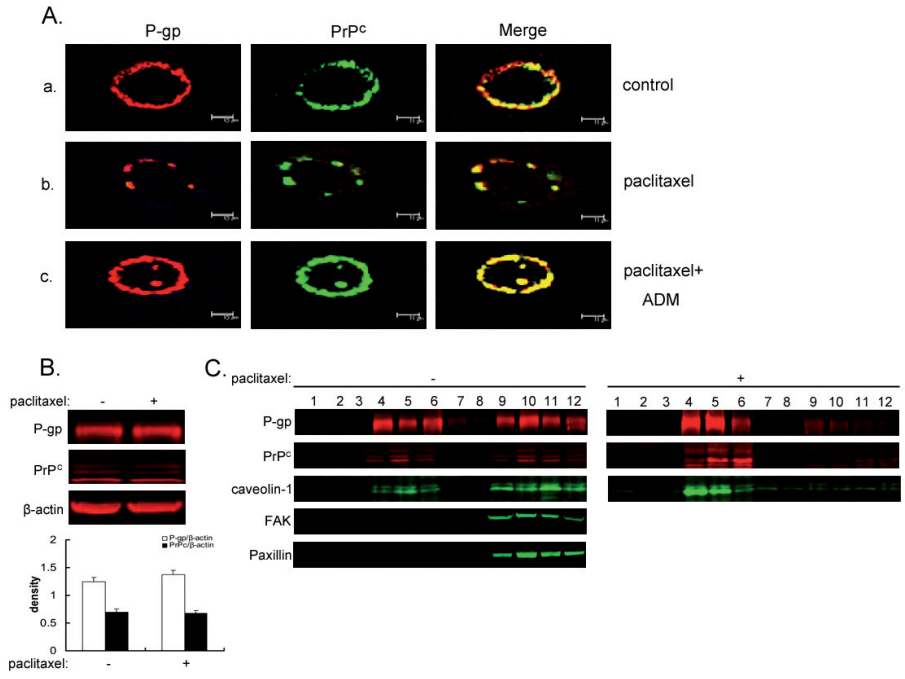

Figure 4. Paclitaxel induces the formation of caveolar domains in which reside the P-gp/PrP^c clusters. (*A*) MCF-7/Adr cells grown in the absence (*a*) or presence of paclitaxel (*b*) or P-gp mAb (1 μg/ml) and paclitaxel together (*c*) were double-stained for P-gp (red) and PrP^c (green). Bar 10 μm. (*B*) P-gp and PrP^c expression were detected in lysates from paclitaxel-exposed MCF7/Adr cells by immunoblotting. Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ *versus* corresponding control cells (Student's *t*-test). (*C*) Fractionation of paclitaxel-treated cells was carried out using the carbonate-based procedure. Distributions of P-gp, PrP^c, caveolin-1, FAK and paxillin in fractions collected from the gradients were evaluated by immunoblotting.

is also dependent on the structural integrity of caveolae.

P-gp/PrP^c complex plays an important role in anti-apoptotic activity of MCF7/Adr cells. P-gp possesses an anti-apoptotic function that protects cells from death by a wide range of stimuli [19, 20]. To investigate whether the P-gp/PrP^c complex is concerned in this process, we transfected MCF7Adr cells with PrP^c siRNA and found that PrP^c depletion greatly enhanced the apoptosis rate (Fig. 8A) as well as caspase activity (Fig. 8B) after paclitaxel treatment. We also observed that paclitaxel raised the expression of bax but reduced that of bcl-2, and that more cytochrome c was released into cytoplasm in PrP^c-depleted cells (Fig. 8D). Meanwhile the Fas and Fas-ligand expressions remained unchanged (data not shown). Z-VAD-FMK (caspase inhibitor) reversed the effects of PrP^c RNAi, as indicated by FCM assays (Fig. 8C). These results demonstrated a causative linkage between P-gp/PrP^c complex and the anti-apoptotic effects in a caspase-dependent manner.

