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Upregulation of Drug Transporter Expression by Osteopontin in Prostate Cancer Cells

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Osteopontin regulates drug transporter expression

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Abbreviations

ABC transporter, ATP-binding cassette transporter; ABCB1, ATP-binding cassette sub-family B member 1; CHX, cycloheximide; DAB, 3,3' diaminobenzidine; DMSO, dimethylsulfoxide; DUN, daunomycin; ev, empty vector; FAK, Focal adhesion kinase; Hx, hypoxia; LRP, lung resistance protein; LY335979, 4-(1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl)-[(5-quinolinyloxy)methyl]-1-piperazineethanol; MDR, multidrug resistance; MRP, multidrug resistance protein; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Nx, normoxia; OPN, osteopontin; P-gp, p-glycoprotein; RGD, Arg-Gly-Asp; RIPA, radioimmunoprecipitation assay; shOPN, OPN knockdown; shRNA, short-hairpin RNA; WT, wild-type.

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Abstract

Multidrug resistance (MDR) is a major cause leading to chemotherapy failure. Recent studies indicate that drug resistance can be rapidly induced by some soluble factors, such as cytokines, chemokines, growth factors and cell adhesion factors in tumor microenvironment. Osteopontin (OPN), an extracellular matrix protein, has a functional RGD domain for binding to integrin. Here we found that OPN expression was up-regulated by hypoxic condition in PC-3 prostate tumor cells. OPN increased the mRNA and protein expression of p-glycoprotein (P-gp), a subfamily of ATP-binding cassette transporter (ABC transporter), in a concentration- and time-dependent manner. The increase of P-gp transporter by OPN was mediated by binding to αvβ3 integrin. Daunomycin (DUN), a chemotherapeutic agent with autofluorescence, was used to evaluate the pump activity and OPN was found to increase the drug pumping-out activity. OPN inhibited DUN-induced cell death, which was antagonized by αvβ3 monoclonal antibody. Long-term treatment with DUN further enhanced the expression of OPN. Knockdown of endogenous OPN potentiated the DUN-induced apoptosis of PC-3 cells. Furthermore, knockdown of OPN also enhanced the cell death caused by other drugs, including paclitaxel, doxorubicin, actinomycin-D and rapamycin, which are also the P-gp substrate. The animal studies also showed that OPN knockdown enhanced the cytotoxic action of DUN. These results indicate that

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OPN is a potential therapeutic target for cancer therapy to reduce the drug resistance in sensitive tumors.

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Introduction

While chemotherapy provides useful palliation, advanced cancer remains incurable since those tumors are initially sensitive to therapy but rapidly develop drug resistance. Tumor-associated microenvironment regulates tumor cell cycle and apoptosis and induces de novo drug resistance (Gonda et al., 2009; Jemal et al., 2009; Meads et al., 2009). Under the threatening of chemotherapy, the cancer cells will turn on self-protection mechanism and overexpress the ABC transporters that actively pump out a variety of amphipathic compounds from cells (Gottesman, 2002; Gottesman et al., 2002) and thus decrease the therapeutic effects of chemotherapeutic agents, i.e., induction of Multidrug resistance (MDR) (Bodor et al., 2005; Spoelstra et al., 1991; Wu et al., 2008). The patients developing MDR will have recurrence and rapid growth of tumor, leading to multiple metastases and high morbidity and mortality. Various membrane efflux pumps, including ABCG2, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, P-gp (ABCB1) and the lung resistance protein (LRP) have been reported to play a role in reducing drug intracellular concentrations leading to MDR in tumor cells in vitro (Marquez and Van Bambeke, 2011). Therefore, the strategies for inhibition of the drug transporter are urgently needed. However, administration of drug transporter inhibitors (in particular of P-gp) to prevent or antagonize drug resistance in cancer patients is unsuccessful partially due

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to the toxicity of these compounds. Additional mechanism may play important roles in clinical resistance to chemotherapy drugs. Discovery of new targets may be beneficial in combating drug resistance in patients.

Osteopontin (OPN) is an acidic and secreted non-collagenous protein with cytokine and chemokine-like functions and this extracellular matrix protein is found initially in osteoblasts (Chellaiah et al., 2003; Denhardt and Noda, 1998; Oldberg et al., 1986). OPN has a functional Arg-Gly-Asp (RGD) domain, which interacts with the integrins. In addition, OPN also interacts with CD44 receptor in an RGD-independent manner (Wai and Kuo, 2004; Wang and Denhardt, 2008). Multiple cellular functions are influenced by OPN including cell proliferation, cell migration, angiogenesis and cell survival (Weilbaecher et al., 2011). OPN has been reported to be associated with malignant transformation and is up-regulated in many kinds of human cancer cell lines. OPN is experimentally demonstrated to play a crucial role in tumor models of progression, invasion and metastasis (Bussard et al., 2008). Clinically elevated levels of circulating OPN in cancer patients are correlated to the increase of metastasis and poor prognosis in many solid tumors including breast, liver, head and neck (Bramwell et al., 2006; Caruso et al., 2008; Khodavirdi et al., 2006; Li et al., 2011; Mack et al., 2008). Among patients with prostate cancer, high serum levels of OPN correlate to a high rate of metastasis to bone (Bonfil et al., 2007; Hotte et al., 2002; Ramankulov et

al., 2007). The precise mechanisms through which OPN promotes metastasis and correlates with poor prognosis in cancer patients remain unsolved.

Hypoxia is an important tumor microenvironmental factor. Mammalian cells take on a variety of responses to maintain oxygen homeostasis, an exact balance between the need for oxygen as an energy substrate for oxidative phosphorylation and other essential metabolic reactions and the intrinsic risk of oxidative damage to cells (Vaupel et al., 2001). Hypoxia in tumor tissue results from the reduced tissue perfusion and deterioration of the diffusion geometry. Oxygen is only able to diffuse 100 - 180 μm from the end of the nearest capillary to cells before it is completely metabolized. Therefore, rapidly growing tumors quickly exceed the vascular supply and result in a microenvironment characterized by hypoxia, low pH, and nutrient starvation. Tumor cells have adaptive mechanisms for changing to a glycolytic metabolism, promoting proliferation, becoming resistant to apoptosis, inducing angiogenesis, evading immune attack, and migrating to less hypoxia areas of body. In addition, hypoxia also decreases the effectiveness of conventional chemotherapy and radiotherapy (Kizaka-Kondoh et al., 2003; Shannon et al., 2003). The chemotherapy-resistant is considered to be due to the limited delivery of drugs via the circulation (Wilson and Hay, 2011). Hypoxia also increases malignant tumor progression (Graeber et al., 1996), enhances tumor cell invasion and metastasis, and

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is prognostic for tumor control by conventional treatment modalities (Brizel et al., 1996; Kimbro and Simons, 2006).

We here investigated the role of OPN in the regulation of drug transporter and development of drug resistance during chemotherapy in PC-3. We are the first to demonstrate that OPN could upregulate the expression of drug transporters. Osteopontin may thus be a potential drug target for the reduction of chemoresistance.

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Materials and Methods

Cell Culture

The human prostate cancer cell lines (PC-3, DU145 and LNCaP) were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in 10 cm^2 dishes with RPMI (Gibco; Grand Island, NY) supplemented with 10 % Fetal bovine serum (FBS; Hyclone, Logan, UT) and maintained at 37 ℃ in a humidified atmosphere of 5 % CO_2.

RNA interference

The OPN-shRNA conjugated on the vector of pLKO.1 with puromycin resistant region was provided by National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center (Supplementary Materials Table 4). shRNA plasmids and Lipofectamine 2000 (LF2000; 10 μg/ml; Invitrogen) were premixed with Opti-MEM I (Gibco, Grand Island, NY) separately for 5 min and then mixed with each other for 25 min and then applied to PC-3 prostate cancer cells. The control shRNA (empty vector, ev) was used as negative control. For transient transfection, cells were transfected with five different OPN-shRNA plasmids for 24 h. For stable transfection, medium was changed to RPMI growth medium after 24 h transfection and PC-3 prostate cancer cells were recovered for 6 h, and puromycin (10 μg/ml; MDBio, Inc.,

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Taipei, Taiwan) was then added to the cultures for the selection of stable clone. Within 15 days, puromycin-resistant colonies appeared and two colonies (sh1 and sh2) were selected.

Western blot

After washing with cold phosphate-buffered saline (PBS), cells were lysed with 50 µl radioimmunoprecipitation assay buffer [RIPA; 50 mM HEPES, 150 m MNaCl, 4 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Triton X-100, 0.25% sodium deoxycholate, 50 mM 4-(2-aminoethyl)-benzene sulfonylfluoride, 50 µg/ml leupeptin, 20 µg/ml aprotinin, pH 7.4] on ice for 30 min. After centrifugation at 14,500 r.p.m. for 1h, the supernatant was used for Western blotting. Protein concentration was measured by BCA assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard. Equal proteins were separated on sodium dodecyl sulphate-polyacrylamide (SDS) gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h with 5% dry skim milk in PBS buffer to block nonspecific binding and then incubated overnight at4℃ with following primary antibodies:rabbit anti-ABCB1 (1:500; Lifespan Biosciences), anti-OPN (1:1000; Abcam Inc., Cambridge, MA) and mouse anti-β-actin (1:10000; Santa Cruz Biotechnology). After washing with phosphate buffered saline Tween (PBST), the membranes were then incubated with mouse anti-rabbit or goat anti-mouse

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peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 h. The blots were visualized by enhanced chemiluminescence (ECL; Millipore, Billerica, MA) using UVP bioimaging system (UVP, Upland, CA). For normalization purpose, the same blot was also probed with mouse anti-β-actin (1:10000; Santa Cruz Biotechnology).

