Upregulation of Drug Transporter Expression by Osteopontin in Prostate Cancer Cells

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ABSTRACT
Multidrug resistance is a major cause of 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 the tumor microenvironment. Osteopontin (OPN), an extracellular matrix protein, has a functional arginine-glycine-aspartic acid (RGD) domain for binding to integrin. Here we found OPN expression to be upregulated 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 in a concentration- and time-dependent manner. The increase in P-gp transporter by OPN was mediated by binding to \( \alpha_v\beta_3 \) integrin. Daunomycin (DUN), a chemotherapeutic agent with autofluorescence, was used to evaluate the pump activity, and OPN increased the drug pumping-out activity. OPN inhibited DUN-induced cell death, which was antagonized by \( \alpha_v\beta_3 \) 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 enhanced cell death caused by other drugs, including paclitaxel, doxorubicin, actinomycin-D, and rapamycin, which are also P-gp substrates. The animal studies also showed that OPN knockdown enhanced the cytotoxic action of DUN. These results indicate that OPN is a potential therapeutic target for cancer therapy to reduce drug resistance in sensitive tumors.

Introduction

Whereas chemotherapy provides useful palliation, advanced cancer remains incurable since those tumors are initially sensitive to therapy but rapidly develop drug resistance. A 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 threat of chemotherapy, the cancer cells will turn on their 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, that is, induction of multidrug resistance (MDR) (Spoelstra et al., 1991; Bodor et al., 2005; Wu et al., 2008). Patients who develop MDR will have recurrence and rapid growth of tumor, leading to multiple metastases and high morbidity and mortality rates. Various membrane efflux pumps, including ABCG2, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, p-glycoprotein (P-gp; ABCB1, ATP-binding cassette sub-family B member 1), and the lung resistance protein 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 in part as a result of the toxicity of these compounds. Additional mechanisms 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 noncollagenous protein with cytokine and chemokine-like functions, and this extracellular matrix protein is found initially in osteoblasts (Oldberg et al., 1986; Denhardt and Noda, 1998; Chellaiah et al., 2003). OPN has a functional Arg-Gly-Asp (RGD) domain that 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 upregulated 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 increased metastasis and poor prognosis in many solid tumors, including breast, liver, head, and neck (Bramwell et al., 2006; Khodavirdi et al., 2006; Caruso et al., 2008; Mack et al., 2008; Li et al., 2012). Among patients with prostate cancer, high serum levels of OPN correlate to a high rate of metastasis to bone (Hotte et al., 2002; Bonfil et al., 2007; Ramankulov et al., 2007). The precise mechanisms through which OPN promotes metastasis and correlates with poor prognosis in cancer patients remain undetermined.

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 reduced tissue perfusion and deterioration of the diffusion geometry. Oxygen is able to diffuse only 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 hypoxic areas of the body. In addition, hypoxia decreases the effectiveness of conventional chemotherapy and radiotherapy (Kizaka-Kondoh et al., 2003; Shannon et al., 2003). This chemotherapy resistance 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 is prognostic for tumor control by conventional treatment modalities (Brizel et al., 1996; Kimbro and Simons, 2006).

We 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.

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-cm² dishes with RPMI (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and maintained at 37°C in a humidified atmosphere of 5% CO₂.

RNA Interference. The OPN-shRNA (short-hairpin RNA) was provided by National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center (Eugene, OR) (Supplemental Table 4). The shRNA plasmids and Lipofectamine 2000 (LF2000; 10 μg/ml; Invitrogen, Carlsbad, CA) were premixed with Opti-MEM I (Gibco) separately for 5 minutes, mixed with each other for 25 minutes, and applied to PC-3 prostate cancer cells. The control shRNA (empty vector) was used as negative control. For transient transfection, cells were transfected with different OPN-shRNA plasmids for 24 hours. For stable transfection, the medium was changed to RPMI growth medium after 24 hours of transfection; PC-3 prostate cancer cells were recovered for 6 hours, and puromycin (10 μg/ml; MDBio, Inc., 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 of RIPA (radioimmunoprecipitation assay) buffer [50 mM HEPES, 150 mM NaCl, 4 mM EDTA, 10 mM Na₃P₂O₇, 100 mM NaF, 2 mM Na₃VO₄, 1% Triton X-100, 0.25% sodium deoxycholate, 50 mM 4-(2-aminoethyl)-benzene sulfonylfluoride, 50 μg/ml of leupeptin, 20 μg/ml of aprotinin, pH 7.4] on ice for 30 minutes. After centrifugation at 14,500 rpm for 1 hour, the supernatant was used for Western blotting. Protein concentration was measured by BCA assay kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standard. Equal proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were incubated for 1 hour with 5% dry skim milk in

