

# The p53 Upregulated Modulator of Apoptosis (PUMA) Chemosensitizes Intrinsically Resistant Ovarian Cancer Cells to Cisplatin by Lowering the Threshold Set by Bcl-x<sub>L</sub> and Mcl-1

Zhu Yuan,<sup>1,\*</sup> Kang Cao,<sup>2,\*</sup> Chao Lin,<sup>1</sup> Lei Li,<sup>1</sup> Huan-yi Liu,<sup>3</sup> Xin-yu Zhao,<sup>1</sup> Lei Liu,<sup>1</sup> Hong-xin Deng,<sup>1</sup> Jiong Li,<sup>1</sup> Chun-lai Nie,<sup>1</sup> and Yu-quan Wei<sup>1</sup>

<sup>1</sup>State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, China; <sup>2</sup>Department of Pathogen Biology, Chengdu Medical College, Chengdu, China; and <sup>3</sup>Cancer Center, Chengdu Military General Hospital, Chengdu, China

Ovarian cancer is the number one cause of death from gynecologic malignancy. A defective p53 pathway is a hallmark of ovarian carcinoma. The p53 mutation correlates significantly with resistance to platinum-based chemotherapy, early relapse and shortened overall survival in ovarian cancer patients. PUMA (p53 upregulated modulator of apoptosis), a BH3-only Bcl-2 family protein, was recently identified as a transcriptional target of p53 and a potent apoptosis inducer in various cancer cells. In this study, we showed that the induction of PUMA by cisplatin was abolished in p53-deficient SKOV3 cells. Elevated expression of PUMA-induced apoptosis and sensitized A2780s and SKOV3 ovarian cancer cells to cisplatin, and the combination of PUMA and low-dose cisplatin, significantly suppressed xenograft tumor growth *in vivo* through enhanced induction of apoptosis compared with treatment with PUMA or cisplatin alone. The effects of PUMA were mediated by enhanced caspase activation and release of cytochrome c and Smac (second mitochondria-derived activator of caspase) into the cytosol. Furthermore, PUMA chemosensitized intrinsically resistant SKOV3 cells to cisplatin through downregulation of B-cell lymphoma-extra large (Bcl-x<sub>L</sub>) and myeloid cell leukemia sequence 1 (Mcl-1). PUMA-mediated Bcl-x<sub>L</sub> downregulation mainly happened at the transcription level, whereas PUMA-induced Mcl-1 downregulation was associated with caspase-dependent cleavage and proteasome-mediated degradation. To our knowledge, these data suggest a new mechanism by which overexpression of PUMA enhances sensitivity of SKOV3 cells to cisplatin by lowering the threshold set simultaneously by Bcl-x<sub>L</sub> and Mcl-1. Taken together, our findings indicate that PUMA is an important modulator of therapeutic responses of ovarian cancer cells and is potentially useful as a chemosensitizer in ovarian cancer therapy.

© 2011 The Feinstein Institute for Medical Research, [www.feinsteininstitute.org](http://www.feinsteininstitute.org)

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2011.00176

## INTRODUCTION

Ovarian cancer is the most deadly of gynecologic malignancies (1). Because the disease is essentially asymptomatic early in its progression, approximately 70% of all ovarian cancers are not diagnosed until advanced stages (International Federation of Gynecology and Obstetrics [FIGO] stage III or IV), when long-term prognosis is poor (2). The current standard treatment for ovarian cancer is cy-

torreductive surgery followed by platinum/taxane combination therapy (3). Cisplatin, which inhibits cell proliferation and induces cell cycle arrest by forming interstrand and intrastrand DNA cross-links (4), is one of the most widely used chemotherapeutic agents in the treatment of ovarian cancer. However, the efficacy of cisplatin is limited in curing most tumors because of dose-dependent toxicity and development of cisplatin resistance

(5,6). Emerging evidence suggests that deregulated programmed cell death or apoptosis is a major contributor to tumor initiation, progression and development of acquired resistance to anticancer therapies (7–9). Therefore, therapeutic manipulation of the apoptotic pathways may be an attractive avenue to improve the clinical response of ovarian cancer patients.

The defective p53 pathway is a hallmark of human cancer. p53 mutation is a common genetic event in ovarian carcinoma (10) and correlates significantly with resistance to platinum-based chemotherapy, early relapse and shortened overall survival in ovarian cancer patients (11). A major physiological function of p53 is to kill damaged or stressed cells through induction of apoptosis (12). p53 induces apoptosis by transactivation

---

\*ZY and KC contributed equally to this work.

**Address correspondence and reprint requests to** Zhu Yuan or Chun-lai Nie, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, #1 Keyuan Road 4, Gaopeng, 610041, China. Phone: +86-28-85164063; Fax: +86-28-85164060; E-mails: [yuanzhujyx@gmail.com](mailto:yuanzhujyx@gmail.com), [niecl1022@scu.edu.cn](mailto:niecl1022@scu.edu.cn). Submitted May 15, 2011; Accepted for publication August 17, 2011; Epub ([www.molmed.org](http://www.molmed.org)) ahead of print August 19, 2011.

of its downstream apoptotic regulators. p53 mutations in cancer cells almost invariably abolish this activity, implying that the apoptotic function of p53 is important for its tumor suppressor activity (12). Restoration of the p53 pathway by activating p53 itself or p53 downstream targets has been explored to improve efficacy of anticancer therapies (13).

The p53 upregulated modulator of apoptosis (PUMA) was recently identified as a transcriptional target of p53 and a potent apoptosis inducer in various cancer cells (14–16). PUMA is a member of the “BH3-only” branch of the Bcl-2 protein family, members of which are shown to initiate apoptosis in a tissue- and stimulus-specific manner (8,17). Recently, PUMA was found to be a critical mediator of p53-dependent and -independent apoptosis induced by a wide variety of stimuli, including genotoxic stress, deregulated oncogene expression, toxins, altered redox status, growth factor/cytokine withdrawal and infection (18). PUMA is localized in the mitochondria and functions through Bax and/or Bak by antagonizing the antiapoptotic activities of the Bcl-2-like proteins such as Bcl-2, B-cell lymphoma-extra large (Bcl-x<sub>L</sub>) and myeloid cell leukemia sequence 1 (Mcl-1), to trigger mitochondrial dysfunction and caspase activation and ultimately leading to cell death (19,20). Several animal studies suggest a role for PUMA in tumor suppression. On the one hand, loss of PUMA in Bim-deficient mice exacerbated hyperplasia of lymphatic organs and promoted spontaneous malignancies (18,21). In a hypoxia-induced tumor model, loss of PUMA- and Bax/Bak-dependent apoptosis contributes to chromosomal instability and enhanced tumorigenesis (18,22). In addition, PUMA deficiency increased B-lineage cells and accelerated the development of B lymphoma, accompanied by leukemia (23). On the other hand, elevated PUMA expression, either alone or in combination with chemotherapy or irradiation, induced profound toxicity to a variety of cancer cells, including lung, head and neck, esophagus and breast cancer cells (24–27). More recently, several

studies have shown that PUMA is involved in chemosensitivity via regulating apoptotic signaling pathways (28–30). However, the role of PUMA in the therapeutic responses of ovarian cancer cells to platinum-based anticancer drugs remains unclear.

In this work, to investigate whether PUMA could induce apoptosis of intrinsically resistant ovarian cancer cells, we selected the cisplatin-resistant SKOV3 (p53 double deletion mutant, p53<sup>-/-</sup>) human ovarian carcinoma cell line as a model of intrinsic resistance (31–33) and the cisplatin-sensitive A2780s (p53 wild-type, p53 WT) human ovarian carcinoma cell line, which was derived from an untreated patient with primary ovarian carcinoma (33,34), as a model of intrinsic chemosensitivity, respectively. We examined the regulation of PUMA by cisplatin in both A2780s (p53 WT) and SKOV3 (p53<sup>-/-</sup>) ovarian carcinoma cell lines and evaluated the effect of PUMA on the chemotherapeutic efficacy of cisplatin in A2780s and SKOV3 ovarian cancer models *in vitro* and *in vivo*. We found that the p53 deletion mutation abolishes the induction of PUMA by cisplatin. We also showed that PUMA can cause apoptosis independently of p53 in both A2780s (p53WT) and SKOV3 (p53<sup>-/-</sup>) ovarian cancer cells and that elevated expression of PUMA can enhance the therapeutic responses of ovarian cancer cells to cisplatin by lowering the threshold set by pro-survival Bcl-x<sub>L</sub> and Mcl-1. To our knowledge, we provide new evidence for the potential application of PUMA as a chemosensitizer in ovarian cancer therapy.

## MATERIALS AND METHODS

### Plasmid Construction and Purification

Cultured A2780s ovarian carcinoma cells were harvested, and total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. On the basis of the cDNA sequence of *human PUMA (hPUMA)*, the RNA sample was then subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) for amplification of the encoded

region of *hPUMA*, using a One Step RNA PCR Kit (AMV) (TaKaRa) with upstream primer 5’-**GCGGATCCATGAAATTTGGCATGGGGTC**-3’ and downstream primer 5’-**CCGCTCGAGCTACATGGTGCAGAGAAAGTC**-3’. The incorporated 5’*Bam*HI and 3’*Xho*I restriction sites are shown in bold, whereas the protective base is shown in italics. The amplified cDNA fragment (about 800 bp) was then cloned into the expression plasmid pcDNA3.1 (Invitrogen). The resulting recombinant plasmid was named as pcDNA3.1-hPUMA. pcDNA3.1 vector was used as a control.

pcDNA3.1-hPUMA, pcDNA3.1 and pGL3-control luciferase reporter plasmid (Promega, Madison, WI, USA) were purified by two rounds of passage over EndoFree columns (Qiagen, Chatsworth, CA, USA), as reported previously (35,36). The purified plasmids were mixed with liposome to form a DNA-liposome complex and were then used for subsequent animal experiments.