Reverse transcription –PCR and Quantitative Real Time-PCR

Total RNA was extracted using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). 2 μg of RNA was used for reverse transcription by using a commercial kit (Invitrogen, Carlsbad, CA). PCR was performed using an initial step of denaturation (5 min at 95°C), 30 cycles of amplification (95°C for 45 sec, 56°C for 1 min, and 72°C for 45 sec), and an extension (72°C for 2 min). PCR products were analyzed on 2% agarose gels. Quantitative real time-PCR was performed using TaqMan/ SYBR Master Mix and analyzed with a model StepOne plus System (Applied Biosystems; Foster City, CA). After pre-incubation at 50°C for 2 min and 95°C for 10 min, the PCR was performed as 40 cycles of 95°C for 15 sec and 60°C for 1 min. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT) (Supplementary Materials Table 1-3).

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Hypoxia treatment

To induce hypoxia (Hx), confluent monolayers of PC-3 cell cultures were placed into special chamber (Anaerobic System PROOX model 110; BioSpherix), which was closed and placed inside a CO_2 incubator at 37℃ and gassing the special chamber with a gas mixture consisting of 94% N_2, 5% CO_2 and 1% O_2.

Cell viability assay

Cell viability was assessed by MTT assay [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrisch, St. Louis, MO). Culture medium was aspirated 24 h after treatment and MTT (0.5 mg/ml) was added to each well. MTT was then removed 30 min later and cells were lysed by 100 µl dimethylsulfoxide (DMSO). The absorbance was measured at 550 nm and 630 nm using a microplate reader (Bio-Tek, Winooski, VT).

Immunofluorescence

Glass coverslips were coated by poly-D-lysine for 1 hr at room temperature and then rinsed with sterile d.d. H_2O (3 times/ 5 min). Cells were seeded onto coverslips for one day. The cells were then incubated in hypoxia for 16 h and were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The cultures were then incubated with 10% BSA and 0.1 % Triton X-100 for 1 h. The cells were treated

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overnight at 4°C with OPN primary rabbit antibody (1:1000; Abcam Inc., Cambridge, MA) and then with Alexa 488-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, California). The images were obtained from fluorescent microscope using excitation wavelength of 488 nm and emission wavelength of 520 nm (model SP5 TCS; Leica, Heidelberg, Germany).

Immunohistochemistry

Tissue section (2 µm thick) were cut from paraffin-embedded blocks on a microtome and mounted from warm water (60°C) onto adhesive microscope slides. Sections were dried overnight at 45°C. After deparaffinization and rehydration, antigen retrieval was performed using Tris-EDTA (pH 9.0) buffer at 100°C for 3 min. The sections were washed and incubated with 0.025% Triton X-100 and 1% BSA in TBS for 1 h. The sections were then incubated with primary rabbit anti-OPN antibody (1:100; Abcam Inc., Cambridge, MA) overnight at 4°C and then 0.3% H_2O_2 in TBS for 15 min. After washing with TBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector laboratories Inc., Burlingame, CA) for 1h. Staining was performed using VECTASTAIN ABC kits (Vector laboratories Inc., Burlingame, CA) and DAB (3,3' Diaminobenzidine) reaction.

Evaluation of daunomycin pumping out activity

Cells were seeded onto glass coverslips. Pretreatment of OPN (10 ng/ml) with or without anti-αvβ3 antibody (2 μg/ml, Merck Millipore, Darmstadt, German) for 24h, the cells were then treated with DUN (100 μM) for 1h, and then washed with PBS, mounted and visualized with confocal microscope to examine the autofluorescence of DUN.

The effect of OPN on the cellular accumulation of DUN was also measured by flow cytometry. Briefly, $5x10^5$ cells were incubated in 6-well plates. The cells were treated with OPN (10 ng/ml) with or without anti-αvβ3 antibody (2 μg/ml) at 37°C for 24 h. The cells were then detached by trypsin and DUN was added and incubated at 37°C for another 1.5 h. The cells were thus collected and washed twice with ice-cold PBS buffer. Finally, the cells were resuspended in PBS buffer for flow cytometric analysis. The relative values were obtained by dividing the fluorescence intensity of each measurement by that of control cells.

Evaluation of P-gp degradation

New protein synthesis was inhibited by cells with 100 μg/ml cycloheximide (CHX, Sigma-Adeich), and the P-gp protein was measured at serial time points by Western blot.

Xenograft animal model

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4-5 week-old male NOD-SCID mice were purchased from Laboratory Animal Center of the National Taiwan University. All mice were kept under standard temperature, humidity, and timed lighting conditions and provided mouse show and water ad libitum. All animal experiments were conducted in accordance with the Guidelines for Animals Research of Agriculture Council, ROC and approved by the Ethical Committee for Animal Research of the National Taiwan University. PC-3 tumor cells at 10^7 in serum-free medium were injected subcutaneously in the flank of male NOD-SCID mice. Drug treatment began when the tumors reached a group average of 100 mm^3 about 1 week later. Tumor volume (mm^3) was calculated using the following equation : 0.52 x(width $(mm)^2$ x length (mm)).

Statistics

Values are expressed as mean ± SEM from at least three experiments. Results were analyzed with one-way analysis of variance (ANOVA), followed by Student's *t* test. Significance was defined as $p< 0.05$.

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Results

Upregulation of osteopontin expression by hypoxia in PC-3 cells

OPN has been shown to play an important role in the tumor progression. Increase of OPN expression in tumor cells leads to a high risk of tumor metastasis and poor outcome in patients (Le et al., 2003). In addition, hypoxia (Hx) also enhances the tumor progression (Graeber et al., 1996). However, the relationship between Hx and OPN on prostate cancer is not clear. We thus examined the effect of Hx on the production of OPN. Cancer cells were put into the chamber containing 1% O_2, 5% CO_2 and 94% N_2. It was found that OPN mRNA level was upregulated following Hx treatment for 2 h and 4 h (Fig. 1A). The OPN protein level is also increased to 1.6 ± 0.1 and 2.1 ± 0.4 fold of control at 16 and 24 h, respectively (Fig. 1B). These results indicate that Hx upregulated both mRNA and protein levels of OPN in PC-3.

Osteopontin induces p-glycoprotein expression in PC-3 cells

P-glycoprotein (P-gp) also known as multidrug resistance protein1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) is a well-characterized ABC transporters. It is responsible for high levels of drug resistance to many chemotherapeutic drugs such as paclitaxel, anthracyclines, vinca alkaloids,

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camptothecins, and epipodophyllotoxins (Leslie et al., 2005). Real time PCR was used to evaluate the effect of OPN on mRNA expression. OPN concentration- and time-dependently increased P-gp expression at both mRNA (Fig. 2A and 2B) and protein (Fig. 2C and 2D) levels in PC-3 cells. The mRNA (6h) and protein (24h) levels were increased to 2.2 ± 0.2-fold and 2.5 ± 0.3-fold of control, respectively at 100 ng/ml of OPN.

Osteopontin upregulates p-glycoprotein expression through αvβ3 integrin in PC-3 cancer cells

OPN, a secreted adhesive glycoprotein with a functional RGD cell-binding domain, interacts mainly with αvβ3 integrin and has been identified in several studies as a key protein in carcinogenesis and metastasis. As shown in Fig. 3A and 3B, OPN-induced increase of mRNA and protein expression of P-gp was significantly antagonized by αvβ3 monoclonal antibody (2 μg/ml). These results indicated that OPN increased P-gp mainly via the αvβ3 integrin. Focal adhesion kinase (FAK) plays an important role in integrin-mediated signaling pathways. Pretreatment of PF573228 (FAK inhibitor) markedly inhibited OPN-induced phosphorylation of FAK and P-gp protein expression (Fig. 3C). These results indicate thatbinding of OPN to αvβ3 integrin and

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enhancement of FAK phosphorylation is involved in OPN-induced P-gp expression.

Osteopontin inhibits daunomycin-induced cell death and cellular accumulation of daunomycin

Drugs affected by P-gp include vinca alkaloids, anthracyclines, RNA transcription inhibitor actinomycin-D and microtubule-stabilizing drug paclitaxel. Daunomycin (DUN; daunorubicin), a natural fluorescent anthracycline drug, is used for the treatment of many types of cancer and is also a substrate of P-gp. As shown in Fig. 4A, DUN concentration-dependently induced cell death in PC-3 cells (viability was 0.48 ± 0.02-fold of control at 10 μM). Pretreatment of OPN, αvβ3 integrin monoclonal antibody (2 μg/ml) or LY335979 (P-gp inhibitor, 5 μg/ml) alone did not affect viability. However, OPN (10 ng/ml) markedly inhibited DUN-induced cell death. Pretreatment with αvβ3monoclonal antibody (2 μg/ml) significantly antagonized the effect of OPN (Fig. 4B), indicating that αvβ3 integrin was involved in the action of OPN to protect cancer cell from DUN-induced cell death. Pretreatment with LY335979 (5 μg/ml), a highly selective P-gp inhibitor, also antagonized the protection effect of OPN, indicating that P-gp was involved in the action of OPN. These results also suggest that P-gp may be an important drug transporter in PC-3 cancer cells after treatment with

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DUN although OPN also regulated the expression of other drug transporters (Supplemental Figure 1).

The fluorescent property of DUN provides the advantage for the measurement of drug pumping-out activity. The confocal microscope was used to examine the intracellular fluorescent intensity of DUN and evaluate the accumulation of drug. The fluorescent intensity was markedly decreased by the treatment of OPN (10 ng/ml), which was reversed by the addition of αvβ3 integrin monoclonal antibody (2 μg/ml) (Fig. 4C). Using flow cytometric analysis, the cellular fluorescence of DUN (uptake in 100 μM DUN) was left-shifted by the treatment with OPN (10 ng/ml). The inhibitory effect of OPN was further antagonized by the concomitant treatment of αvβ3 integrin monoclonal antibody (2 μg/ml) (Fig. 4D). The summarized results were shown in Fig. 4E.

Daunomycin up-regulates osteopontin expression in PC-3 cells

Since OPN upregulates drug transporter to pump out DUN, we then examined whether the long-term treatment of DUN affects the expression of OPN. It was found that incubation of cells with DUN for 48 h significantly increased OPN mRNA and protein levels in a concentration-dependent manner (Fig. 5A and 5B). In addition,

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treatment with DUN (1 μM) upregulated the expression of OPN mRNA and protein time-dependently (Fig. 5C and 5D). The protein expression was increased up to 3.4 ± 0.7-fold (n=3) 48 h after treatment of DUN. These results further indicate that long-term therapy with cytotoxic drug may induce drug resistance via the up-regulation of OPN.