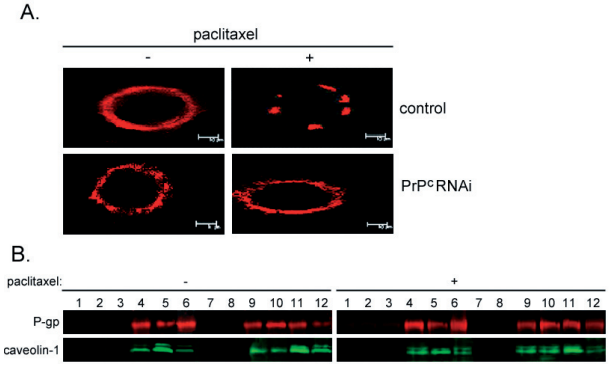

Figure 5. Interaction of P-gp with PrP^c mediates the paclitaxel-induced clustering of P-gp/PrP^c complex. After siPrP^c-transfected MCF7/Adr cells were treated with paclitaxel, immunofluorescence confocal microscopy analyses of P-gp were performed, bar 10 μm (*A*); these cells were also subjected to subcellular fractionation after homogenization, and distributions of P-gp and caveolin-1 in fractions collected from the gradients were evaluated by immunoblotting (*B*).

Discussion

Recently, emerging roles for PrP^c in cancers have drawn much attention, but relatively little is known about its function. In the present study, we confirmed that PrP^c expression was increased in MCF7/Adr cells that also overexpress P-gp. Similar results have been reported in MDR gastric cancer cells [15]. P-gp, an ATP-binding cassette (ABC) drug efflux pump, has been found to be associated with multiple molecules and alteration of their cellular properties have been attributed to this interaction [21–24]. For example, a physical and genetic interaction between CD44 s and P-gp contributes in part to the association between MDR and invasive potential in cancer cells. Interfering with the interaction between CD19 and P-gp chemosensitizes the MDR B-lymphoma cell line. Using immunoprecipitation and confocal microscopy analysis, we found that PrP^c was physically linked with P-gp in MCF7/Adr cells. MCF7/Adr cells treated with

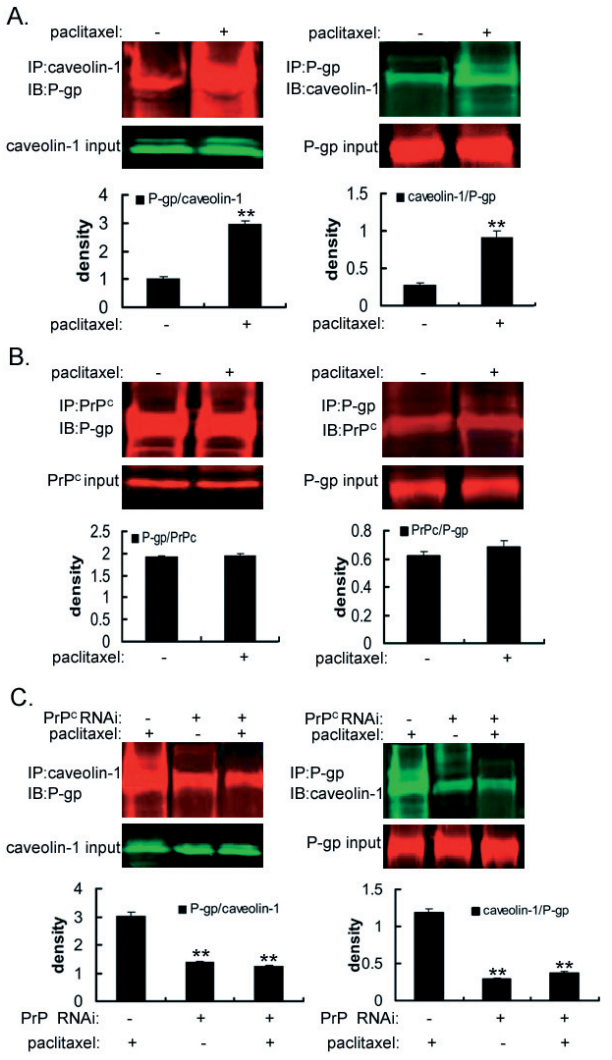

Figure 6. Effects of paclitaxel treatment on the P-gp/PrPc/caveolin-1 complex formation. (*A, B*) Lysates from paclitaxel-treated MCF7/Adr cells were immunoprecipitated with the indicated antibodies and then used for immunoblotting with the corresponding antibodies. (*C*) After siPrP^c-transfected MCF7/Adr cells were exposed to paclitaxel for 48 h, lysates immunoprecipitated with anti-caveolin-1 were used for immunoblotting with anti-P-gp and *vice versa*. Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ *versus* corresponding control cells (Student's *t*-test).

paclitaxel produce more CD147 through mediation of EGFR activity and, thereby, exhibit an elevated capacity to invade [4]. This effect was initiated by P-gp pump function and could be primarily reversed by either disrupting P-gp function or depleting PrP^c expression, indicating that the P-gp/PrP^c complex may be responsible for the association of advanced malignancy with drug treatment.