### Cell Culture and Transfection

Human ovarian cancer A2780s and SKOV3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) and RPMI (Roswell Park Memorial Institute medium) 1640 (GIBCO) containing 10% fetal bovine serum (FBS), respectively, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Transfection was performed with Lipofectamine™ 2000 according to the manufacturer’s instruction. Briefly, aliquots of 2 × 10<sup>5</sup>/2 × 10<sup>3</sup> cells were grown in each well of 6/96-well plates in triplicate and incubated overnight to 70% confluence. DNA (pcDNA3.1, pcDNA3.1-hPUMA, 2 µg/mL)/Lipofectamine 2000 (5 µL/mL) was complexed in DMEM/RPMI 1640 medium and left at room temperature for 20 min. A2780s and SKOV3 cells were incubated for 4 h with the above complexes, followed by rinsing three times, and then 1.5 mL/100 µL DMEM supplemented with fetal calf serum was added to each well of

6/96-well plates and incubated for a further 48 h.

### Treatments of Cells in the *In Vitro* Experiments

A2780s and SKOV3 cells were classified into the following five groups and treated as follows. Group 1, control: the cells were left untreated, and when cultured for 72 h, cells were harvested for subsequent experiments. Group 2, pcDNA3.1 (empty vector) alone: the cells were first incubated for 24 h and then transfected with pcDNA3.1 plasmid; 48 h after transfection, cells were harvested for subsequent experiments. Group 3, hPUMA alone: the cells were first incubated for 24 h and then transfected with pcDNA3.1-hPUMA plasmid; 48 h after transfection, cells were harvested for subsequent experiments. Group 4, cisplatin alone: when the cells were cultured for 48 h, cisplatin was added at a concentration of 5  $\mu\text{g}/\text{mL}$ ; 24 h later, cells were harvested for subsequent experiments. Group 5, hPUMA plus cisplatin (combination): the cells were first incubated for 24 h and then transfected with pcDNA3.1-hPUMA plasmid. At 24 h posttransfection, cisplatin was added at a concentration of 5  $\mu\text{g}/\text{mL}$ ; 48 h after transfection, cells were harvested for subsequent experiments.

The harvested cells above were used for the following *in vitro* experiments including 3-(4,5)-dimethylthiazolium (-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) assay, flow cytometric analysis, Hoechst 33258 staining, RT-PCR, real-time RT-PCR and Western blotting analysis.

### MTT Assay

A2780s and SKOV3 cells were treated according to the schedules as described above. Survival of cells after treatment was quantified using the MTT assay (37). Data represent the average of three wells, and the experiment was repeated three times. Media only-treated cells served as the indicator of 100% cell viability.

### Flow Cytometric Analysis

Flow cytometric analysis was performed to identify sub-G1 cells/apoptotic cells and to measure the percentage of

sub-G1 cells in hypotonic buffer, as described previously (38). Briefly, cells were suspended in 1 mL hypotonic fluorochrome solution containing 50  $\mu\text{g}$  propidium iodide/mL in 0.1% sodium citrate plus 0.1% Triton X-100, and the cells were analyzed by the use of a flow cytometer (ESP Elite; Coulter, Miami, FL, USA). Apoptotic cells appeared in the cell cycle distribution as cells with a DNA content of less than that of G1 cells and was estimated with Listmode software.

### Hoechst 33258 Staining

A2780s and SKOV3 cells treated as described above were harvested, fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) and then washed in PBS twice. Cells were stained with Hoechst 33258 for 5 min and washed with PBS. Finally, apoptosis was visualized with a ZEISS fluorescence microscope (Zeiss, Jena, Germany).

### Semiquantitative RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the instructions of the manufacturer. First-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Semiquantitative RT-PCR was done to amplify *PUMA*, *p53*, *Bcl-x<sub>L</sub>*, *Mcl-1* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). These molecules were amplified using the following pairs of primers: *PUMA* forward 5'-CTGCTGCCCGCTGCC TACCT-3' and *PUMA* reverse 5'-CCGCT CGTACTGTGCG TTGAG-3'; *p53* forward 5'-GTCATCTTCTGTCCCTTCCC-3' and *p53* reverse 5'-ACCTCAGGCGGC TCATAG-3'; *Bcl-x<sub>L</sub>* forward 5'-CAACC CATCCTGGCACCT-3' and *Bcl-x<sub>L</sub>* reverse 5'-GCATCTCCTT GTCTACGCTTT-3'; *Mcl-1* forward 5'-CGGTAATCGGACTCA ACCTC-3' and *Mcl-1* reverse 5'-ACCC ATCCCAGCCTCTTT-3'; *GAPDH* forward 5'-AATCCCATCACCATCTTCC-3' and *GAPDH* reverse 5'-CATCACGCCA CAGTTTCC-3'.

### Real-Time RT-PCR

Total RNA was reverse-transcribed using random primers and the Superscript

II-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) at 42°C for 60 min, according to the manufacturer's instructions. Real-time PCR analysis of *Bcl-x<sub>L</sub>* expression was performed in an ABI Prism 7000 Sequence Detector (Applied Biosystems, Darmstadt, Germany) using SYBR Green PCR Master mix and the thermocycler conditions recommended by the manufacturer. Human  $\beta$ -actin was used as reference gene to normalize for differences in the amount of total RNA in each sample. Amplification of human  $\beta$ -actin cDNA was evaluated using the primer sequences 5'-TGACGTGGACATCCGCAAAG (forward primer) and 5'-CTGGAAGGTG GACAGCGAGG-3' (reverse primer) to exons 5 and 6 of the  $\beta$ -actin gene. Primer pairs for amplification of human *Bcl-x<sub>L</sub>* cDNA were 5'-ACTGTGCGTGGAAG CGTAG-3' (forward primer) and 5'-GCATTGTTCCCATAGAGTT CCA-3' (reverse primer) to exons 2 and 3 of the *Bcl-x<sub>L</sub>* gene. Melting curve analysis showed a single sharp peak with the expected melting temperature ( $T_m$ ) for all samples. mRNA relative quantities were obtained using the  $2^{-\Delta\Delta C_t}$  method (39).

### Subcellular Fractionation

Subcellular fractionation was done as previously described (40). Briefly, cells were harvested after different treatments, washed in ice-cold PBS and then resuspended in an isotonic buffer (250 mmol/L sucrose, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES] [pH 7.5], 10 mmol/L KCl, 1.5 mmol/L  $\text{MgCl}_2$ , 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 1 mmol/L ethyleneglycotetraacetic acid [EGTA], 1 mmol/L phenylmethylsulfonyl fluoride [PMSF] and protease inhibitors [Complete; Roche, Basel, Switzerland]) on ice for 20 min. After incubation, cells were subjected to 40 strokes of homogenization on ice in a 2-mL Dounce homogenizer and then centrifuged at 800g for 10 min at 4°C. The resulting supernatant was centrifuged at 8,000g for 20 min at 4°C to obtain mitochondrial and cytosolic fractions. These fractions were used to monitor cytochrome c and Smac (second mitochondria-derived



activator of caspase) release from mitochondria. Mitochondrial fractions were lysed in 1% Chaps buffer for Western blot analysis.

### Western Blotting

Total cell lysates were prepared in 1% Chaps buffer (5 mmol/L  $MgCl_2$ , 137 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Chaps, 20 mmol/L Tris-HCl [pH 7.5] and protease inhibitors [Complete]) as described previously (41). Total cell lysates and subfractionation lysates were used for Western blot analysis. Western blotting was done as previously described (42). The following primary antibodies were used: anti-caspase-3, anti-cleaved caspase-3, anti-caspase-9 and its cleaved form (Cell Signaling Technology, Danvers, MA, USA); anti-Smac (clone FKE02; R&D Systems, Minneapolis, MN, USA); anti-cytochrome c, cytochrome oxidase subunit IV (Molecular Probes); anti-PUMA, p53, anti-Bcl-2, anti-Bcl-x<sub>L</sub>, anti-Mcl-1 and anti-β-actin (Santa Cruz Technology, Santa Cruz, CA, USA).

### Animal Tumor Models and Treatment

*In vivo* experiments were performed according to our previous report with some modifications (36). Briefly, A2780s and SKOV3 cells ( $2 \times 10^6$  cells) were implanted subcutaneously into the right flanks of 6- to 8-week-old female nude mice, respectively. To explore the therapeutic efficacy of hPUMA plus cisplatin, we treated the mice on d 10 after the implantation of tumor cells, when tumor diameter reached ~5 mm in diameter. The mice were randomly divided into the following five groups (five mice per group) and treated with the following: (i) 100 μL PBS; (ii) 10 μg pcDNA3.1 plasmid/30 μg liposome complexes in 100 μL PBS; (iii) 10 μg pcDNA3.1-hPUMA plasmid/30 μg liposome complexes in 100 μL PBS; (iv) 100 μL of 0.1 mg cisplatin (5 mg/kg body weight); and (v) 10 μg pcDNA3.1-hPUMA plasmid/30 μg liposome complexes in 100 μL PBS and 100 μL of 0.1 mg cisplatin. Because we showed previously that liposome has no effect on

tumor growth *in vivo* (43), we did not set the liposome group as a control. The mice were treated with DNA-liposome complex by intravenous administration via the tail vein twice a week and received cisplatin by intraperitoneal route once a week for 4 wks. Tumor size was monitored by measuring the longest dimension (length) and shortest dimension (width) in a 3-d interval with a dial caliper, and tumor volume was calculated by the following formula: tumor volume ( $mm^3$ ) =  $0.52 \times$  length (mm)  $\times$  width (mm)  $\times$  width (mm). At the end of the experiment, mice were sacrificed. The tumor tissues were collected for subsequent terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) experiments (see below). All studies involving mice were approved by the Institutional Animal Care and Treatment Committee of Sichuan University.