Knockdown of endogenous osteopontin enhances the cytotoxicity of chemotherapeutic drugs

To examine the effect of endogenous OPN expression on DUN-induced cell death, knockdown of OPN was performed by using shRNA transfection. Five different sequences of OPN-shRNA were used to evaluate the knockdown efficacy (Fig. 6A). Two sequences of shRNA (#1 and #3) down-regulated OPN to 0.56 ± 0.03-fold and 0.47 ± 0.03 -fold of empty vector, respectively and were used to select puromycin-resistant stable clones (sh1 and sh2). As shown in Fig. 6B, sh1 and sh2 exhibited lower expression levels of both mRNA and protein. We further used sh1 and sh2 to examine the cell viability exposed to DUN. PC-3 cells with OPN-knockdown were more sensitive to DUN-induced cell death (Fig. 6C). The cytotoxicity of several chemotherapeutic drugs was also examined in both wild-type and OPN-knockdown

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cells. Paclitaxel, doxorubicin, actinomycin-D and rapamycin all induced more apoptotic cells in OPN-knockdown cells than that in empty vector-transfected PC-3 cells. However, estramutine, which is not P-gp substrate, was not sensitive to OPN-knockdown (Fig. 6D). After long-term treatment with DUN for 48h, OPN and drug transporter expression increased, which were antagonized by αvβ3 integrin monoclonal antibody. In addition, knockdown of OPN also exerted similar inhibitory effects (Fig. 6E and 6F). These results indicate that endogenously released OPN from tumor cells could protect cells against apoptosis induced by chemotherapeutic drugs.

Knockdown of osteopontin enhances the cytotoxicity of daunomycin in Xenograft animal model

In order to confirm the effect of OPN on the sensitivity to chemotherapeutic drug, the *in vivo* Xenograft model in NOD-SCID mice was performed. Two kinds of PC-3cells were used, PC-3 cells with OPN-knockdown or with empty vector transfection. As shown in Fig. 7A and 7B, OPN knockdown increased the sensitivity to DUN. Nineteen days after tumor cell implantation, it was found that the tumor volume in OPN-knockdown group was not significantly different from that transfected with empty vector (tumor volume was 3098 ± 178 mm^3 and 2428 ± 360 mm^3 for empty vector and

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OPN knockdown group, respectively). However, the OPN-knockdown group was more sensitive to the cytotoxic effect of DUN (tumor volume was 2428 ± 360 mm^3 and 909 ± 289 mm^3 for empty vector and OPN knockdown group, respectively). The expression levels of OPN and P-gp in tumor tissues of these four groups were also measured after sacrifice of mice. After long-term treatment of DUN, P-gp levels were upregulated. However, tumor tissues with OPN knockdown had lower levels of OPN and P-gp (Fig. 7C). The immunohistochemistry also showed that OPN immunoreactivity increased in tumor tissue after long-term treatment with daunomycin, which was antagonized by OPN knockdown (Fig. 7D).

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Discussion

Resistance of chemotherapy is always a major problem in progressive cancer disease and tumor cells begin to amplify its proliferation, metastasis and invasion to distant organs, leading to low outcome and survival rate (Raguz and Yague, 2008). The classical MDR is mainly caused by the overexpression of multidrug resistance gene (MDR1) encoding the P-gp, which is supposed to act as an energy-dependent drug efflux pump (Endicott and Ling, 1989; Germann et al., 1993; Sharom, 2008). P-gp expression in tumor may increase the drug resistance and impair the patients' response to chemotherapy (Leslie et al., 2005). Long-term exposure to chemotherapy drugs, like doxorubicin, mitoxantrone and docetaxel, induces high levels of MDR proteins, such as P-gp, MRP1 (multidrug resistance protein 1) and LRP protein, and lowers the drug sensitivity in primary prostate tumor and prostate cancer cell lines. These primary prostate tumors are highly resistant to mitoxantrone and maintain the cell survival over 80% despite the administration of high dose of cytotoxic drug (Sanchez et al., 2009). OPN is a multifunctional phosphoprotein (Sodek et al., 2000). It can bind type I collagen, fibronectin, osteocalcin and other OPN molecules. OPN uses a conserved RGD sequence to bind to multiple integrin receptors and trigger cell signaling, promote cell adhesion, migration and flattening (Denhardt and Guo, 1993; Luedtke et al., 2002).

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Here we examined the relation between OPN and drug resistance in PC-3 prostate cancer cells. We found that chemotherapy drug could markedly increase OPN and P-gp expression in PC-3 and DU145 (Supplemental Figure 2D) prostate cancer cells and the P-gp expression was less in OPN knockdown PC-3 cells. Compared with PC-3 cells, non-invasive LNCaP cells have much lower expression of αvβ3 integrin (Zheng et al., 2000), which may explain the lower sensitivity of LNCaP to osteopontin. It has been reported that OPN expression increases in many malignancies and increase of plasma OPN levelsis negatively associated with the survival in patients with tumor metastasis (Graessmann et al., 2007; Senger et al., 1988). OPN seems to play an important role in chemoresistance. In our study, OPN could up-regulate P-gp expression, which is present in PC-3 cell membrane and cytoplasm (Moriyama-Gonda et al., 1998) and also increased P-gp expression in DU145 prostate cancer cells (Supplemental Fig. S2). Since mRNA of P-gp was increased by OPN, OPN may upregulate the transporter by increasing protein synthesis, In addition, the P-gp degradation was not significantly affected by OPN (Supplemental Figure 3). Furthermore, OPN also increased the expression of several otherdrug transporters (Supplemental Figure 1). αvβ3 integrin plays an important role in OPN-induced P-gp upregulation in PC-3 prostate cancer cells. The production of OPN protected the tumor cells from chemotherapy drugs-induced cell apoptosis. This

protective function of OPN was antagonized by pretreatment with αvβ3 integrin monoclonal antibody or P-gp inhibitor (LY335979) (Abu Ajaj et al., 2012; Ekins et al., 2002). These results indicate that the increase of cell survival following chemotherapy by OPN was resulting from the up-regulation of P-gp expression via acting through αvβ3 integrin. FAK is a downstream protein after extracellular binding to integrin. It was found that OPN acted through binding to αvβ3 and induced downstream FAK activation leading to promotion of tumor P-gp drug transporter expression in PC-3 cells. Using daunomycin as a model, which is a substrate of P-gp, it was found that the transporter pumped out daunomycin from cells and the fluorescent intensity decreased markedly by OPN treatment. Both confocal microscopy and flow cytometry assay showed that OPN could enhance the drug efflux through increasing P-gp expression in PC-3 cancer cells.

Hypoxia (Hx) is an independent prognostic indicator of poor clinical outcome for patients with cancer. This Hx microenvironment also correlates with the increase of tumor invasiveness metastases and resistance to chemotherapy (Stewart et al., 2010). Under Hx conditions, OPN level was upregulated in PC-3 cancer cells, indicating that OPN may be involved in Hx-induced tumor progression. Endogenous release of OPN may also affect the sensitivity of chemotherapy drugs. Although the clinically relevant concentrations of the DUN are 0.2-1 μM, here we used 10 μM DUN incubation to

accelerate cell death within 12-24 hr. Knockdown of endogenous OPN enhanced the apoptosis effect of cytotoxic drugs. Chemotherapy drugs like paclitaxel, doxorubicin, actionmycin-D and rapamycin all exhibited higher cytotoxicity to OPN-knockdown cells. However, the cytotoxic action of estramustine, which is not a substrate for P-gp, was not affected by OPN. P-gp is thus involved in resistance induction of chemotherapy drugs in PC-3 cells. In animal studies, osteopontin knockdown group was more sensitive to DUN treatment and the P-gp and OPN expression was less than normal group treated with DUN.

In conclusion, we are the first to demonstrate that OPN plays an important role to regulate chemotherapeutic drug resistance via the increase of drug transporter expression. Furthermore, long-term treatment of cytotoxic drug could further up-regulate OPN secretion from tumor cells. *In vivo* animal studies also show that knockdown of OPN inhibited the P-gp expression and enhanced the cytotoxicity of chemotherapeutic drug. These results indicate that OPN could be a potential drug target for reducing the drug resistance in prostate cancer therapy. Fig. 8 is the schematic diagram to show that endogenously released OPN binds to αvβ3 integrin, leading to the activation of FAK, and up-regulates P-gp expression to cause the chemoresistance.

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Authorship Contributions.

Participated in research design: Hsieh, Liou, Huang, Chuang, Yang and Fu

Conducted experiments: Hsieh, Liou, Huang and Fu

Performed data analysis: Hsieh and Huang

Wrote or contributed to the writing of the manuscript: Hsieh and Fu

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Figures Legends.

Fig. 1. Upregulation of osteopontin mRNA and protein levels by hypoxia in PC-3 human prostate cancer cells.

PC-3 cancer cells were exposed to hypoxia environment (1% O_2, 5% CO_2 and 94% N_2) for different time intervals. OPN mRNA (A) and protein (B) levels increased time-dependently. Data are presented as Mean ± S.E (n=4), *p ≤ 0.05, as compared with control normoxia group. Nx, normoxia; Hx, hypoxia.

Fig. 2. Osteopontin increases p-glycoprotein expression in PC-3 cancer cells.

The mRNA (A, B) and protein (C, D) levels of P-gp were upregulated by OPN in a time- and concentration-dependent manner in PC-3 cells. The expression levels of mRNA (A) or protein (C) were evaluated in 6h and 24h, respectively. The mRNA was evaluated using quantitative PCR. Data are presented as Mean ± S.E. (n=4), *p ≤ 0.05, as compared with control group (con).

Fig. 3. αvβ3 integrin is involved in osteopontin-induced p-glycoprotein expression in PC-3 cancer cells.