However, expression of P-gp or of PrP^c has been reported to be correlated with metastatic phenotype of cancer cells [14, 25, 26]. The question was then raised of whther it is possible that P-gp or PrP^c alone but not the P-gp/PrP^c complex contributes to the drug-induced invasion. Consistent with the findings in gastric cancer, we noticed that, although introduction of PrP^c in MCF7 cells elevated the capacity to invade, it was dominantly through up-regulation of MMP11, but not due to the consequences of P-gp/PrP^c interaction. Furthermore, paclitaxel had no effect on invasion in both P-gp(+)PrP^c(-) and P-gp(-)PrP^c(+) transfectants. These results clearly confirmed that paclitaxel promotes the invasive rate in MDR breast cancer cells through the interaction of P-gp with PrP^c.

PrP^c is regarded as a cell-surface receptor for combination with some interactors, such as some glycosaminoglycans, laminin, N-CAM, laminin receptor, and laminin receptor precursor, involved in cell adhesion and migration [17, 27–29]. However, to date there is no functional ligand of PrP^c that has been clearly identified and widely accepted. Taken together with the data on immunoprecipitation and confocal microscopy, our studies present a model in which P-gp may play a role as one of the ligand of PrP^c receptors and interaction between the ligand/receptor accounts for drug-induced invasion.

Formation of P-gp clusters may be a general property, and the clusters of P-gp play an important role in MDR [30]. Also, PrP^c dimerization activates signaling pathways and results in further clustering of the protein. Clustering is thus regarded as a sign of PrP^c activation [17, 31].Using an immunofluorescence method, we revealed the clustering of P-gp/PrP^c complexes induced by paclitaxel. Antibodies that interfere with the function of P-gp also interfered with the formation of P-gp/PrP^c clusters. We postulate that the capacity of P-gp to sequestrate the hydrophobic substrates initiates conformational changes of PrP^c and sequentially activates its dimerization. Furthermore, paclitaxel treatments modifies the subcellular localization of P-gp/PrP^c complex, which is in accordance with caveolin-1 redistribution, suggesting an increased number of caveolar domains in which P-gp/PrP^c clusters reside. As this relocalization could be interrupted by PrP^c depletion, it is possible that paclitaxel effect on P-gp/PrP^c clustering in caveolae-like domains is secondary to the drug effects on P-gp/PrP^c interaction. Many studies have provided evidence suggesting a role of caveolae in tumor invasion. Caveolae are cholesterol and sphingolipid-rich plasma membrane invaginations distinct from other coated vesicles. Multiple signaling cascades have been shown to be regulated by the localization of key cascade elements in caveolae membrane microenvironments. Caveolae compartmentalize intracellular signaling pathways to orchestrate tumor metastasis [32, 33]. Our findings indicated that the inte-