### TUNEL Detection of Apoptotic Tumor Cells

Dissected tumors were weighed and each was divided in half; one-half was fixed in 4% paraformaldehyde in PBS, embedded in paraffin and cut into 3- to 5-μm sections, and the other half was frozen at  $-80^\circ C$ . The tumor tissues frozen at  $-80^\circ C$  were used for detection of hPUMA expression *in vivo* by RT-PCR (see below), whereas the paraffin sections were used for TUNEL experiments. TUNEL was performed with an In Situ Cell Death Detection Kit (Roche). Cell apoptosis was quantified by determining the percentage of positively stained cells for all of the nuclei in 20 randomly chosen fields/section at 200 $\times$  magnification. Slides of the apoptosis studies were quantified in a blind manner by two independent reviewers two different times.

### Detection of hPUMA Expression within the Tumor Tissues

Overexpression of hPUMA *in vivo* was first confirmed indirectly by analyzing the expression of luciferase within the tumor tissues. Briefly, the A2780s tumor-bearing mice were divided into two groups (five mice per group) and treated with 100 μL

PBS or 10 μg pGL3-control luciferase reporter plasmid/30 μg liposome complexes in 100 μL PBS via the tail vein twice a week for 4 wks. At 48 h after the last injection, tumors were collected for sample preparation, and luciferase values were measured using the Luciferase Reporter Assay Kit (Promega) and luminometer. Luciferase activity of the samples was assayed as follows: 100 μL luciferase substrate was added by the luminometer injection system to 20 μL of tumor tissues extracts, and sample light units were recorded several times within 5 s after substrate addition. The resulting luciferase values per 20-μL sample volume were normalized to those of luciferase per 1 mL of tumor extracts. The expression level of luciferase within the tumor tissues was shown as relative luciferase activity, which was calculated from relative light units (RLUs) of the samples.

Then, overexpression of hPUMA *in vivo* was further verified by RT-PCR. The primers used for amplification of hPUMA and GAPDH were the same as those described in the semiquantitative RT-PCR section.

### Data Analysis and Statistics

The statistical analysis was performed with SPSS software (version 17.0 for Windows). All the values were expressed as means  $\pm$  standard deviation (SD). Data were analyzed by one-way analysis of variance, and then differences among the means were analyzed using the Tukey-Kramer multiple comparison test. Survival curves were constructed according to the Kaplan-Meier method (44). Statistical significance was determined by the log-rank test (45).  $P < 0.05$  was considered significant. Error bars represent the standard error of the mean unless otherwise indicated.

## RESULTS

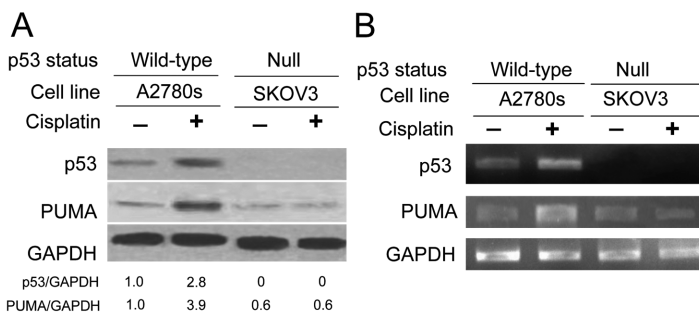
### PUMA Induction by Cisplatin Is Abolished in p53 Double Deletion Mutant SKOV3 Ovarian Cancer

PUMA is normally expressed at low basal levels and can be induced by p53 or

DNA damaging agents (14,15,46). To investigate the regulation of PUMA by cisplatin in ovarian cancer cells with different p53 status, both A2780s (p53 WT) and SKOV3 (p53<sup>-/-</sup>) cells were treated with 5 ng/mL cisplatin. The expressions of PUMA and p53 were analyzed by Western blotting. As shown in Figure 1A, both PUMA and p53 were found to be induced by cisplatin in the A2780s cell line (p53WT), but not in the p53-null SKOV3 cell line. To determine whether the deficiency in PUMA induction is at the mRNA level, semiquantitative RT-PCR was used to examine PUMA in A2780s and SKOV3 cells with and without cisplatin treatment. Similarly, PUMA transcripts were found to be increased in the wild-type p53 A2780s but not in the p53-null SKOV3 cells (Figure 1B). These results indicate that the induction of PUMA by cisplatin in ovarian cancer cells is mediated by p53 and that this induction is abolished in p53-deficient ovarian cancer cells.

**Inhibition of Cell Proliferation *In Vitro* by hPUMA and Cisplatin**

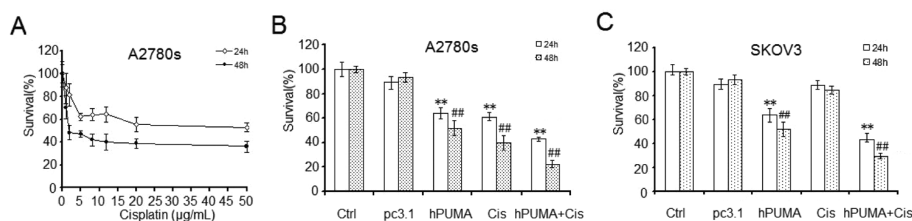
The proapoptotic function of PUMA and lack of PUMA induction in intrinsically cisplatin-resistant SKOV3 (p53<sup>-/-</sup>) ovarian cells prompted us to investigate whether elevated expression of PUMA suppresses ovarian cancer cell growth. As expected, our observations showed that elevated expression of hPUMA in A2780s and SKOV3 cells could result in their proliferation inhibition (Figure 2). Considering the important role of PUMA in DNA damage-induced apoptosis and the known result that the cytotoxic action of cisplatin is mediated by DNA damage, we predict that elevated PUMA expression can sensitize ovarian cancer cells to cisplatin. To test this hypothesis, we first treated A2780s cells with cisplatin at indicated concentrations, with a 24-h or 48-h interval, and found that the dose of half-maximal inhibitory concentration (IC<sub>50</sub>) of cisplatin ranged from 5 to 10 μg/mL (Figure 2A). Then, in the following *in vitro* experiments, we treated cells with cisplatin at a suboptimal dose (5 μg/mL), with a 24/48-h in-



**Figure 1.** p53-dependent activation of PUMA by cisplatin in ovarian cancer cells. (A) A2780s (p53 WT) and SKOV3 (p53<sup>-/-</sup>) cells were treated with cisplatin (5 μg/mL) for 24 h and analyzed for the expression of p53 and PUMA by Western blotting. GAPDH was used as a loading control. Densitometric analysis of the Western blots was performed, and the amount of p53 and PUMA was compared with the protein loading control. The relative amount of p53 and PUMA from the untreated A2780s cells was set as 1. (B) Semiquantitative RT-PCR was done for PUMA, p53 and the control GAPDH (25 cycles) using the cDNA prepared from cells with or without cisplatin treatment.

terval, according to the various schedules, as described in Materials and Methods. After treatment, viability of cells was determined by MTT assay. As shown in Figure 2B, compared with the control, either hPUMA or cisplatin significantly reduced A2780s cell viability by 36%/49% (P < 0.001) and 39%/61% (P < 0.001), respectively. hPUMA plus cisplatin significantly reduced A2780 cell viability by 58%/78% (P < 0.001). In a p53-deficient SKOV3 tumor model, com-

pared with the control, hPUMA also significantly reduced cell viability (P < 0.001), while cisplatin showed only a slight, but not statistically significant, effect on SKOV3 cell growth (P = 0.917, P = 0.762). However, the combination of hPUMA and cisplatin significantly reduced SKOV3 cell viability by 57%/71% (P < 0.001) (Figure 2C). These results indicate that hPUMA can suppress proliferation independently of p53 in both A2780s and SKOV3 ovarian cancer cells



**Figure 2.** Inhibition of tumor cell proliferation *in vitro* by hPUMA and cisplatin. The MTT assay was performed as described in Materials and Methods. (A) The treatment of cisplatin at indicated concentrations and periods reduced A2780s cell viability, showing that the dose of IC<sub>50</sub> ranged from 5 to 10 μg/mL. (B) The treatment of hPUMA plus cisplatin reduced A2780s cell viability more significantly than the treatment of hPUMA alone or cisplatin alone. Significant differences compared with the control group: 24 h, \*\*P ≤ 0.001; 48 h, ###P ≤ 0.001. (C) The treatment of cisplatin alone had little effect on survival of SKOV3 cells, and the combination of hPUMA plus cisplatin reduced SKOV3 cell viability more significantly than the treatment of hPUMA alone or cisplatin alone. Significant differences compared with the control group: 24 h, \*\*P ≤ 0.001; 48 h, ###P ≤ 0.001. Percentage of survival was calculated. Results are shown as means ± SD of six wells and triplicate experiments. In each experiment, the medium-only treatment (untreated) indicates 100% cell viability.

and enhances the response of A2780s and/or SKOV3 cells to cisplatin.