The mRNA (A) and protein (B) levels of P-gp were evaluated in 6h and 12h, respectively. The increase of mRNA (A) and protein (B) levels was significantly antagonized by pretreatment of anti-αvβ3 monoclonal antibody (2 μg/ml). The mRNA

was evaluated using quantitative PCR. (C) Pretreatment of the FAKinhibitor PF573228 for 30 min significantly inhibited OPN-induced phosphorylation of FAK (upper panel) and P-gp protein expression (lower panel) in a concentration-dependent manner. Data are presented as Mean ± S.E. (n=4), *$p \leq 0.05$, as compared with control group (con). $^{\#}p \leq 0.05$, as compared with OPN treatment alone.

Fig. 4. Osteopontin inhibits daunomycin-induced cell death and cellular accumulation of daunomycin.

(A) Daunomycin (DUN), a substrate of P-gp, concentration-dependently induced cell death after 24 h's treatment. (B) DUN(10 μM) -induced cell death was inhibited by OPN (10 ng/ml), which was further antagonized by anti-αvβ3 antibody and P-gp inhibitor LY335979.OPN, anti-αvβ3 antibody and LY335979 alone exerted no effect on cell viability. DUN was exported from cytoplasm, which was inhibited by OPN. Treatment of anti-αvβ3 antibody antagonized the action of OPN as shown by immunofluorescent confocal microscopy (C) and flow cytometry (D). The summarized results were shown in (E). pink, DUN (100 μM); purple, DUN (100 μM) + OPN (10 ng/ml); blue, DUN + OPN + anti-αvβ3 antibody. Data are presented as Mean ± S.E. (n=5), *$p \leq 0.05$, as compared with control group (con). $^{\#}p \leq 0.05$, as compared with daunomycin treatment alone. $^{+}p \leq 0.05$, as compared with daunomycin + OPN group.

Fig. 5. Long-term treatment of daunomycin increases osteopontin expression in PC-3 cells.

OPN mRNA (A) and protein levels (B) were upregulated by the treatment of DUN for 48 h in a concentration-dependent manner. DUN (1 μM) also increased OPN mRNA and protein expression in a time-dependent manner (C, D). Data are presented as Mean ± S.E (n=3), *p ≤ 0.05, as compared with control group (con).

Fig. 6. Osteopontin knockdown potentiates the cytotoxic effect of chemotherapy drugs and inhibits daunomycin-induced p-glycoprotein expression.

(A) Five OPN-shRNA plasmids (#1~ #5) and one empty vector (ev) plasmid were transiently transfected in PC-3 cells to examine the silencing efficacy. (B) Using puromycin (10 μg/ml) to select two puromycin-resistant stable clones designated as OPN-shRNA 1 (#1) and OPN-shRNA 2 (#3). (C) Cell viability assay was performed by using sh1 and sh2 stable clones. Note that DUN (10 μM)-induced cell death was markedly increased in OPN-knockdown cells after drug treatment for 12 h. (D) Two stable clones of OPN-knockdown cells were used to examine the cytotoxic sensitivity to other chemotherapy drugs, like palictaxel (0.1 μM), doxorubicin (10 μM), actinomycin-D (5 μg/ml), rapamycin (1 μM) and estramustine (25 μM). Note that knockdown of OPN enhanced the cell death induced by these cytotoxic drugs except

estramustine. DUN treatment (48 h) increased mRNA levels of OPN (E) and P-gp (ABCB1) (F), which was antagonized by αvβ3 integrin monoclonal antibody or knockdown of OPN.Data are presented as Mean ± S.E (n=4), *p ≤ 0.05, as compared with control group (con). #p ≤ 0.05, as compared with respective ev group.

Fig. 7. Osteopontin knockdown enhances the cytotoxicity of chemotherapeutic drug in Xenograft animal model.

(A) PC-3 tumor cells (at 10^7) with empty vector or shOPN cells were injected subcutaneously in the flank of 4-5 week-old male NOD-SCID mice. One week after cell implantation, mice were intravenously injected with saline or DUN (0.5 mg/kg) at two days intervals. Tumor volume was measured every two days. (B) Tumors were excised and weighed after 19 days of drug treatment. Note that OPN knockdown enhanced the cytotoxic effect of DUN. (C) OPN and P-gp expression in tumor was examined by Western blot. (D) Immunohistochemistry was used to examine the distribution of OPN following daunomycin treatment. Note that DUN (ev+DUN) increased OPN expression, which was antagonized by OPN knockdown (shOPN+DUN). Data are presented as Mean ± S.E (n=6 for each group). *p ≤ 0.05, as compared with ev group. #p ≤ 0.05, as compared with ev+DUN.

Fig.8. The schematic diagram to show the signaling pathways of osteopontin in PC-3

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cells.

Osteopontin (OPN) expression was increased by hypoxia condition. Endogenously released OPN binds to αvβ3 integrin, leading to the activation of FAK and up-regulates P-gp expression to cause the chemoresistance.

Figure 1

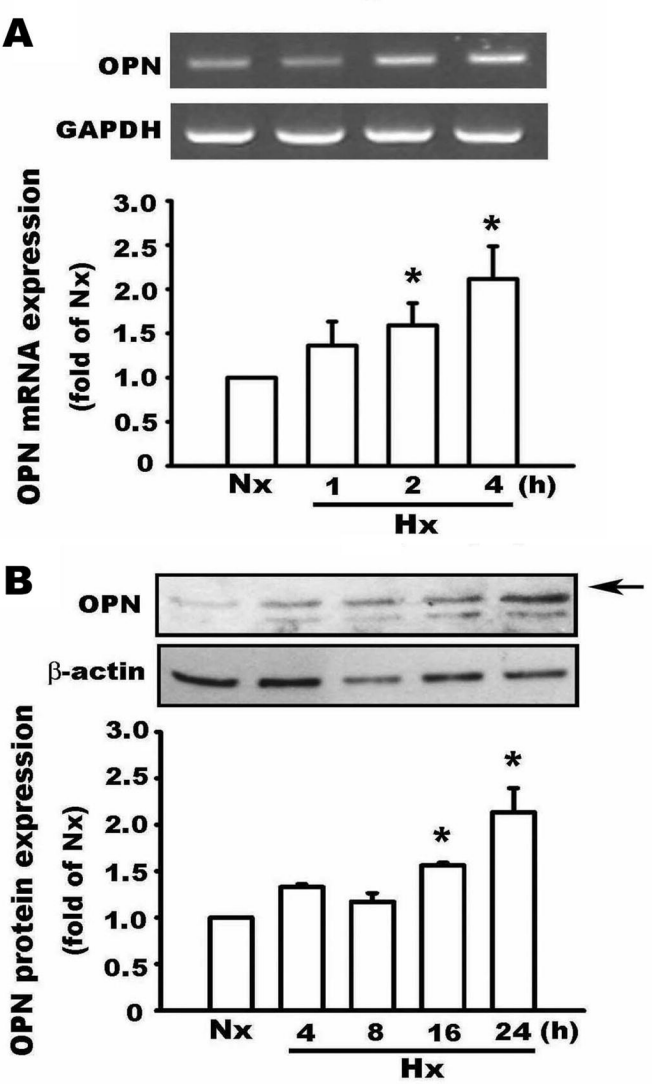
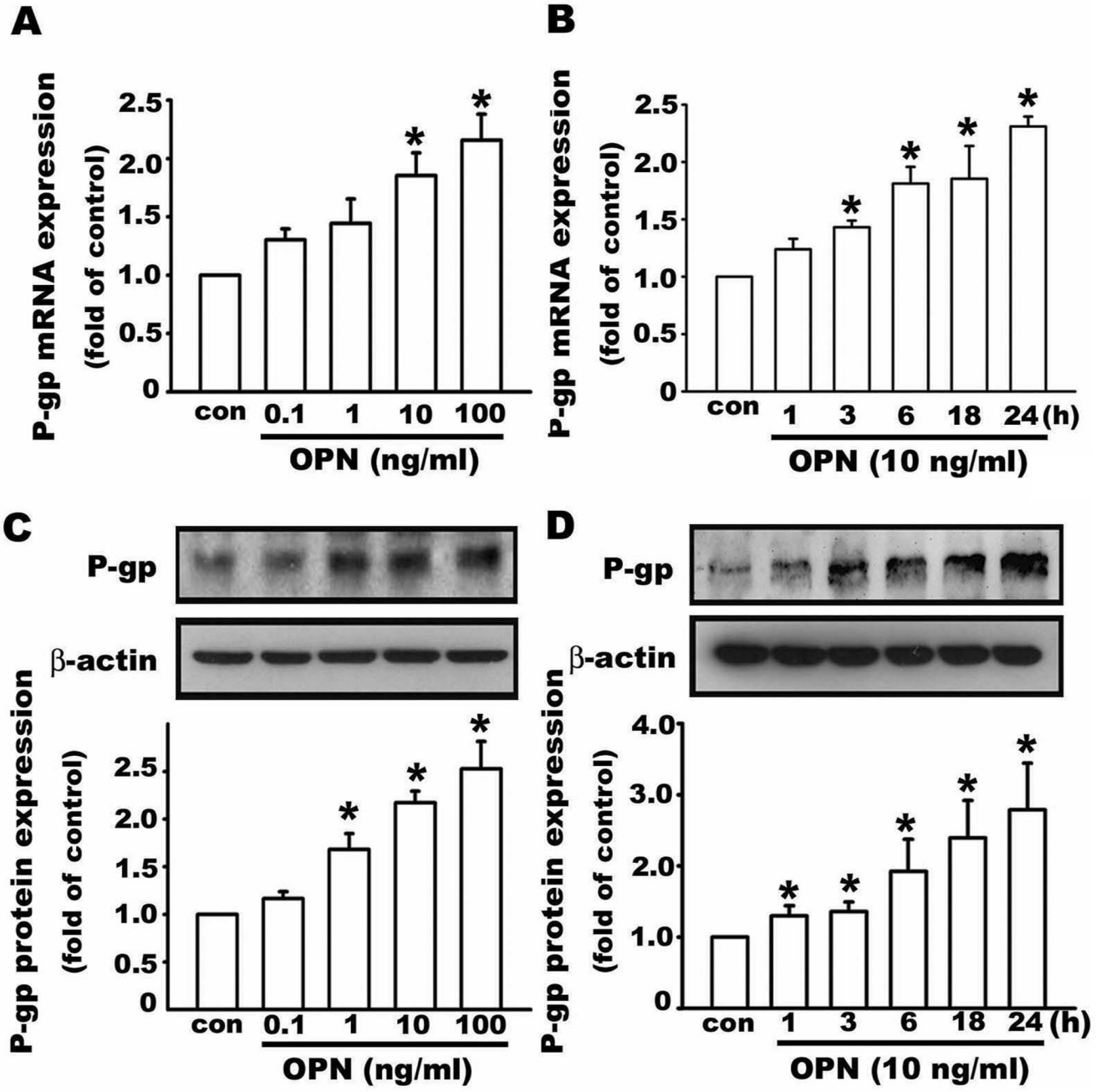