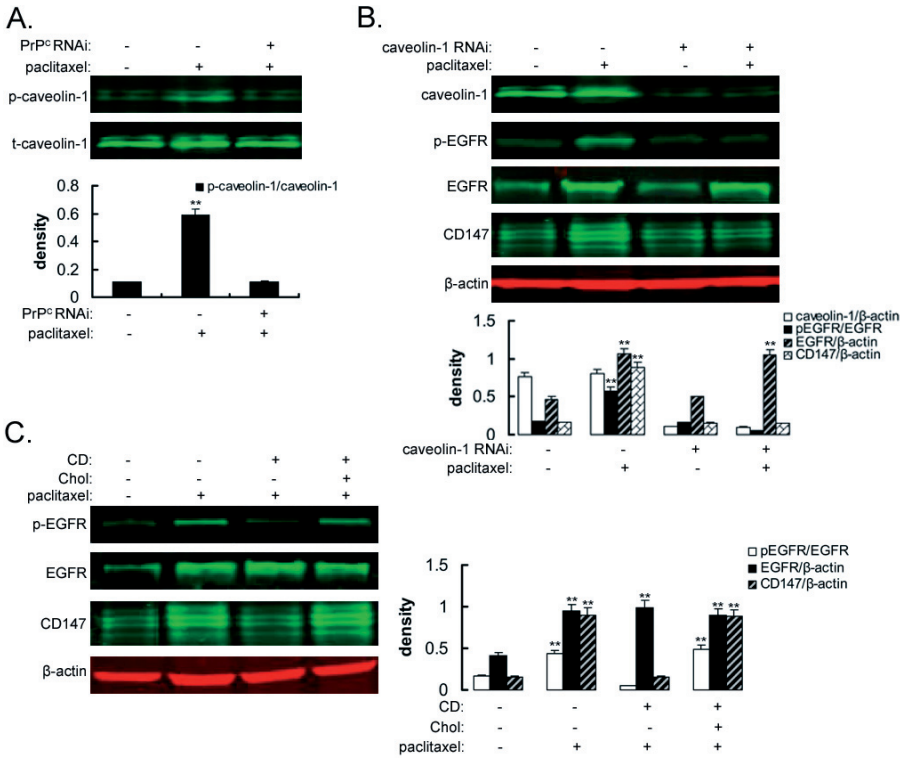

Figure 7. Augmented interaction of caveolin-1 with P-gp/PrP^c dictates downstream responses induced by paclitaxel. (*A*) Caveolin-1 phosphorylation in response to paclitaxel treatment was assessed by immunoblotting. (*B*) Effects of caveolin-1 depletion on paclitaxel-induced p-EGFR and CD147 expression were determined by immunoblotting. (*C*) After pretreatment with cyclodextrin (CD, 5 nM) and/or exogenous cholesterol (15 μg/ml), expressions of pEGFR, EGFR and CD147 in MCF7/Adr cells incubated with paclitaxel were determined by immunoblotting. Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ *versus* corresponding control cells (Student's *t*-test).

grality of caveolar domains containing P-gp/PrP^c clusters is essential to both EGFR transactivation and CD147 up-regulation and, therefore, paclitaxel-related cancer invasion.

Caveolin-1, a 21-24-kDa molecule, is a principal component of caveolae structure [34]. A portion of P-gp and PrP^c has been characterized to be associated with caveolin-1 within the cell membrane. In MCF7/Adr cells grown in the presence of paclitaxel, we demonstrated the formation of a P-gp/PrP^c/caveolin-1 complex. The role of the P-gp/PrP^c interaction induced by external stimuli mainly reflected the effect on the P-gp/caveolin-1 interaction. Remarkably, the increased levels of the P-gp/caveolin-1 complex after paclitaxel treatment showed good correlation with the extent of P-gp/PrP^c-dependent enhancement of EGFR activity and CD147 expression under this condition. Therefore, it is likely that distinct effects of paclitaxel on tumor invasion, at least in part, depend on how it impacts on the P-gp/PrP^c/caveolin-1 interaction. Caveolin-1 is a metastasis-related gene [35, 36], and the physiological consequences of its overexpression remain controversial and vary according to cancer type. In addition, caveolin-1 plays a regulatory role in many signaling pathways, such as Ras/MAPK, PI3K/Akt, Src, PKC and G proteins [37]. The functional interaction between P-gp and PrP^c seems to be the first step in a complex molecular mechanism that results in drug-induced invasion. Other steps in this process, probably downstream of the interaction, have to be optimized for the cancer cell to invade. For example, the clustering of P-gp/PrP^c at caveolar domains, increased binding of caveolae-1 to P-gp/PrP^c complex, recruitment of other signaling proteins by caveolin-1 are contributing steps.

P-gp overexpression is the major molecular mechanism mediating MDR to efflux pump for various anticancer agents. However, it is difficult to fully explain this drug resistance *via* altered drug transport alone. P-gp has also been suggested to be involved in the inhibition of apoptosis and survival of tumor cells. P-gp delays the apoptotic cascade in Chinese hamster ovary fibroblasts [38]. The effect of P-gp blocking on apoptosis of PHA-activated mononuclear cells has also been validated [19]. We demonstrated the P-gp/PrP^c inhibition on paclitaxel-induced apoptosis in MCF7/Adr cells through the mitochondrial pathway, implying a possible mechanism through which P-gp exerts its anti-apoptotic effects. Combined with the role of P-gp/PrP^c complex in drug-induced invasion, we suggest that under treatment with P-gp substrates, P-gp/PrP^c complex not only elevates the invasive capacity, but also assists MDR tumor cells to elude the cytotoxic attacks *via* its anti-apoptotic activity.