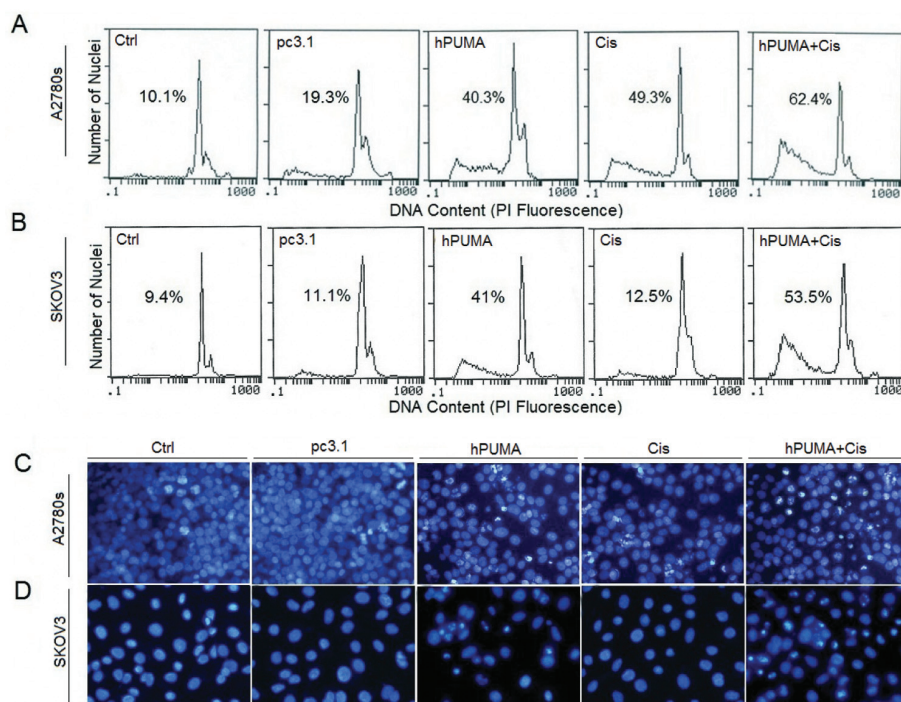
### Induction of Apoptosis of Ovarian Cancer Cells *In Vitro* by hPUMA and Cisplatin

Several lines of evidence have shown that PUMA can induce apoptosis of various cancer cells (14,20,24–26). We then tested whether induction of apoptosis is a major mechanism of PUMA-mediated chemosensitization. The quantitative assessment of sub-G1 cells by flow cytometry was used to estimate the number of apoptotic cells. As shown in Figure 3A, in the cisplatin-sensitive A2780s tumor model, the apoptotic cells accounted for 40.3% in the hPUMA-treated group versus 19.3% in pcDNA3.1-treated group and 10.1% in the control group. The apoptotic cells accounted for 62.4% in the combination treatment group versus 49.3% in cisplatin-treated group. These results suggested that either cisplatin or hPUMA alone significantly induced apoptosis of A2780s cells, and hPUMA plus cisplatin further augmented the induction of apoptosis. Similar results were obtained in an intrinsically resistant SKOV3 tumor model, except that cisplatin alone was found to have little ability to induce apoptosis of SKOV3 cells (Figure 3B).

Apoptosis was further evaluated by Hoechst 33258 staining. Similar to the above results obtained with flow cytometry analysis, in both A2780s and SKOV3 tumor models, the number of condensed nuclei (intact or fragmented), which are characteristic of apoptosis, in the combination treatment group were observed than that in hPUMA- or cisplatin-treated group cells. There was no significantly condensed nuclei in medium-only and pcDNA3.1-treated control groups. However, it should be noted that cisplatin-treated SKOV3 cells showed no similar apoptotic signs (Figures 3C, D).

### The Sensitizing Effects of PUMA Are Mediated by Release of Cytochrome c and Smac into the Cytosol

Previous studies have shown that PUMA causes apoptosis through Bax

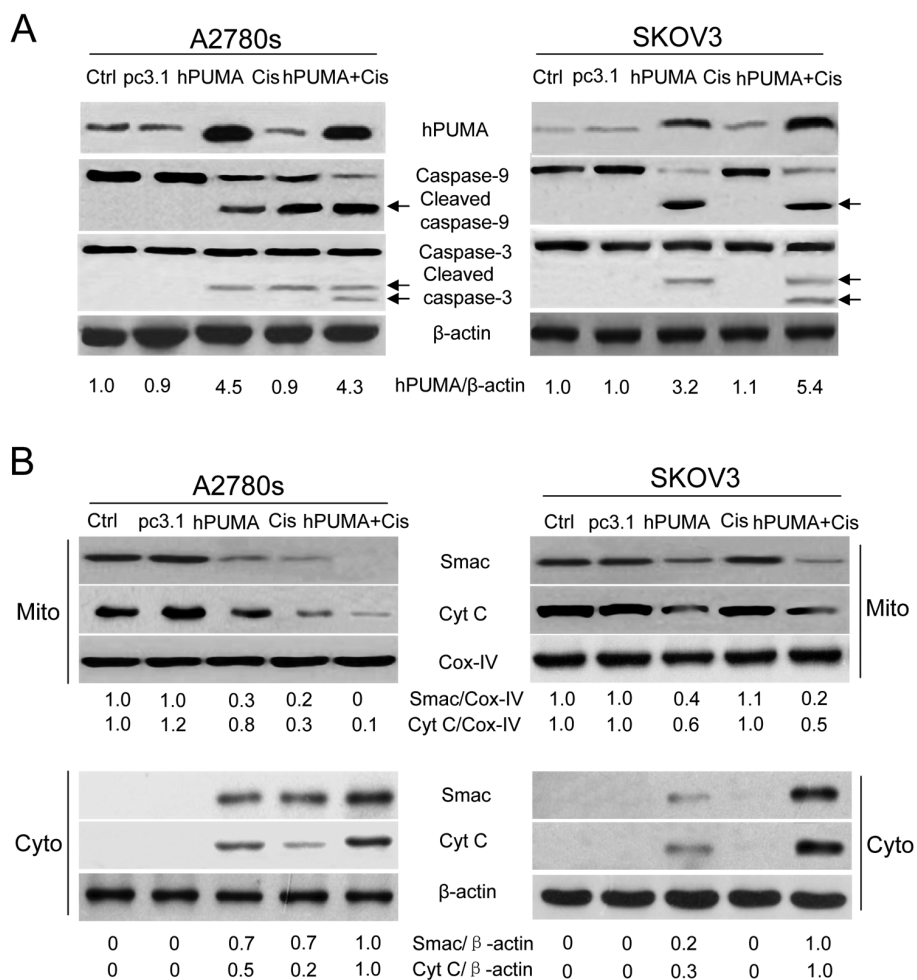


**Figure 3.** Induction of apoptosis of tumor cells *in vitro* by hPUMA and cisplatin. (A) Representative DNA fluorescence histograms of propidium iodide (PI)-stained cells. A2780s cells were treated with hPUMA for 24 h and then with 5  $\mu\text{g}/\text{mL}$  cisplatin for an additional 24 h. A2780s cells were untreated or treated with pcDNA3.1 or hPUMA, cisplatin alone or hPUMA plus cisplatin, and groups Ctrl, pc3.1, hPUMA, Cis (cisplatin) and hPUMA + Cis correspond to these five treatments (the same as shown in the subsequent panels), with 10.1% (Ctrl), 19.3% (pc3.1), 40.3% (hPUMA), 49.3% (Cis) and 62.4% (hPUMA + Cis) sub-G1 cells (apoptotic cells), respectively, as assessed by flow cytometry. (B) SKOV3 cells were treated with hPUMA for 24 h and then with 5  $\mu\text{g}/\text{mL}$  cisplatin for an additional 24 h. SKOV3 cells were untreated, treated with empty vector or hPUMA, cisplatin alone or hPUMA plus cisplatin, with 9.4% (Ctrl), 11.1% (pc3.1), 41% (hPUMA), 12.5% (Cis) and 53.5% (hPUMA + Cis) sub-G1 cells (apoptotic cells), respectively, as assessed by flow cytometry. (C) Normal and apoptotic nuclear morphology of A2780s cells was analyzed by Hoechst 33258 staining. A2780s cells were treated with the same conditions as described above. (D) Normal and apoptotic nuclear morphology of SKOV3 cells was analyzed by Hoechst 33258 staining. SKOV3 cells were treated with the same conditions as mentioned above.

and/or Bak by antagonizing the anti-apoptotic Bcl-2-like proteins to trigger mitochondrial dysfunction and caspase activation (18–20). We then tested whether mitochondrial dysfunction and caspase activation were involved in the induction of apoptosis by PUMA. As shown in Figure 4A, in the cisplatin-sensitive A2780s tumor model, either hPUMA or cisplatin alone resulted in significant activation of caspases 3 and 9, and their combination further enhanced activation of the caspases. Furthermore, the apoptosis was accompa-

nied by release of cytochrome c and Smac into the cytosol (Figure 4B). Similar results were also obtained in the intrinsically resistant SKOV3 tumor model, except that cisplatin alone was found not to cause activation of the caspases and release of cytochrome c and Smac (Figures 4A, B). These data indicate that PUMA can synergize with cisplatin to trigger cytochrome c and Smac release and caspase activation to initiate apoptosis in intrinsically cisplatin-sensitive and -resistant ovarian cancer cells.





**Figure 4.** The sensitizing effects of hPUMA are mediated by enhanced caspase activation and release of cytochrome c and Smac into the cytosol. (A) A2780s and SKOV3 cells were subjected to the indicated treatments as described in Materials and Methods. PUMA expression and caspase activation were analyzed by Western blotting. Arrows indicate active forms of caspases. β-Actin was used as a loading control. Densitometric analysis of the Western blots was performed and the amounts of hPUMA were compared with the protein loading control. Relative amount of hPUMA from the untreated cells was set as 1. (B) A2780s and SKOV3 cells were subjected to the indicated treatments as described in Materials and Methods. Release of cytochrome c and Smac into the cytosol were analyzed by Western blotting. Cox-IV blots indicate mitochondrial loading controls, whereas β-actin was used as a loading control for the cytosolic fraction. Densitometric analysis of the Western blots was performed, and the amounts of Smac and cytochrome c were compared with the protein loading control. Relative amount of cytosol Smac and cytochrome c from the cells treated with hPUMA plus Cis was set as 1, whereas a relative amount from untreated cells was set as 1 for mitochondria Smac and cytochrome c. Mito, mitochondria; Cyto, cytosol.