A
OPN
GAPDH
OPN mRNA expression
(fold of Nx)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
Nx
1
2
4 (h)
Hx
B
OPN
β-actin
OPN protein expression
(fold of Nx)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
Nx
4
8
16
24 (h)
Hx

Figure 2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P-gp mRNA expression
(fold of control)
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2.0
1.5
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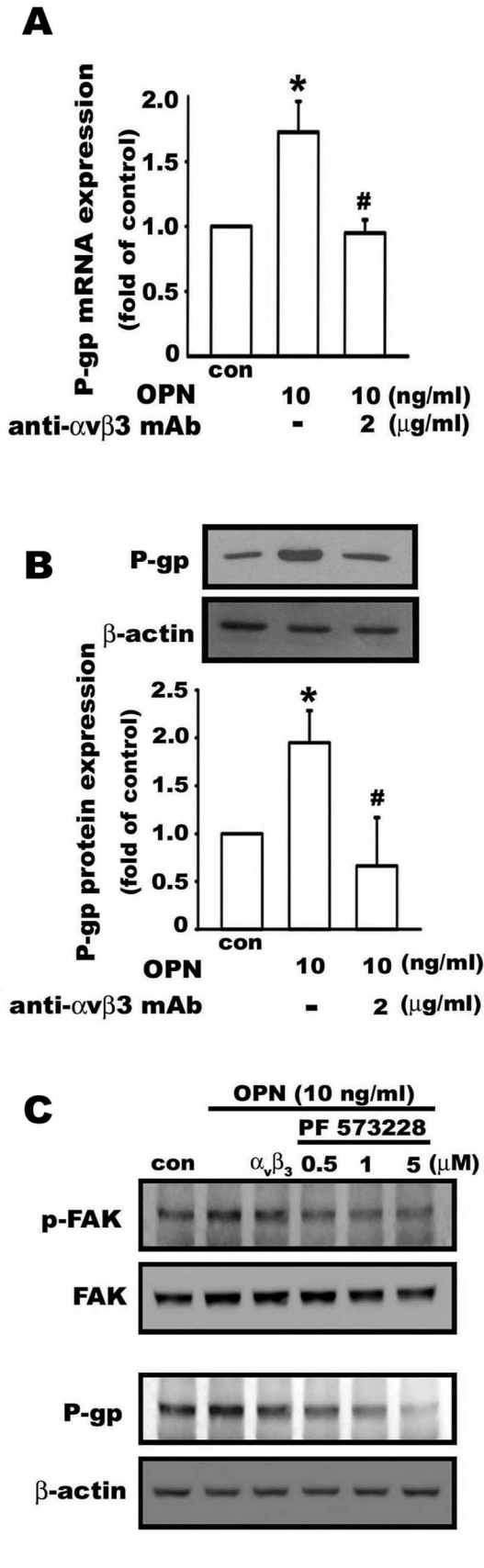
0.5
0
con
0.1
1
10
100
OPN (ng/ml)
B
P-gp mRNA expression
(fold of control)
con
1
3
6
18
24
(h)
OPN (10 ng/ml)
C
P-gp
β-actin
P-gp protein expression
(fold of control)
D
P-gp
β-actin
P-gp protein expression
(fold of control)
4.0
3.0

Figure 3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P-gp mRNA expression
(fold of control)
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1.5
1.0
0.5
0
*
#
con
OPN
10
10 (ng/ml)
anti-αvβ3 mAb
-
2 (μg/ml)
B
P-gp
β-actin
P-gp protein expression
(fold of control)
2.5
2.0
1.5
1.0
0.5
0
*
#
con
OPN
10
10 (ng/ml)
anti-αvβ3 mAb
-
2 (μg/ml)
C
OPN (10 ng/ml)
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con
αvβ3
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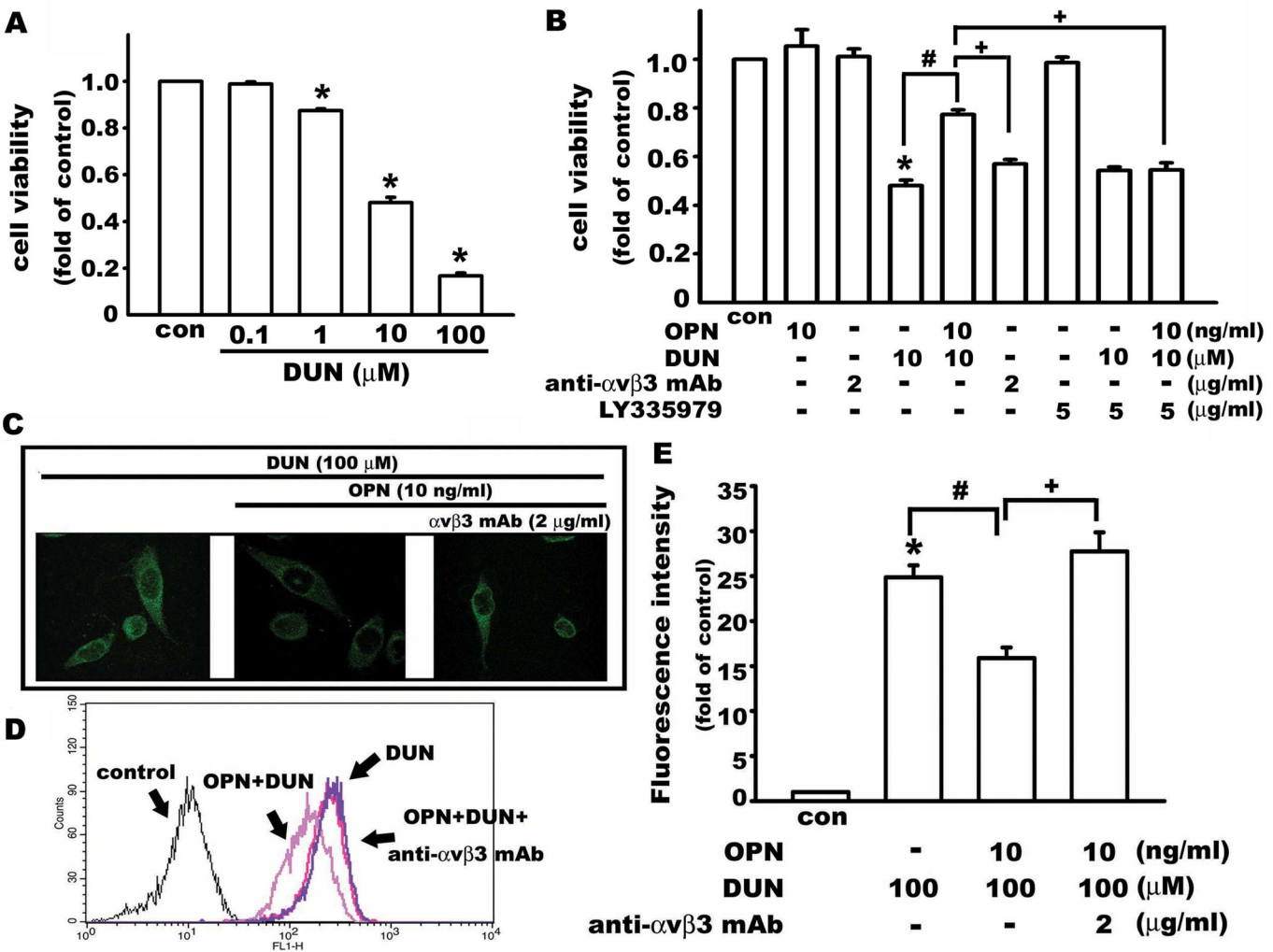
1
5 (μM)
p-FAK
FAK
P-gp
β-actin

Figure 4


A
cell viability
(fold of control)
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0.8
0.6
0.4
0.2
0
con
0.1
1
10
100
DUN (μM)
B
cell viability
(fold of control)
1.0
0.8
0.6
0.4
0.2
0
con
OPN 10 - - 10 - - - 10 (ng/ml)
DUN - - 10 10 - - 10 10 (μM)
anti-αvβ3 mAb - 2 - - 2 - - - (μg/ml)
LY335979 - - - - - 5 5 5 (μg/ml)
C
DUN (100 μM)
OPN (10 ng/ml)
αvβ3 mAb (2 μg/ml)
D
control
OPN+DUN
DUN
OPN+DUN+
anti-αvβ3 mAb
Counts
FL1-H
E
Fluorescence intensity
(fold of control)
35
30
25
20
15
10
5
0
con
OPN - 10 10 (ng/ml)
DUN 100 100 100 (μM)
anti-αvβ3 mAb - - 2 (μg/ml)