![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% O₂, 5% CO₂, and 94% N₂) 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, compared with control normoxia group. Nx, normoxia; Hx, hypoxia.]
PBS buffer to block nonspecific binding and then incubated overnight at 4°C with following primary antibodies: rabbit anti-ABCB1 (1:500; Lifespan Biosciences, Seattle, WA), anti-OPN (1:1000; Abcam Inc., Cambridge, MA), and mouse anti-β-actin (1:10,000; Santa Cruz Biotechnology, Dallas, TX). After washing with phosphate-buffered saline Tween (PBST), the membranes were incubated with mouse anti-rabbit or goat anti-mouse peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 hour. The blots were visualized by enhanced chemiluminescence (Millipore, Billerica, MA) using UVP bioimaging system (UVP, Upland, CA). For the purpose of normalization, the same blot was also probed with mouse anti-β-actin (1:10,000; Santa Cruz Biotechnology).

Reverse Transcription-Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted using a TRIzol kit (MDBio, Inc.). Two micrograms of RNA was used for reverse transcription using a commercial kit (Invitrogen). Polymerase chain reaction (PCR) was performed using an initial step of denaturation (5 minutes at 95°C), 30 cycles of amplification (95°C for 45 seconds, 56°C for 1 minute, and 72°C for 45 seconds), and an extension (72°C for 2 minutes). 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 preincubation at 50°C for 2 minutes and 95°C for 10 minutes, PCR was performed as 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. 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) (Supplemental Tables 1-3).

Hypoxia Treatment. To induce hypoxia, confluent monolayers of PC-3 cell cultures were placed in a special chamber (Anaerobic System PROOX model 110; BioSpherix, Lacona, NY), which was closed and placed inside a CO₂ incubator at 37°C, and the special chamber was gassed with a gas mixture consisting of 94% N₂, 5% CO₂, and 1% O₂.

Cell Viability Assay. Cell viability was assessed by MTT assay [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO). Culture medium was aspirated 24 hours after treatment, and MTT (0.5 mg/ml) was added to each well. MTT was removed 30 minutes later, and cells were lysed with 100 µl of dimethylsulfoxide. 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 hour at room temperature and then rinsed with sterile distilled deionized H₂O (3 times/5 minutes). Cells were seeded onto coverslips for 1 day and then incubated in hypoxia for 16 hours and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The cultures were then incubated with 10% BSA and 0.1% Triton X-100 for 1 hour. The cells were treated overnight at 4°C with OPN primary rabbit antibody (1:1000; Abcam Inc.) and then with Alexa 488-conjugated goat anti-rabbit secondary antibody (Invitrogen). The images were obtained from fluorescent microscope using an excitation wavelength of 488 nm and an emission wavelength of 520 nm (model SP5 TCS; Leica, Heidelberg, Germany).

Immunohistochemistry. Tissue sections (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 minutes. The sections were washed and incubated with 0.025% Triton X-100 and 1% BSA in Tris-buffered saline (TBS) for 1 hour. The sections were then incubated with primary rabbit anti-OPN antibody (1:100; Abcam Inc.) overnight at 4°C and then in 0.3% H₂O₂ in TBS for 15 minutes. After washing with TBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody.
(Vector Laboratories Inc., Burlingame, CA) for 1 hour. Staining was performed using VECTASTAIN ABC kits (Vector Laboratories Inc.) and DAB (3,3′-diaminobenzidine) reaction.

Evaluation of Daunomycin Pumping-Out Activity. Cells were seeded onto glass coverslips. After pretreatment of OPN (10 ng/ml) with or without anti-αvβ3 antibody (2 μg/ml; Merck Millipore, Darmstadt, German) for 24 hours, the cells were then treated with daunomycin (DUN; 100 μM) for 1 hour 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, 5 × 10⁶ cells were incubated in six-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 hours. The cells were then detached by trypsin, and DUN was added and incubated at 37°C for another 1.5 hours. 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 (Sigma-Aldrich), and the P-gp protein was measured at serial time points by Western blot.