**PUMA Chemosensitizes SKOV3 Cells by Lowering the Threshold Set Simultaneously by Bcl-x<sub>L</sub> and Mcl-1**

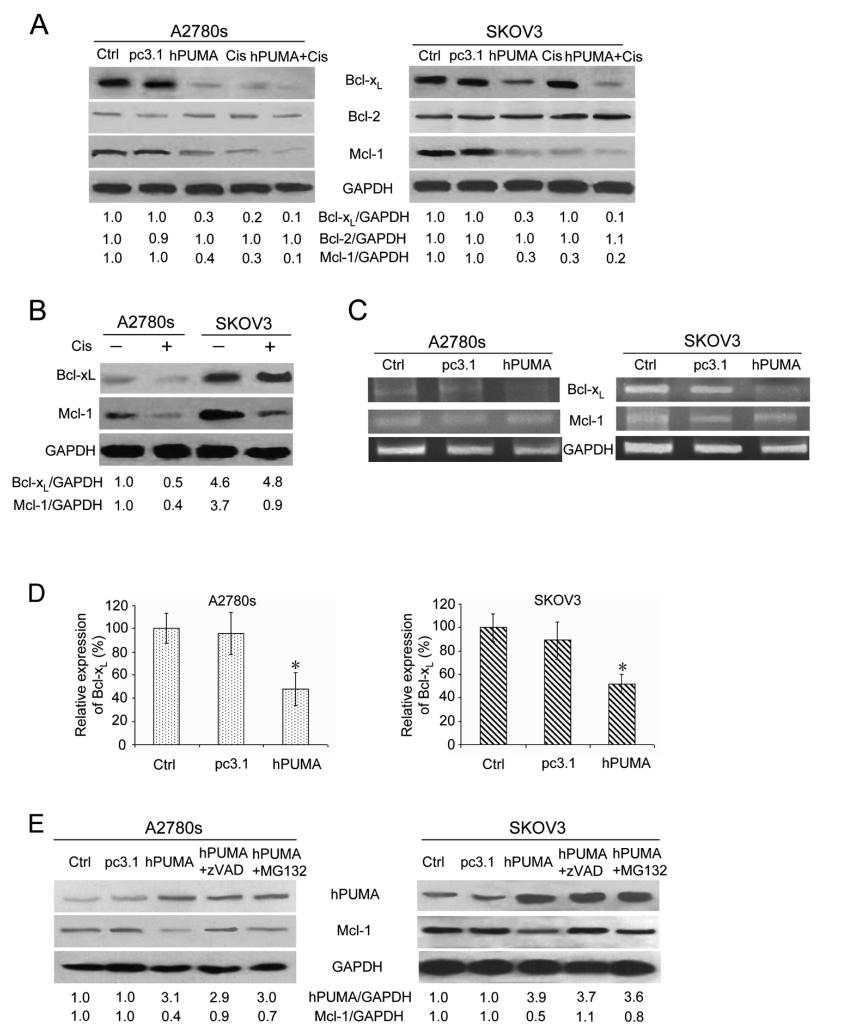
Considering that expression variation of chemosensitivity determinants may exist between the cisplatin-sensitive

A2780s cell line and intrinsically cisplatin-resistant SKOV3 cell line, we decided to investigate whether PUMA-induced apoptosis in SKOV3 cells was associated with expression changes of some important determinants of

chemosensitivity. Previous studies have shown that Bcl-2 and Bcl-x<sub>L</sub> proteins are frequently overexpressed (34,47–49) and appear to be involved in chemoresistance in ovarian carcinoma (34,47,49–51). Furthermore, chemoresistance of ovarian carcinoma has been shown to associate with the absence of Bcl-x<sub>L</sub> expression downregulation in response to cisplatin (32,34). More recently, it was reported that Bcl-x<sub>L</sub> and Mcl-1 are able to cooperate to protect ovarian carcinoma cells against oncogenic stress or chemotherapy-induced apoptosis (52). On the basis of these observations, we examined the expression of the three antiapoptotic molecules (that is, Bcl-2, Bcl-x<sub>L</sub> and Mcl-1) in both A2780s and SKOV3 cells after treatment, as described in Materials and Methods. As shown in Figure 5A, in the cisplatin-sensitive A2780s tumor model, either hPUMA or cisplatin alone resulted in downregulation of Bcl-x<sub>L</sub> and Mcl-1, and their combination further enhanced downregulation of the two molecules. In the cisplatin-resistant SKOV3 tumor model, cisplatin resulted in downregulation of Mcl-1, but not Bcl-x<sub>L</sub>, whereas hPUMA caused downregulation of Bcl-x<sub>L</sub> and Mcl-1, and hPUMA plus cisplatin further enhanced the downregulation of the two molecules. It is worth noting that, compared with the basal expression level, no modification of Bcl-2 was observed in both A2780s and SKOV3 tumor models.

We further examined the expression variation of Bcl-x<sub>L</sub> and Mcl-1 in chemosensitive- and chemoresistant-tumor models before and after treatment with cisplatin. As shown in Figure 5B, A2780s cells expressed low endogenous levels of Bcl-x<sub>L</sub> and Mcl-1, whereas SKOV3 cells expressed relatively high endogenous levels of the two molecules. When treated with cisplatin, both Bcl-x<sub>L</sub> and Mcl-1 were downregulated in A2780s cells, but in SKOV3 cells, only Mcl-1 was downregulated. These data indicate that the threshold set simultaneously by Bcl-x<sub>L</sub> and Mcl-1 is important for apoptosis of ovarian cancer cells.

In addition, we analyzed the transcript variation of *Bcl-x<sub>L</sub>* and *Mcl-1* in hPUMA-



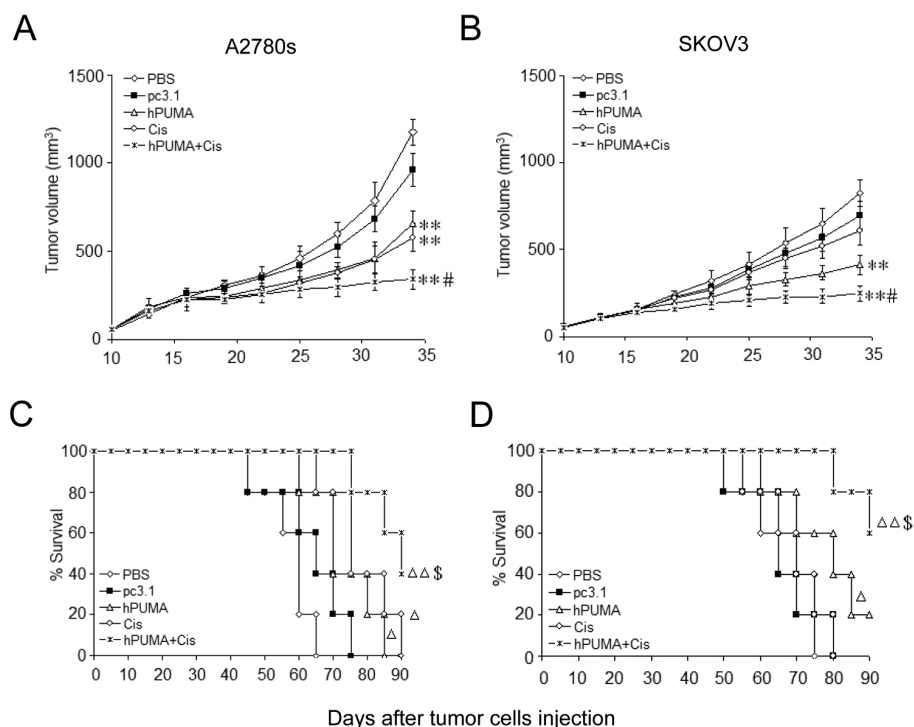
**Figure 5.** PUMA chemosensitizes ovarian cancer cells through downregulation of Bcl-x<sub>L</sub> and Mcl-1. (A) A2780s and SKOV3 cells were subjected to the indicated treatments as described in Materials and Methods. Expression changes of Bcl-x<sub>L</sub>, Bcl-2 and Mcl-1 were analyzed by Western blotting. GAPDH was used as a loading control. Individual protein levels were measured by densitometric analysis of the Western blots and compared with GAPDH levels. Relative amount of individual proteins from control group cells was set as 1. (B) A2780s and SKOV3 cells were treated with cisplatin (5 μg/mL) for 24 h and analyzed for the expression of Bcl-x<sub>L</sub> and Mcl-1 by Western blotting. GAPDH was used as a loading control. Bcl-x<sub>L</sub> and Mcl-1 levels were measured by densitometric analysis of the Western blots and compared with GAPDH levels. A relative amount of Bcl-x<sub>L</sub> and Mcl-1 from untreated A2780s cells was considered as 1. (C) Semiquantitative RT-PCR was done for Bcl-x<sub>L</sub>, Mcl-1 and the control GAPDH (25 cycles) using the cDNA prepared from untreated or pcDNA3.1/hPUMA-treated A2780s and SKOV3 cells. (D) Real-time RT-PCR was carried out with Bcl-x<sub>L</sub> and β-actin-specific primers to quantify Bcl-x<sub>L</sub> mRNA levels. Columns, ratio of Bcl-x<sub>L</sub> mRNA relative to β-actin in untreated or pcDNA3.1/hPUMA-treated A2780s and SKOV3 cells. Bars, SD of each sample measured in triplicate (\*P ≤ 0.05). (E) Both A2780s and SKOV3 cells were untreated or treated with pcDNA3.1/hPUMA in the presence or absence of zVAD-fmk (10 μmol/L) and MG132 (20 μmol/L). GAPDH was used as a loading control. hPUMA and Mcl-1 levels were measured by densitometric analysis of the Western blots and compared with GAPDH levels. Relative amount of hPUMA and Mcl-1 from untreated cells was considered as 1. zVAD-fmk almost completely blocked the disappearance of Mcl-1. The proteasome inhibitor MG132 also partially protected Mcl-1 from decay.