Figure 5

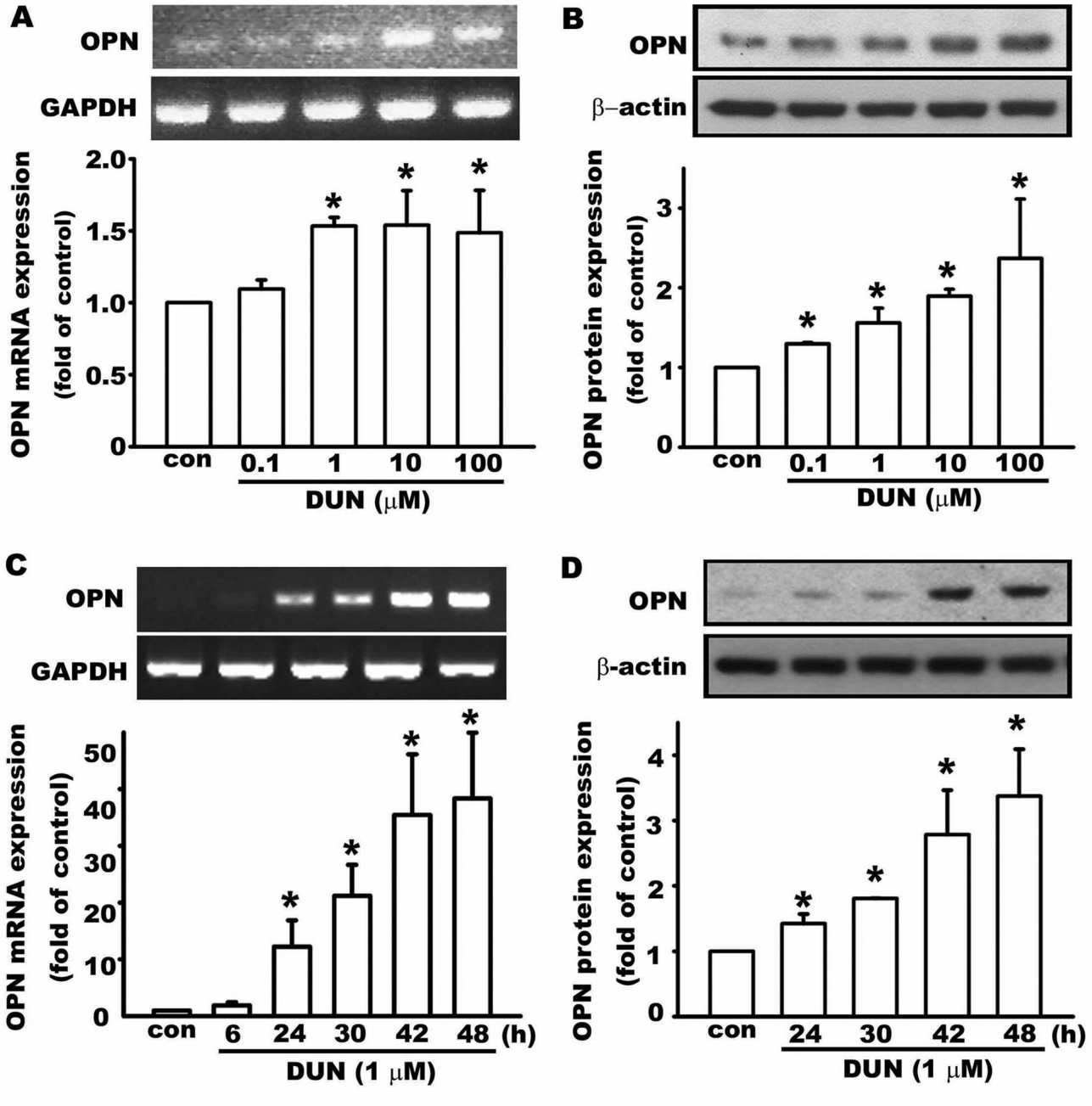
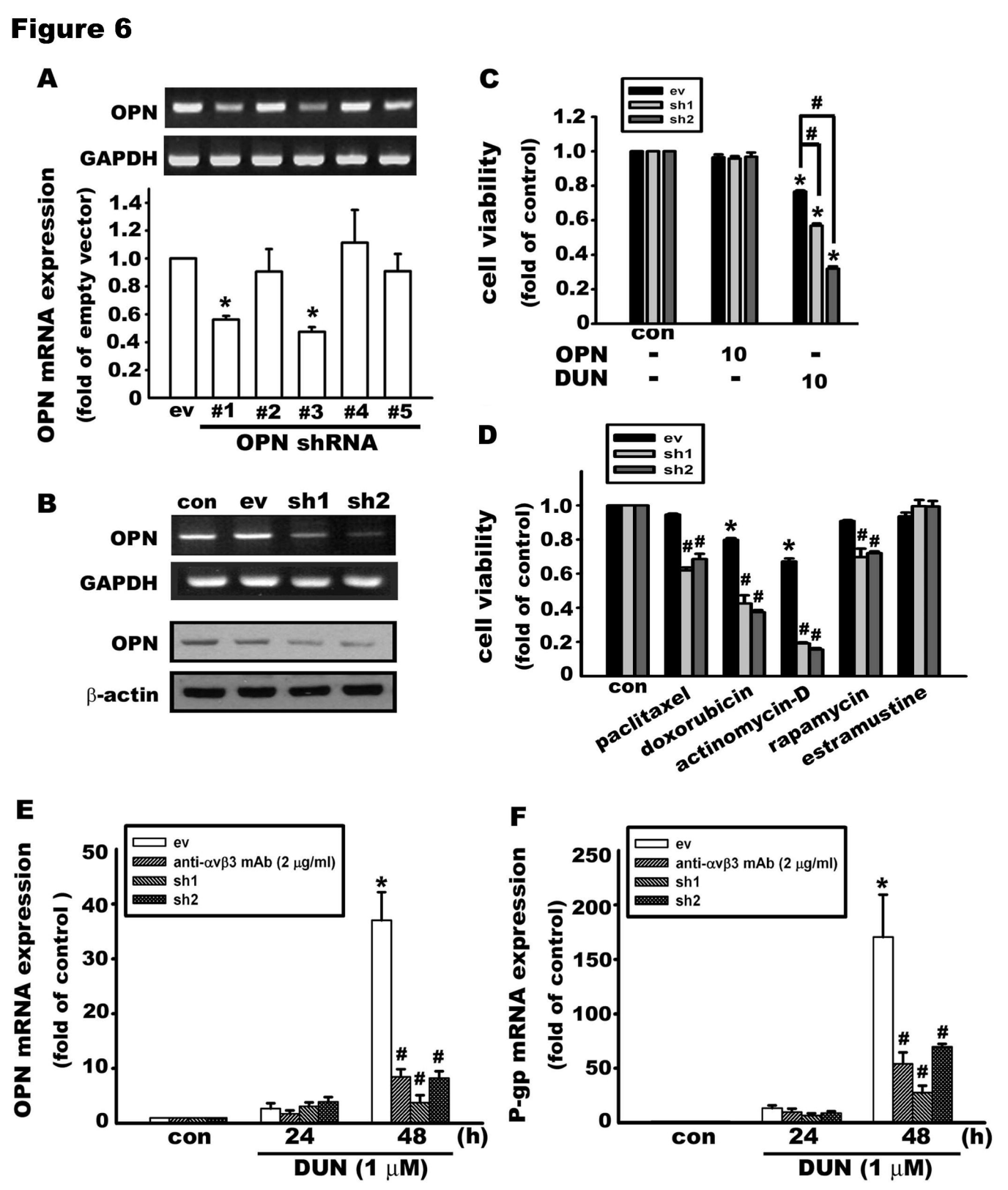

A
OPN
GAPDH
OPN mRNA expression
(fold of control)
2.0
1.5
1.0
0.5
0
con
0.1
1
10
100
DUN (μM)
B
OPN
β–actin
OPN protein expression
(fold of control)
3
2
1
0
con
0.1
1
10
100
DUN (μM)
C
OPN
GAPDH
OPN mRNA expression
(fold of control)
50
40
30
20
10
0
con
6
24
30
42
48
(h)
DUN (1 μM)
D
OPN
β-actin
OPN protein expression
(fold of control)
4
3
2
1
0
con
24
30
42
48
(h)
DUN (1 μM)

Figure 6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OPN
GAPDH
OPN mRNA expression
(fold of empty vector)
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1.2
1.0
0.8
0.6
0.4
0.2
0
ev
#1
#2
#3
#4
#5
OPN shRNA
B
con
ev
sh1
sh2
OPN
GAPDH
OPN
β-actin
C
ev
sh1
sh2
cell viability
(fold of control)
1.2
1.0
0.8
0.6
0.4
0.2
0
con
OPN - 10 -
DUN - - 10
D
ev
sh1
sh2
cell viability
(fold of control)
1.0
0.8
0.6
0.4
0.2
0
con
paclitaxel
doxorubicin
actinomycin-D
rapamycin
estramustine
E
ev
anti-αvβ3 mAb (2 μg/ml)
sh1
sh2
OPN mRNA expression
(fold of control)
50
40
30
20
10
0
con
24
48
(h)
DUN (1 μM)
F
ev
anti-αvβ3 mAb (2 μg/ml)
sh1
sh2
P-gp mRNA expression
(fold of control)
250
200
150
100
50
0
con
24
48
(h)
DUN (1 μM)