Xenograft Animal Model. We purchased 4- to 5-week-old male non obese diabetic/severe combined Immunodeficiency mice from the Laboratory Animal Center of the National Taiwan University. All mice were kept under standard temperature, humidity, and timed lighting conditions and provided mouse chow and water ad libitum. All animal experiments were conducted in accordance with the Guidelines for Animal Research of Agriculture Council, Republic of China, and approved by the Ethical Committee for Animal Research of the National Taiwan University. PC-3 tumor cells at 10⁷ in serum-free medium were injected subcutaneously in the flank of male non obese diabetic/severe combined Immunodeficiency mice. Drug treatment began when the tumors reached a group average of 100 mm³ about 1 week later. Tumor volume (mm³) was calculated using the following equation: 0.52 × [width (mm)]² × length (mm).

Statistics. Values are expressed as mean ± S.E.M. from at least three experiments. Results were analyzed with one-way analysis of variance, followed by Student’s t test. Significance was defined as *P < 0.05.

Results

Upregulation of Osteopontin Expression by Hypoxia in PC-3 Cells. OPN has been shown to play an important role in the tumor progression. An increase in 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 enhances tumor progression (Graeber et al., 1996). However, the relationship between hypoxia and OPN on prostate cancer is not clear. We thus examined the effect of hypoxia on the production of OPN. Cancer cells were put into a chamber containing 1% O₂, 5% CO₂, and 94% N₂. It was found that the OPN mRNA level was upregulated after hypoxia treatment of 2 hours and 4 hours (Fig. 1A). The OPN protein level also increased to 1.6 ± 0.1- and 2.1 ± 0.4-fold of control at 16 and 24 hours, respectively (Fig. 1B). These results indicate that hypoxia upregulates both mRNA and protein levels of OPN in PC-3.

Osteopontin Induces P-gp Expression in PC-3 Cells. P-glycoprotein, also known as multidrug resistance protein1 (MDR1) or ABCB1, is a well-characterized ABC transporter. It is responsible for high levels of drug resistance to many chemotherapeutic drugs such as paclitaxel, anthracyclines, vinca alkaloids, 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. 2, A and B) and protein (Fig. 2, C and D) levels in PC-3.
cells. The mRNA (6 hours) and protein (24 hours) levels were increased to $2.2 \pm 0.2$- and $2.5 \pm 0.3$-fold of control, respectively, at 100 ng/ml of OPN.

**Osteopontin Upregulates P-gp Expression through $\alpha\nu\beta3$ Integrin in PC-3 Cancer Cells.** OPN, a secreted adhesive glycoprotein with a functional RGD cell-binding domain, interacts mainly with $\alpha\nu\beta3$ integrin and has been identified in several studies as a key protein in carcinogenesis and metastasis. As shown in Fig. 3, A and B, OPN-induced increase of mRNA and protein expression of P-gp was significantly antagonized by $\alpha\nu\beta3$ monoclonal antibody (2 $\mu$g/ml). These results indicated that OPN increased P-gp mainly via the $\alpha\nu\beta3$ 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 that binding of OPN to $\alpha\nu\beta3$ integrin and 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 $0.48 \pm 0.02$-fold of control at 10 $\mu$M). Pretreatment of OPN, $\alpha\nu\beta3$ integrin monoclonal antibody (2 $\mu$g/ml), or LY335979 (4-(1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl)-[5-quinolinlyoxy]methyl]-1-piperazineethanol) (P-gp inhibitor, 5 $\mu$g/ml) alone did not affect viability. However, OPN (10 ng/ml) markedly inhibited DUN-induced cell death. Pretreatment with $\alpha\nu\beta3$ monoclonal antibody (2 $\mu$g/ml) significantly antagonized the effect of OPN (Fig. 4B), indicating that $\alpha\nu\beta3$ integrin was involved in the action of OPN to protect cancer cell from DUN-induced cell death. Pretreatment with LY335979 (5 $\mu$g/ml), a highly selective P-gp inhibitor, also antagonized the protective 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 DUN, although OPN also regulated the expression of other drug transporters (Supplemental Fig. 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 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 are shown in Fig. 4E.