treated A2780s and SKOV3 cells. In both tumor models, Bcl-x<sub>L</sub> expression was strongly downregulated, whereas Mcl-1 expression remained unchanged (Figure 5C). Real-time PCR further verified that Bcl-x<sub>L</sub> mRNA expressions in both hPUMA-treated A2780s and SKOV3 cells were downregulated by 52.2% and 48.1%, respectively (Figure 5D). These results indicated that PUMA mediated Bcl-x<sub>L</sub> downregulation mainly at the transcription level, whereas Mcl-1 was downregulated possibly at the posttranscription level. Because Mcl-1 protein can be cleaved by caspases (53,54) and degraded by the proteasome (55,56), the levels of Mcl-1 in hPUMA-treated A2780s and SKOV3 cells were determined in the presence or absence of the pan-caspase inhibitor zVAD-fmk or proteasome inhibitor MG132. As shown in Figure 5E, the pan-caspase inhibitor zVAD-fmk almost completely blocked the disappearance of Mcl-1. The proteasome inhibitor MG132 also partially protected Mcl-1 from decay. These results indicate that both caspase-dependent and proteasome pathways are involved during apoptosis of ovarian cancer cells.

### Enhanced Antitumor Efficacy of the Combination of hPUMA and Low-dose Cisplatin

On the basis of the *in vitro* growth-inhibitory and proapoptotic effects of hPUMA and cisplatin, we further examined the combined antineoplastic effect of hPUMA and cisplatin on A2780s and SKOV3 tumors *in vivo*. As shown in Figures 6A and B, on d 34 after implantation, the A2780s (Figure 6A) and SKOV3 (Figure 6B) tumors of mice treated with PBS reached 1,174.28 ± 70.43 and 823.82 ± 73.27 mm<sup>3</sup> in volume, respectively. The A2780s and SKOV3 tumors treated with hPUMA were significantly (A2780s model, P < 0.001; SKOV3 model, P < 0.001) smaller than those treated with PBS, reaching only 654.56 ± 76.71 and 410.11 ± 53.17 mm<sup>3</sup> in volume, respectively. The combination of hPUMA and cisplatin further suppressed tumor growth such that the A2780s and SKOV3 tumors reached 340.24 ± 57.4 and





**Figure 6.** Combined effect of hPUMA and cisplatin on two xenograft models: tumor suppression and survival advantage in mice. A2780s cells (A, C) or SKOV3 cells (B, D) of  $2 \times 10^6$  were inoculated subcutaneously into female nude mice at 6–8 wks of age. Mice (five per group) were treated with PBS, pc3.1, hPUMA, Cis and hPUMA + Cis. In an A2780s tumor model, significant differences in tumor suppression (\*\* $P < 0.001$ ) and survival time ( $\Delta P < 0.05$ ) in mice treated with hPUMA or cisplatin versus PBS and pcDNA3.1 controls are shown. Also shown are the significant difference for tumors treated with hPUMA + cisplatin versus PBS and pcDNA3.1 controls (\*\* $P < 0.001$ ;  $\Delta\Delta P < 0.01$ ) and the significant difference for the combination therapy versus hPUMA or cisplatin monotherapy ( $\#P < 0.05$ ;  $\$P < 0.05$ ). Similar results were also found in the SKOV3 model, except that no significant differences in tumor suppression ( $P = 0.262$ ) and survival time ( $P = 0.634$ ) between cisplatin- and pcDNA3.1-treated tumors were found.

$248.41 \pm 46.59 \text{ mm}^3$  in volume, respectively. These levels were significantly (A2780s model,  $P < 0.001$ ; SKOV3 model,  $P < 0.001$ ) smaller than those of control tumors and significantly smaller than those of the tumors treated with hPUMA (A2780s model,  $P < 0.001$ ; SKOV3 model,  $P < 0.01$ ) or cisplatin (A2780s model,  $P < 0.01$ ; SKOV3 model,  $P < 0.001$ ). Cisplatin also resulted in a significant reduction in tumor volume ( $577.08 \pm 77.04 \text{ mm}^3$ ) compared with control tumors ( $P < 0.001$ ) in the A2780s model. However, in the SKOV3 model, no significant difference in tumor volume ( $605.44 \pm 80.51 \text{ mm}^3$ ) was observed in the cisplatin-treated group compared with the pcDNA3.1-treated con-

trol group ( $695.57 \pm 79.28 \text{ mm}^3$ ) ( $P = 0.262$ ).

Survival curve analysis (Figure 6C) showed that A2780s tumor-bearing mice in the PBS- or pcDNA3.1-treated groups survived <63 d on average. By contrast, either hPUMA or cisplatin resulted in a significant ( $P < 0.05$ ) increase in lifespan compared with the two control groups, with the mean survival time being 73 and 78 d, respectively. The combination of hPUMA and cisplatin further improved survival to a greater extent than the two control groups ( $P < 0.01$ ), with the mean survival time being 85 d. Except that there was no significant difference in survival time between cisplatin-

treated mice and PBS-treated mice ( $P = 0.750$ ) or pcDNA3.1-treated mice ( $P = 0.634$ ), similar results were also found in the SKOV3 tumor model (Figure 6D).

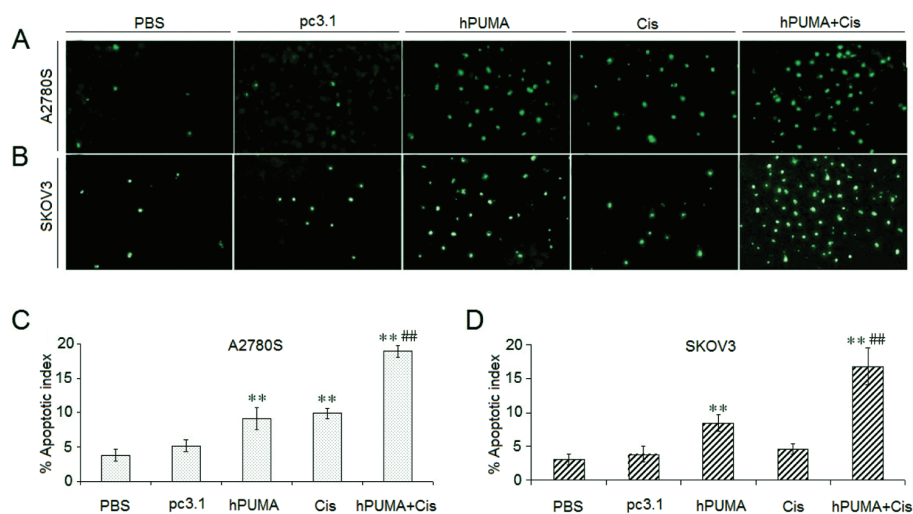
The mice treated with hPUMA, cisplatin or a combination of both have been investigated for potential side effects. No adverse consequences were indicated in gross measures such as weight loss, ruffling of fur, lifespan, behavior and feeding. Furthermore, no pathological changes in heart, liver, spleen, lung, kidney, etc., were found by microscopic examination (data not shown).

### Induction of Apoptosis in Tumor Tissues

To study the antitumor mechanism *in vivo*, the TUNEL assay was carried out to detect apoptosis of tumor cells. As shown in Figure 7A, in the A2780s tumor model, either hPUMA or cisplatin increased the apoptotic rate of tumor cells when compared with PBS or the empty vector. The combination therapy further increased the density of apoptotic cancer cells. The apoptotic index of tumors treated with hPUMA or cisplatin was significantly higher than that of tumors treated with PBS or the pcDNA3.1 empty vector ( $P < 0.001$ ). The apoptotic index for tumors treated with the combination of hPUMA and cisplatin was significantly higher than for tumors treated with the PBS or pcDNA3.1 control vector ( $P < 0.001$ ) and significantly higher than that of tumors treated with the hPUMA and cisplatin monotherapies ( $P < 0.001$ ). No significant differences in the apoptotic index of tumors treated with the two monotherapies were found in A2780s model ( $P = 0.823$ ). Similar results were also found in the SKOV3 tumor model, except that no significant differences in apoptotic index were observed between cisplatin-treated tumors and pcDNA3.1-treated tumors ( $P = 0.951$ ) or PBS-treated tumors ( $P = 0.524$ ) (Figures 7B, D).

### Detection of hPUMA Overexpression In Vivo

The observations that hPUMA chemosensitized A2780s and SKOV3



**Figure 7.** TUNEL staining of tumor tissues. Tumor tissue preparation and procedure for TUNEL staining is described in Materials and Methods. Representative sections were taken from A2780s (A) and SKOV3 (B) tumor tissues of mice receiving PBS, pc3.1, hPUMA, cisplatin (Cis) and hPUMA + Cis. The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each field. Apoptotic index within A2780s (C) and SKOV3 (D) tumor tissues was counted. In the A2780s model, statistically significant difference in the apoptotic index for tumors treated with hPUMA or cisplatin versus PBS and pcDNA3.1 controls (\*\* $P < 0.001$ ) is shown. Also shown are the significant difference for tumors treated with hPUMA + cisplatin versus the two controls (\*\* $P < 0.001$ ) and significant difference for the combination therapy versus hPUMA or cisplatin monotherapy (\*\* $P < 0.001$ ). Similar results were also found in the SKOV3 model, except that no significant differences in the apoptotic index between cisplatin-treated tumor and pcDNA3.1-treated tumor ( $P = 0.951$ ) or PBS-treated tumor ( $P = 0.524$ ) were found.