In summary, study of the P-gp interaction with PrP^c suggested that its importance as a pharmacological target for clinical therapy has far exceeded the role initially found as an export protein in MDR. However,

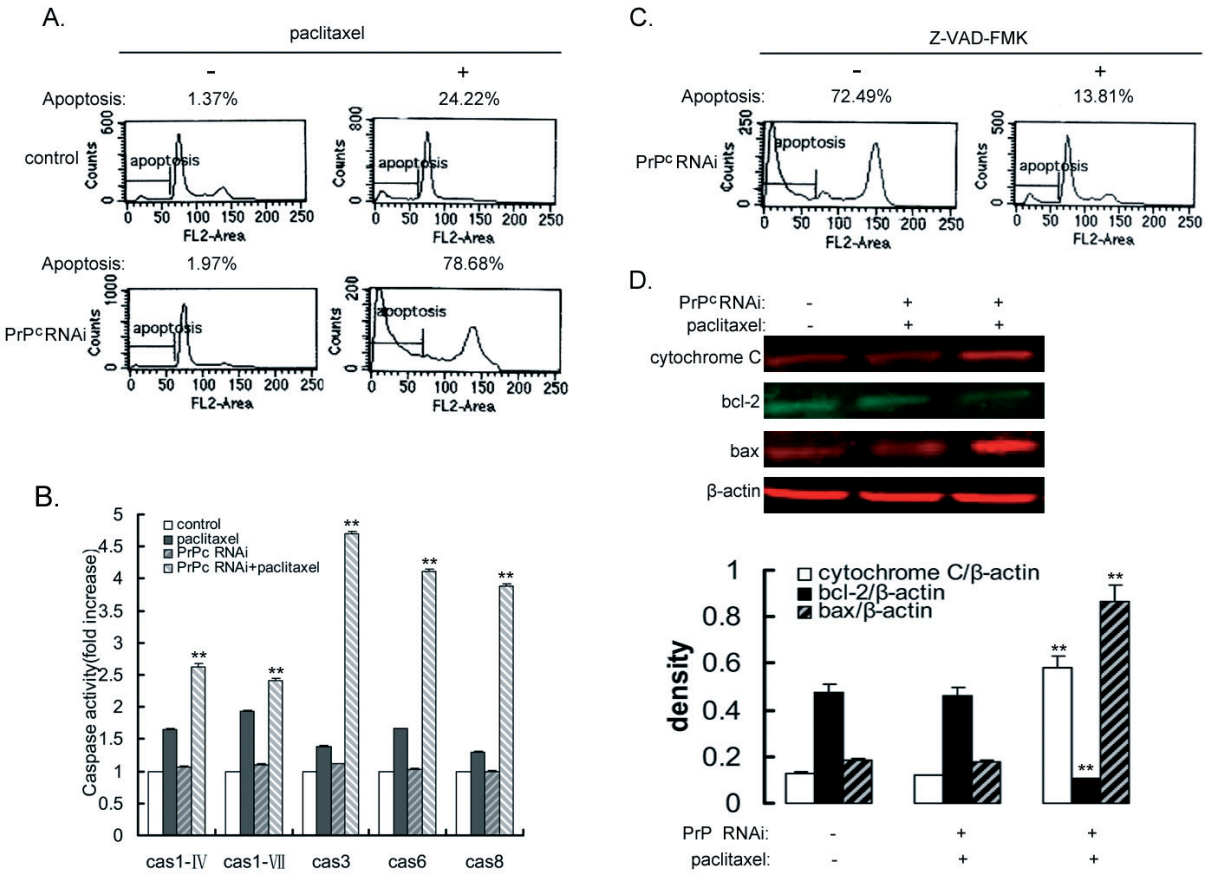

Figure 8. P-gp/ PrP^c complex is involved in the anti-apoptotic activity of MCF7/Adr cells *via* a mitochondrial pathway. Apoptosis, caspase activity and expressions of cytochrome c, bcl-2 and bax in siPrP^c-transfected MCF7/Adr cells treated with paclitaxel were determined by FCM (*A*), caspase activity assay (*B*) and immunoblotting (*D*). Caspase activity in non-treated cells was set as 1 (*C*). After pretreatment of Z-VAD-FMK, apoptosis in siPrP^c-transfected MCF7/Adr cells treated with paclitaxel was determined by FCM. Bars represent the mean of triplicate samples; error bars represent SD. Data are representative of three independent experiments. ** $p<0.05$ *versus* corresponding control cells (Student's *t*-test).

the reversal of P-gp-mediated resistance by most chemosensitizers only occurs optimally at concentrations that are toxic to humans. This raises the necessity of exploring other target molecules downstream of P-gp. Our work puts forward a full explanation for the effects of P-gp substrates on metastatic phenotype in MDR breast cancer cells, and contributes to a better understanding of how MDR tumors escape death induced by chemotherapeutic agents. It pinpoints the potential inhibitive points that may lead to improved effectiveness of clinical chemotherapy. Further work is in progress to evaluate the efficacy of PrP^c RNAi in *in vivo* breast tumor chemotherapy.

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