**Daunomycin Upregulates Osteopontin Expression in PC-3 Cells.** Since OPN upregulates drug transporter to pump out DUN, we then examined whether long-term DUN treatment affects the expression of OPN. Incubation of cells with DUN for 48 hours significantly increased OPN mRNA and protein levels in a concentration-dependent manner (Fig. 5, A and B). In addition, treatment with DUN (1 μM) upregulated the expression of OPN mRNA and protein time dependently (Fig. 5, C and D). The protein expression was increased up to 3.4 ± 0.7-fold (n = 3) 48 hours after treatment of DUN. These results further indicate that long-term therapy with cytotoxic drug may induce drug resistance via the upregulation 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) downregulated 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 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 48 hours, 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. 6, E and F). 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.** 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-3 cells were used, PC-3 cells with OPN-knockdown or with empty vector transfection. As shown in Fig. 7, A and B, OPN knockdown increased the
sensitivity to DUN. Nineteen days after tumor cell implantation, the tumor volume in the OPN knockdown group did not significantly differ from that transfected with empty vector (tumor volume was 3098 ± 178 mm³ and 2428 ± 360 mm³ for empty vector and OPN knockdown group, respectively). However, the OPN knockdown group was more sensitive to the cytotoxic effect of DUN (tumor volume was 2428 ± 360 mm³ and 909 ± 289 mm³ 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). Immunohistochemistry also showed that OPN immunoreactivity increased in tumor tissue after long-term treatment with daunomycin, which was antagonized by OPN knockdown (Fig. 7D).

Discussion

Resistance of chemotherapy is always a major problem in progressive cancer, and tumor cells begin to amplify its proliferation, metastasis, and invasion to distant organs, leading to low outcome and survival rate (Raguz and Yague, 2016).
The classic MDR is caused mainly 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 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, MRPI (multidrug resistance protein 1), and lung resistance 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 greater than 80% despite the administration of high-dose 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).

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 Fig. 2D) prostate cancer cells, and P-gp expression was less in OPN knockdown PC-3 cells. Compared with PC-3 cells, noninvasive 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 an increase in plasma OPN levels is negatively associated with survival in patients

Fig. 7. Osteopontin knockdown enhances the cytotoxicity of chemotherapeutic drug in Xenograft animal model. (A) PC-3 tumor cells (at 10⁶) with empty vector or shOPN (OPN shRNA) cells were injected subcutaneously in the flank of 4- to 50-week-old male non obese diabetic/severe combined Immunodeficiency mice. One week after cell implantation, mice were intravenously injected with saline or DUN (0.5 mg/kg) at 2-day intervals. Tumor volume was measured every 2 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 after daunomycin treatment. Note that DUN (empty vector + 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, compared with the empty vector group; †P ≤ 0.05, compared with empty vector (ev) + DUN.
with tumor metastasis (Senger et al., 1988; Graessmann et al., 2007). OPN seems to play an important role in chemoresistance. In our study, OPN could upregulate 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. 2). 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 Fig. 3). Furthermore, OPN also increased the expression of several other drug transporters (Supplemental Fig. 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) (Ekins et al., 2002; Abu Ajay et al., 2012). These results indicate that the increase in cell survival after chemotherapy by OPN resulted from the upregulation 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 the 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 is an independent prognostic indicator of poor clinical outcome for patients with cancer. This hypoxia microenvironment also correlates with the increase in tumor invasiveness metastases and resistance to chemotherapy (Stewart et al., 2010). Under hypoxia conditions, the OPN level was upregulated in PC-3 cancer cells, indicating that OPN may be involved in hypoxia-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- to 24-hour knockdown of endogenous OPN enhanced the apoptosis effect of cytotoxic drugs. Chemotherapy drugs like paclitaxel, doxorubicin, actinomycin-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, the 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 in regulating chemotherapeutic drug resistance via the increase of drug transporter expression. Furthermore, long-term treatment of cytotoxic drug could further upregulate 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 a chemotherapeutic drug. These results indicate that OPN could be a potential drug target for reducing drug resistance in prostate cancer therapy. Fig. 8 is a schematic diagram showing that endogenously released OPN binds to αvβ3 integrin, leading to activation of FAK, and upregulates P-gp expression to cause the chemoresistance.

**Authorship Contributions**

**Participated in research design:** Hsieh, Liou, Huang, Chang, Yang, Fu.

**Conducted experiments:** Hsieh, Liou, Huang, Fu.

**Performed data analysis:** Hsieh, Huang.

Wrote or contributed to the writing of the manuscript: Hsieh, Fu.

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