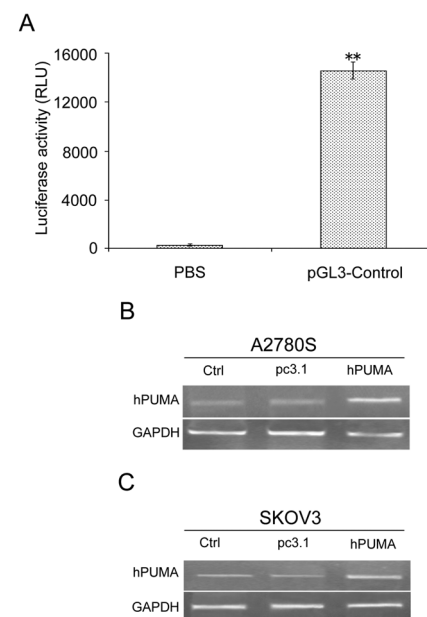
cells to cisplatin besides its antineoplastic effect *in vivo* raised a question whether the enhanced antitumor efficacy resulted from the delivery of hPUMA via tail vein injection. To address this issue, first, we tried to prove that liposome delivery of pGL3-control luciferase reporter plasmid via tail vein injection resulted in the expression of luciferase within the tumor tissues. We examined tumor tissue extracts for luciferase activity 48 h after the last injection. As shown in Figure 8A, the luciferase activity of A2780s tumors in the pGL3-control plasmid-treated group was significantly higher than that of the PBS-treated group ( $P < 0.001$ ), indicating the expression of luciferase within the tumor tissues.

Then, to further confirm whether the treatment using liposome delivery of hPUMA via tail vein injection actually gets to the tumor cells, RT-PCR was performed. As expected, *in vivo* overexpres-

sions of recombinant hPUMA were further verified by RT-PCR in A2780s (Figure 8B) and SKOV3 tumor tissues (Figure 8C). These results indicated directly and indirectly that intravenous injections of pcDNA3.1-hPUMA plasmid led to the expression of exogenous hPUMA within the tumor tissues.

## DISCUSSION

Ovarian cancer is the number one cause of death from gynecologic malignancy. Except for some improvement in survivorship with the introduction of platinum and paclitaxel therapy, the long-term survival remains poor because of eventual tumor recurrence and development of chemotherapy resistance. Resistance to chemotherapeutics has been thoroughly studied, and a number of mechanisms have been proposed. For instance, it is suggested that deregulated programmed cell death or apoptosis is a



**Figure 8.** Detection of hPUMA overexpression *in vivo*. (A) The A2780s tumor-bearing mice (five mice per group) were treated with PBS or liposome-pGL3-control luciferase reporter plasmid complex via the tail vein twice a week for 4 wks. Lysates of tumors were obtained, and luciferase values were read on a luminometer. Luciferase activities 48 h after the last injection of A2780s tumors treated with PBS or pGL3-control expression plasmid were shown in the graph. Statistically significant difference compared with the PBS treatment group (\*\* $P < 0.001$ ) is shown. (B) RT-PCR analysis of exogenous hPUMA expression in A2780s tumor tissues. (C) RT-PCR analysis of exogenous hPUMA expression in SKOV3 tumor tissues. Total RNA samples were isolated from A2780s and SKOV3 tumor tissues in the untreated group, pcDNA3.1 treatment group and pcDNA3.1-hPUMA treatment group, respectively.

major contributor to development of acquired resistance to anticancer therapies (7–9). Because chemotherapeutic drugs can induce apoptosis in tumor cells, enhancement of this process, by directly activating apoptosis or else lowering the threshold for its initiation by cytotoxic drugs, is an attractive strategy (57).

A defective p53 pathway is a hallmark of ovarian carcinoma (10). p53 mutation in ovarian cancer correlates significantly

with resistance to platinum-based chemotherapy and early relapse (11). A major physiological function of p53 is to induce apoptosis of damaged or stressed cells (12). p53 induces apoptosis by transactivating its downstream apoptotic regulators. Therefore, restoration of the p53 pathway by activating p53 itself or p53 downstream targets has been explored to improve efficacy of anticancer therapies (13).

PUMA was initially identified as a transcriptional target of p53 and a potent apoptosis inducer in various cancer cells (14–16). PUMA is localized in the mitochondria and induces apoptosis through the Bcl-2 family proteins Bax/Bak and the mitochondrial pathway (19,20). Previous studies have shown that elevated PUMA expression, either alone or in combination with chemotherapy or irradiation, induced profound toxicity to a variety of cancer cells such as lung, head and neck, esophagus and breast cancer cells (24–27). However, the role of PUMA in the therapeutic responses of ovarian cancer cells to platinum-based anticancer drugs remains unclear. The present study was designed to investigate whether PUMA could induce apoptosis of ovarian cancer cells, especially the intrinsically resistant, p53 double deletion mutant ovarian cancer cells, and whether PUMA could potentiate antineoplastic effects of cisplatin on ovarian cancer cells.

Several observations have been made in the current study concerning induction of apoptosis by PUMA in ovarian cancer cells *in vitro* and *in vivo*. Our data showed that p53 deletion mutation abolishes the induction of PUMA by cisplatin (Figure 1). Our data also showed that delivery of hPUMA into both A2780s (p53WT) and SKOV3 (p53<sup>-/-</sup>) ovarian cancer cells induced apoptosis independently of p53 and enhanced sensitivity to cisplatin, as evidenced by MTT assay (Figure 2), flow cytometry analysis (Figures 3A, B), Hoechst 33258 staining (Figures 3C, D), activation of caspases 3 and 9 (Figure 4A) and release of cytochrome c and Smac into the cytosol (Figure 4B). Furthermore, the *in*

*vitro* enhanced antiproliferative and proapoptotic activities of hPUMA plus cisplatin on ovarian cancer cells correlates well with the *in vivo* improved anti-tumor efficacy. The enhanced antitumor efficacy *in vivo* was associated with the enhanced induction of apoptosis, as verified by TUNEL analysis (Figure 7). These results suggest that adequate levels of PUMA are crucial for triggering apoptotic responses to cisplatin in ovarian cancer cells, especially the intrinsically resistant, p53 double deletion mutant ovarian cancer cells.

Previous studies have shown that cisplatin-induced apoptosis can be initiated through both intrinsic and extrinsic pathways. Cisplatin induces rapid dose-dependent release of cytochrome c from mitochondria to cytosol (58,59). Cytochrome c subsequently activates the caspase cascade, eventually leading to apoptotic cell death (60). In this work, it was observed that cisplatin induces apoptosis of chemosensitive A2780s cells, but not chemoresistant SKOV3 cells. Furthermore, cisplatin-induced apoptosis in A2780s cells is associated with activation of caspase 3 and 9 (Figure 4A), which is consistent with the notion that caspase 3 and 9 are critical for cisplatin-induced apoptosis, and their activation is attenuated in resistant cells (61–63).

We also found that cisplatin induces mitochondrial Smac release in chemosensitive A2780s cells, but not in chemoresistant SKOV3 cells, whereas PUMA induces mitochondrial Smac release and apoptosis in both A2780s and SKOV3 cells (Figure 4B), indicating that Smac release may be a key apoptosis mediator in chemoresistant SKOV3 cells. A recent report demonstrated that cisplatin-induced mitochondrial Smac release is a determinant of chemosensitivity in ovarian cancer cells (64). Therefore, we speculated that a link between PUMA-induced apoptosis and determinants of chemosensitivity may exist.

Previous studies have shown that expression of Bcl-x<sub>L</sub> in ovarian carcinoma is associated with chemoresistance and recurrent disease and that Bcl-x<sub>L</sub> downreg-

ulation in response to cisplatin is absent in chemoresistant ovarian carcinoma cells (32,34). Moreover, in ovarian carcinoma, Bcl-2 and Bcl-x<sub>L</sub> proteins are frequently overexpressed (34,47–49) and appear to be involved in chemoresistance (34,47,49–51). More recently, it was reported that Mcl-1 is an important determinant of the apoptotic response to the BH3-mimetic molecule HA14-1 in cisplatin-resistant ovarian carcinoma cells (52). Our data showed that hPUMA caused downregulation of Bcl-x<sub>L</sub> and Mcl-1 in both cisplatin-sensitive A2780s and cisplatin-resistant SKOV3 models and that cisplatin caused downregulation of Bcl-x<sub>L</sub> and Mcl-1 in the cisplatin-sensitive A2780s model. The combination of hPUMA and cisplatin further enhanced downregulation of the two molecules in the two tumor models, although cisplatin only caused downregulation of Mcl-1, but not Bcl-x<sub>L</sub>, in the cisplatin-resistant SKOV3 model (Figure 5A). Our data also showed that A2780s cells expressed relatively low endogenous levels of both Bcl-x<sub>L</sub> and Mcl-1, whereas SKOV3 cells expressed relatively high endogenous levels of the two molecules. Furthermore, cisplatin caused downregulation of both Bcl-x<sub>L</sub> and Mcl-1 in A2780s cells, but in SKOV3 cells, it only caused Mcl-1 downregulation (Figure 5B). These results indicate that the threshold set simultaneously by Bcl-x<sub>L</sub> and Mcl-1 is important for apoptosis of ovarian cancer cells, which is in agreement with a previous report that Bcl-x<sub>L</sub> and Mcl-1 cooperate to protect ovarian carcinoma cells against oncogenic stress or chemotherapy-induced apoptosis (52). Taken together, we speculated that lowering the apoptotic threshold set simultaneously by Bcl-x<sub>L</sub> and Mcl-1 may be one of the reasons for PUMA-mediated chemosensitivity of SKOV3 cells to cisplatin.