Figure 7

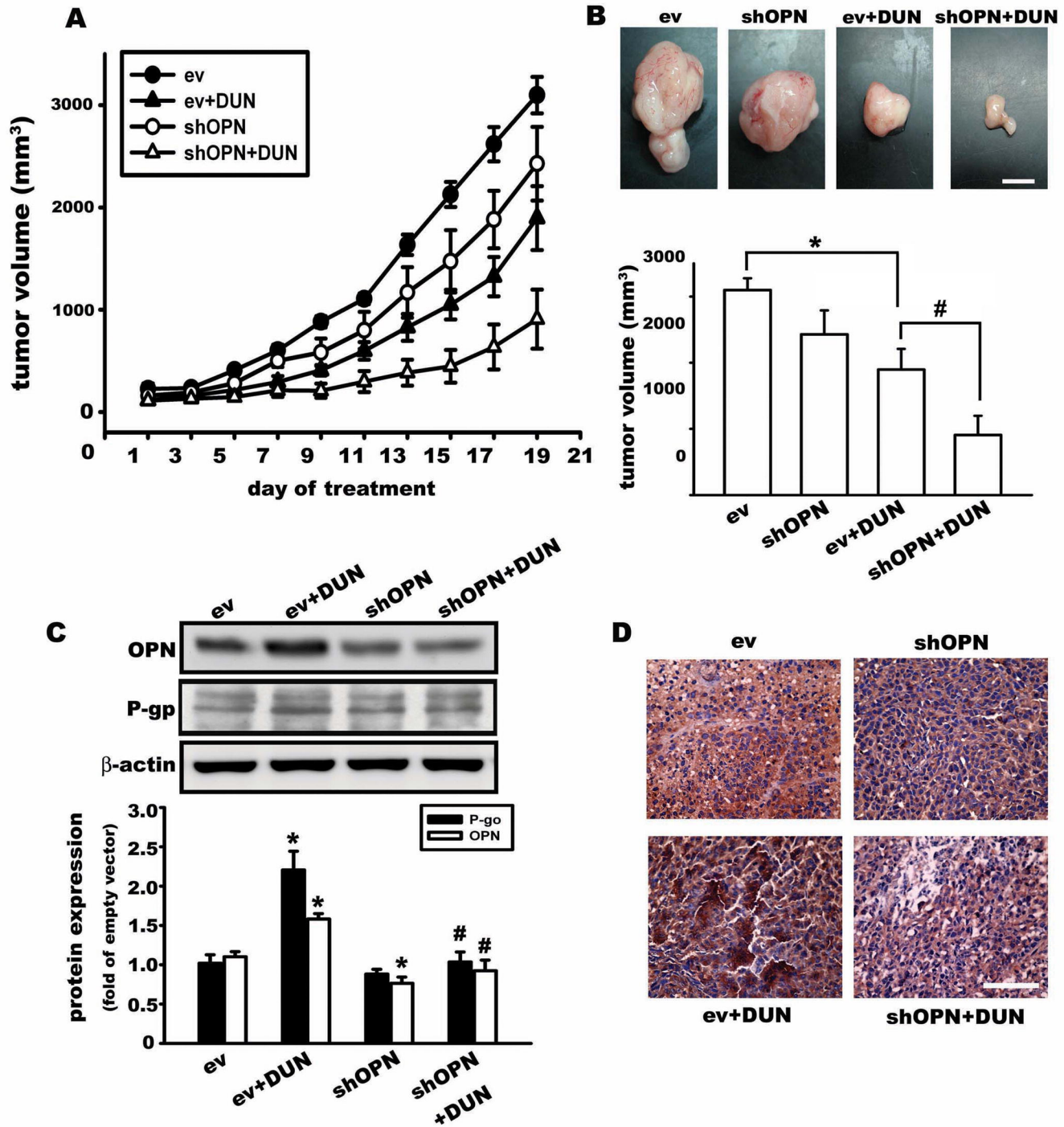
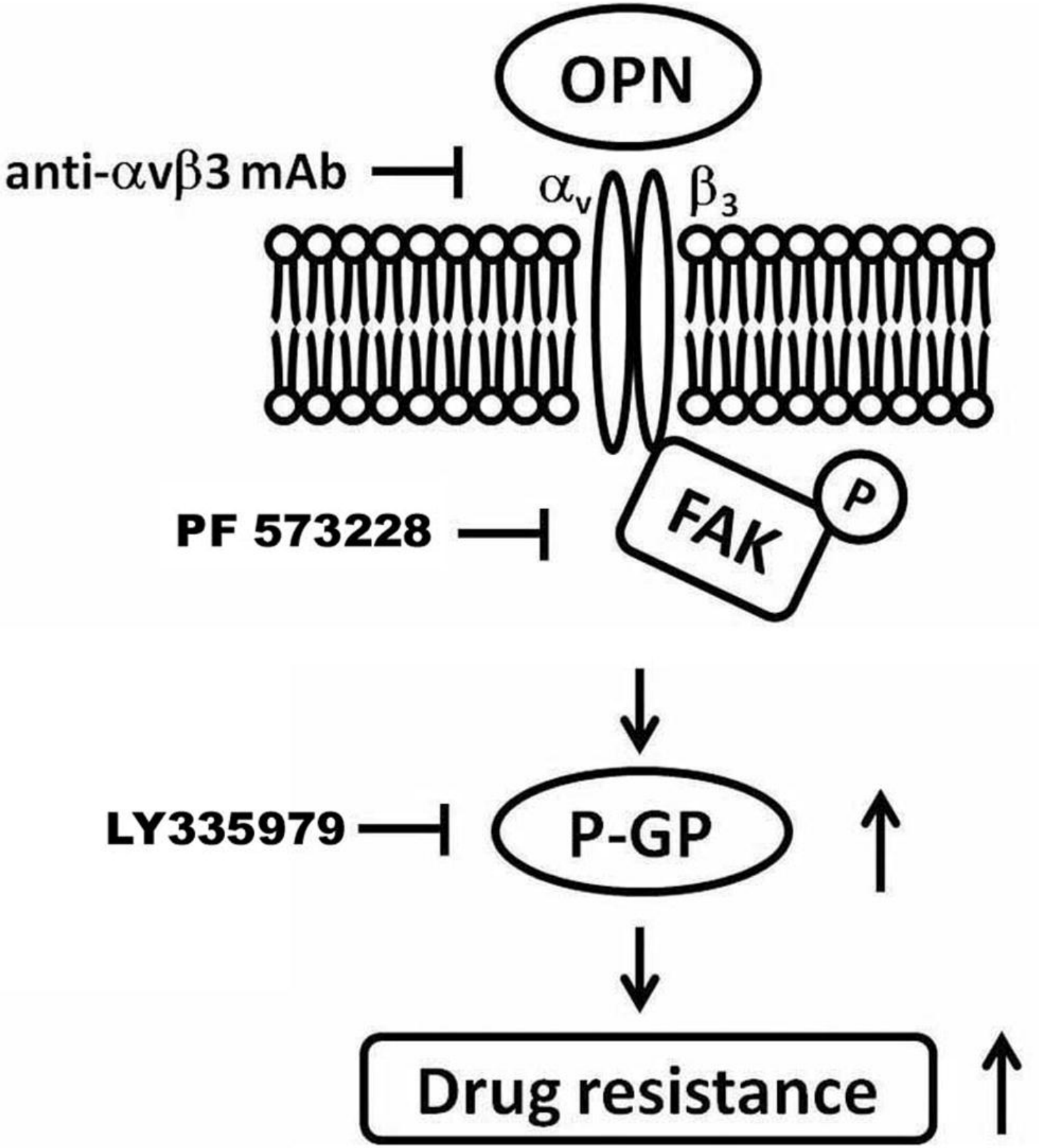

A
ev
ev+DUN
shOPN
shOPN+DUN
tumor volume (mm3)
3000
2000
1000
0
0 1 3 5 7 9 11 13 15 17 19 21
day of treatment
B
ev
shOPN
ev+DUN
shOPN+DUN
tumor volume (mm3)
3000
2000
1000
0
*
#
ev
shOPN
ev+DUN
shOPN+DUN
C
ev
ev+DUN
shOPN
shOPN+DUN
OPN
P-gp
β-actin
protein expression
(fold of empty vector)
3.0
2.5
2.0
1.5
1.0
0.5
0
P-go
OPN
*
*
*
#
#
ev
ev+DUN
shOPN
shOPN +DUN
D
ev
shOPN
ev+DUN
shOPN+DUN

Figure 8


OPN
anti-αvβ3 mAb
αv
β3
PF 573228
FAK
P
LY335979
P-GP
Drug resistance

MOLECULAR PHARMACOLOGY

Upregulation of drug transporter expression by osteopontin in prostate cancer cells

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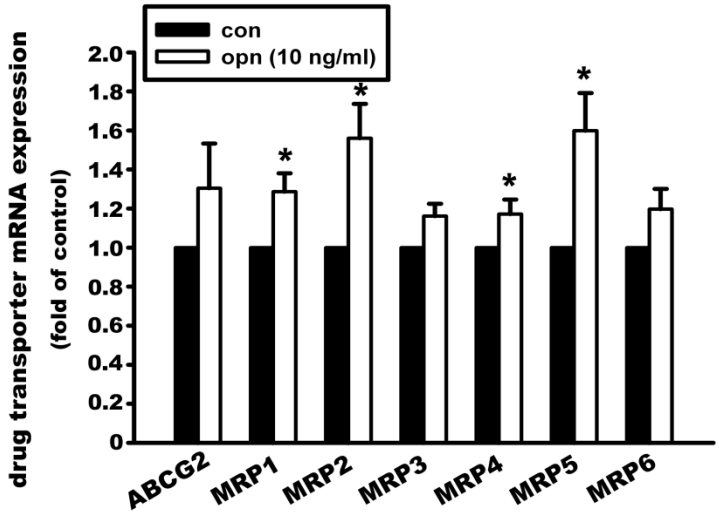
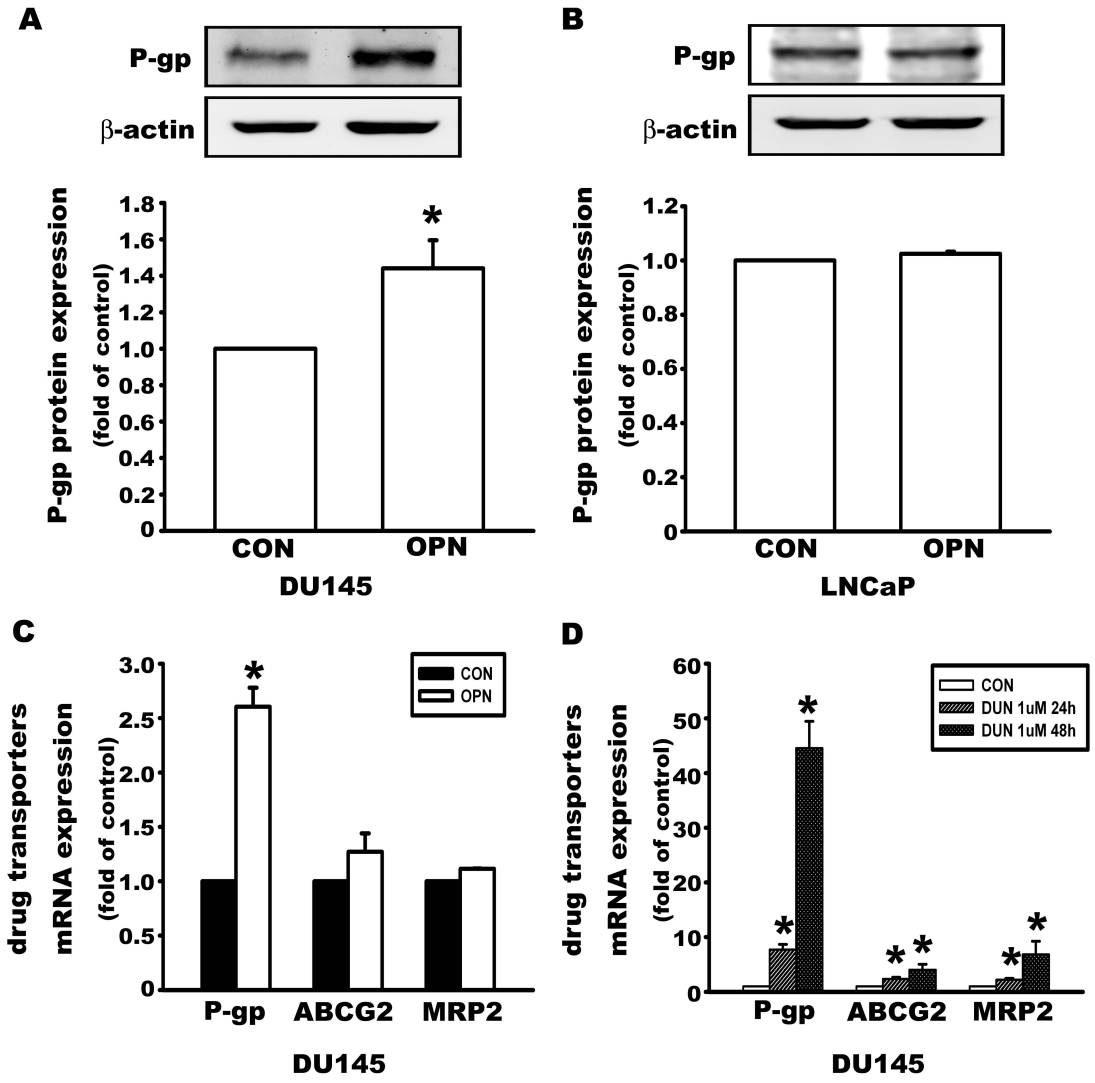
Supplemental Figures



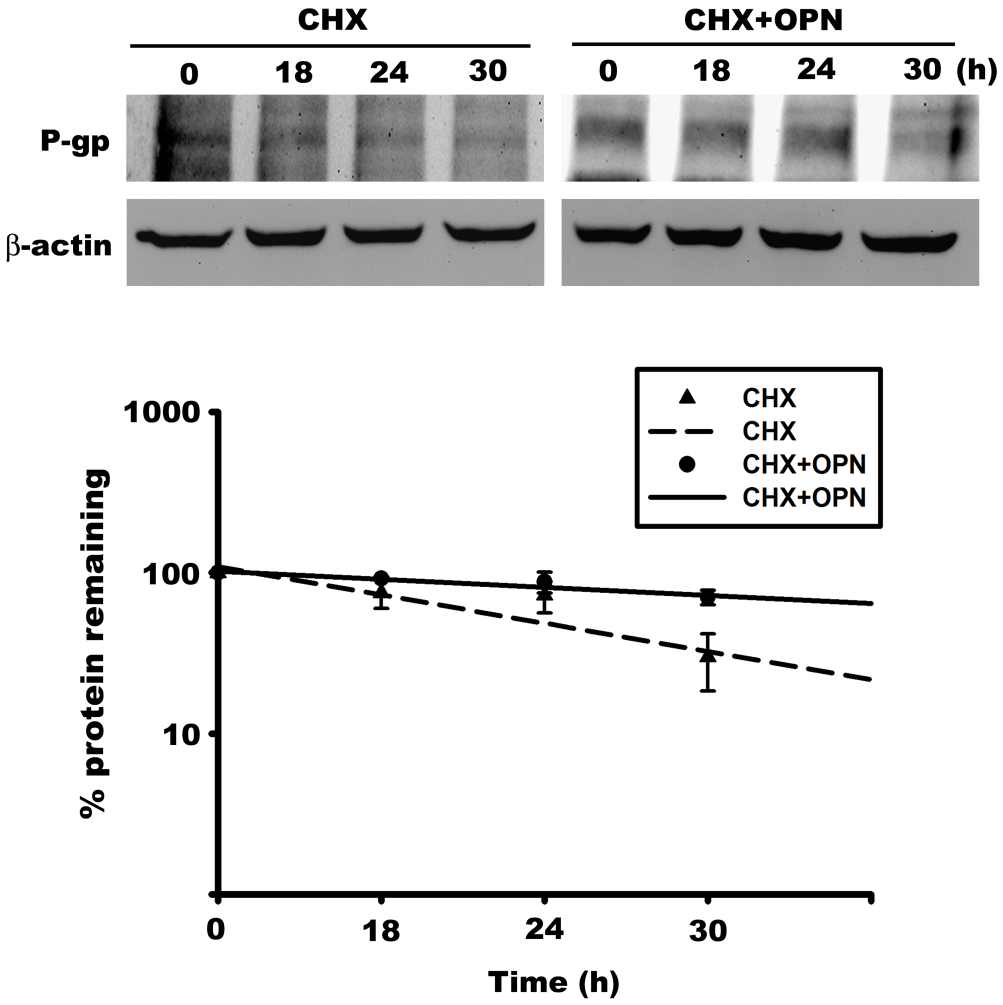
Supplemental Figure 1. Osteopontin upregulates the expression of several drug transporters in PC-3 cancer cells

PC-3 were treated with OPN (10 ng/ml) for 6 h and the mRNA levels of various drug transporters were evaluated by Q-PCR. Note that MRP1, MRP2, MRP4 and MRP5 were upregulated by OPN. Data are presented as Mean ± S.E. (n=4), *, $p<0.05$, as compared with control group (con).



Supplemental Figure 2. P-glycoprotein is upregulated by osteopontin and daunomycin in DU-145 prostate cancer cells

After treatment with OPN (10 ng/ml) for 24h, the P-gp protein level was upregulated in DU145 (A) but not in LNCaP (B) cancer cells. P-gp mRNA levels were upregulated after treatment of OPN (10 ng/ml) for 6h (C). DUN (1 μM) increased P-gp, ABCG2 and MRP2 mRNA expression in DU145 cancer cells in a time-dependent manner (D). Data are presented as Mean ± S.E. (n=4), *, $p<0.05$, as compared with control group (con).



Supplemental Figure 3. Effect of osteopontin on the degradation rate of P-gp in PC-3 cancer cells

PC3 cells were treated with cycloheximide (CHX, 100 µg/ml) and CHX (100 µg/ml) + OPN (10 ng/ml) and P-gp protein content was measured at serial time points by Western blot. Data are presented as Mean ± S.E. (n=4) (p = 0.109; Wilcoxon rank test).

Supplemental Materials

Table 1. RT-PCR primer sequences

	Primer name	Sequences
1.	P-gp	F: 5’-GTGGGGCAAGTCAGTTCATT-3’
		R: 5’-GCTCCTTGACTCTGCCATTC-3’
2.	OPN	F: 5’-CTGTGCCATACCAGTTA-3’
		R: 5’-GATGTCAGGTCTGCGAAA-3’
3.	GAPDH	F: 5’-GCCATCAACGCCCCTTCATTGAC-3’
		R: 5’-ACGGAAGGCCATGCCAGTGAGCTT-3’

Table 2. Real-time PCR primers for SYBR assay

	Primer name	Sequences
1.	ABCG2	F: 5'-TGCAACATGTACTGGCGAAGA-3’
		R: 5'-TCTTCCACAAGCCCCAG-3’
2.	MRP1	F: 5'-GGGCTGCGGAAAGTCGT-3’
		R: 5'-AGCCCTTGATAGCCACGTG-3’
3.	MRP2	F: 5'-TGAGCAAGTTTGAAACGCACAT-3’
		R: 5'-AGCTCTTCTCCTGCCGTCTCT-3’
4.	MRP3	F: 5'-GTCCGCAGAATGGACTTGAT-3’
		R: 5'-TCACCACTTGGGGATCATTT-3’
5.	MRP4	F: 5'-GTCTTCATTTTCCTTATTCTCCTAAACAC-3’
		R: 5'-CCATTTACAGTGACATTTAGCATACTTTGT-3’
6.	MRP5	F: 5'-CGAAGGGTTGTGTGGATCTT-3’
		R: 5'-GTTTCACCATGAAGGCTGGT-3’
7.	MRP6	F: 5'-TGTCGCTCTTTGGAAAATCC-3’
		R: 5'-AGGAACACTGCGAAGCTCAT-3’

Table 3. TaqMan probe (Applied Biosystems; Foster City, CA)

	Probe name	ID
1.	ABCB1	Hs01069047_m1
2.	ABCG2	Hs01053796_m1
3.	ABCC2	Hs00166123_m1
4.	ABCC5	Hs00981087_m1
5.	SPP1(OPN)	Hs00960942_m1
6.	GAPDH	Hs99999905_m1

Table 4. Small RNA sequences

Small RNA	sequences
OPN-shRNA #1	CCGGCTTCAGGGTTATGTCTATGTTCTCGAGAACATAG ACATAACCCTGAAGTTTTT
OPN-shRNA #3	CCGGCCACAAGCAGTCCAGATTATACTCGAGTATAATC TGGACTGCTTGTGGTTTTT
Control shRNA	CCGGTCACAGAATCGTCGTATGCAGCTCGAGCTGCAT ACGACGATTCTGTGATTTTTG