Additionally, it should be noticed that Bcl-2 expression remained unchanged in both hPUMA-treated A2780s and SKOV3 tumor models. More recently, a report showed that exogenous PUMA is phosphorylated on serine residues in HeLa and Bax/Bak double knockout mouse



embryonic fibroblast (DKO MEF) cells; however, the phosphorylation does not affect the interaction of PUMA with Bcl-2 (65). Therefore, we speculated that the phosphorylation of PUMA may exist in ovarian cancer cells transfected with hPUMA, and it does not affect association of PUMA with Bcl-2, eventually resulting in an unchanged expression level of Bcl-2. However, the mechanism by which Bcl-2 expression remained unchanged in hPUMA-treated ovarian cancer cells awaits further elucidation.

Furthermore, we found that both caspase-dependent cleavage and proteasome pathways are involved in PUMA-mediated Mcl-1 downregulation during apoptosis of ovarian cancer cells (Figure 5E), which is consistent with previous reports that Mcl-1 protein can be cleaved by caspases (53,54) and degraded by the proteasome (55,56). A recent report has shown that overexpression of PUMA in interleukin-3-dependent BaF3 cells results in caspase-mediated cleavage of Mcl-1 (66). This appears to be inconsistent with our observation. One possible explanation for this inconsistency is that degradation of Mcl-1 may vary among different cell types under different conditions. We also found that PUMA-mediated *Bcl-x<sub>L</sub>* downregulation mainly happened at the transcription level in ovarian cancer cells (Figures 5C, D), although previous reports have shown that *Bcl-x<sub>L</sub>* can be degraded by proteasome system and proteases such as calpain and lysosomal cysteine cathepsins (67–69). However, the mechanism by which *PUMA* regulates transcriptionally the expression of *Bcl-x<sub>L</sub>* remains to be elucidated.

In summary, our data suggest that overexpression of PUMA can cause apoptosis independently of p53 in both cisplatin-sensitive A2780s (p53<sup>WT</sup>) and cisplatin-resistant SKOV3 (p53<sup>-/-</sup>) ovarian cancer cells and that elevated expression of PUMA can enhance the therapeutic responses of ovarian cancer, especially the intrinsically resistant, p53 double deletion mutant ovarian cancer cells, to cisplatin by lowering the threshold set si-

multaneously by prosurvival *Bcl-x<sub>L</sub>* and Mcl-1. To our knowledge, we provide new evidence for the potential application of PUMA as a chemosensitizer in ovarian cancer therapy.

#### ACKNOWLEDGMENTS

The authors thank Dr. Bing Kan and Yong-qiu Mao for their technical support. This work was supported by the National Key Basic Research Program of China (2010CB529900) and Natural Science Foundation of China (30900744; 81071817/H1609).

#### DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

#### REFERENCES

- Jemal A, et al. (2006) Cancer statistics. *CA Cancer J. Clin.* 56:106–30.
- Banks E. (2000) The Epidemiology of Ovarian Cancer. In: *Ovarian Cancer: Methods and Protocols*. Bartlett JMS (ed.) Totowa (NJ): Humana Press, pp. 3–12.
- Malpas JS. (1979) Chemotherapy in the management of ovarian cancer: a review. *J. R. Soc. Med.* 72:357–61.
- Reed E. (1998) Platinum-DNA adduct, nucleotide excision repair and platinum based anti-cancer chemotherapy. *Cancer Treat. Rev.* 24:331–44.
- Cohen SM, Lippard SJ. (2001) Cisplatin: from DNA damage to cancer chemotherapy. *Prog. Nucleic Acid Res. Mol. Biol.* 67:93–130.
- Tanabe M, et al. (2003) Activating transcription factor 4 increases the cisplatin resistance of human cancer cell lines. *Cancer Res.* 63:8592–5.
- Johnstone RW, Ruefli AA, Lowe SW. (2002) Apoptosis: a link between cancer genetics and chemotherapy. *Cell.* 108:153–64.
- Yu J, Zhang L. (2004) Apoptosis in human cancer cells. *Curr. Opin. Oncol.* 16:19–24.
- Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. *Cell.* 100:57–70.
- Milner BJ, et al. (1993) p53 mutation is a common genetic event in ovarian carcinoma. *Cancer Res.* 53:2128–32.
- Reles A, et al. (2001) Correlation of p53 mutations with resistance to platinum-based chemotherapy and shortened survival in ovarian cancer. *Clin. Cancer Res.* 7:2984–97.
- Vogelstein B, Lane D, Levine AJ. (2000) Surfing the p53 network. *Nature.* 408:307–10.
- Lane DP, Lain S. (2002) Therapeutic exploitation of the p53 pathway. *Trends Mol. Med.* 8:S38–42.
- Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol. Cell.* 7:673–82.
- Nakano K, Vousden KH. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 7:683–94.
- Han J, et al. (2001) Expression of *bbc3*, a proapoptotic BH3-only gene, is regulated by diverse cell death and survival signals. *Proc. Natl. Acad. Sci. U. S. A.* 98:11318–23.
- Labi V, Erlacher M, Kiessling S, Villunger A. (2006) BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. *Cell Death Differ.* 13:1325–38.
- Yu J, Zhang L. (2008) PUMA, a potent killer with or without p53. *Oncogene.* 27 Suppl 1:S71–83.
- Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. (2003) PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 100:1931–6.
- Ming L, Wang P, Bank A, Yu J, Zhang L. (2006) PUMA dissociates Bax and BCL-XL to induce apoptosis in colon cancer cells. *J. Biol. Chem.* 281:16034–42.
- Erlacher M, et al. (2006) Puma cooperates with Bim, the rate-limiting BH3-only protein in cell death during lymphocyte development, in apoptosis induction. *J. Exp. Med.* 203:2939–51.
- Nelson DA, et al. (2004) Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. *Genes Dev.* 18:2095–107.
- Michalak EM, et al. (2009) Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. *Cell Death Differ.* 16:684–96.
- Yu J, Yue W, Wu B, Zhang L. (2006) PUMA sensitizes lung cancer cells to chemotherapeutic agents and irradiation. *Clin. Cancer Res.* 12:2928–36.
- Sun Q, Sakaida T, Yue W, Gollin SM, Yu J. (2007) Chemosensitization of head and neck cancer cells by PUMA. *Mol. Cancer Ther.* 6:3180–8.
- Wang H, et al. (2006) Administration of PUMA adenovirus increases the sensitivity of esophageal cancer cells to anticancer drugs. *Cancer Biol. Ther.* 5:380–5.
- Wang R, et al. (2009) Tumor-specific adenovirus-mediated PUMA gene transfer using the survivin promoter enhances radiosensitivity of breast cancer cells in vitro and in vivo. *Breast Cancer Res. Treat.* 117:45–54.
- Al-Bahlani S, et al. (2011) P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. *Oncogene.* Apr 25. [Epub ahead of print]
- Liu YY, et al. (2011) Suppression of glucosylceramide synthase restores p53-dependent apoptosis in mutant p53 cancer cells. *Cancer Res.* 71:2276–85.
- Yang G, et al. (2010) CXCR2 promotes ovarian cancer growth through dysregulated cell cycle, diminished apoptosis, and enhanced angiogenesis. *Clin. Cancer Res.* 16:3875–86.
- Gallardo D, Drazan KE, McBride WH. (1996) Adenovirus-based transfer of wild-type p53 gene

- increases ovarian tumor radiosensitivity. *Cancer Res.* 56:4891–3.
32. Villedieu M, et al. (2007) Absence of Bcl-xL down-regulation in response to cisplatin is associated with chemoresistance in ovarian carcinoma cells. *Gynecol. Oncol.* 105:31–44.
  33. Sasaki H, Sheng YL, Kotsuji F, Tsang BK. (2000) Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. *Cancer Res.* 60:5659–66.
  34. Williams J, et al. (2005) Expression of Bcl-xL in ovarian carcinoma is associated with chemoresistance and recurrent disease. *Gynecol. Oncol.* 96:287–95.
  35. Su JM, et al. (2003) Active immunogene therapy of cancer with vaccine on the basis of chicken homologous matrix metalloproteinase-2. *Cancer Res.* 63:600–7.
  36. Yuan Z, et al. (2009) Improved therapeutic efficacy against murine carcinoma by combining honokiol with gene therapy of PNAS-4, a novel pro-apoptotic gene. *Cancer Sci.* 100:1757–66.
  37. Blezinger P, et al. (1999) Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene. *Nat. Biotechnol.* 17:343–8.
  38. Wei YQ, et al. (1994) Induction of apoptosis by quercetin: involvement of heat shock protein. *Cancer Res.* 54:4952–7.
  39. Livak K, Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔCt</sup> method. *Methods.* 25:402–8.
  40. Ruiz-Vela A, Opferman JT, Cheng EH, Korsmeyer SJ. (2005) Proapoptotic BAX and BAK control multiple initiator caspases. *EMBO Rep.* 6:379–85.
  41. Leu JJ, Dumont P, Hafey M, Murphy ME, George DL. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat. Cell Biol.* 6:443–50.
  42. Zhang R, et al. (2006) Combination of MIG (CXCL9) chemokine gene therapy with low-dose cisplatin improves therapeutic efficacy against murine carcinoma. *Gene Ther.* 13:1263–71.
  43. Lin X, et al. (2007) Efficient inhibition of intraperitoneal human ovarian cancer growth and prolonged survival by gene transfer of vesicular stomatitis virus matrix protein in nude mice. *Gynecol. Oncol.* 104:540–6.
  44. Kaplanand EL, Meier P. (1958) Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* 53:457–81.
  45. Peto R, Peto J. (1972) Asymptotically efficient rank invariant test procedures. *J. Roy. Stat. Soc. Ser. A. Gene.* 135:185–206
  46. Jeffers JR, et al. (2003) Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell.* 4:321–8.
  47. Liu JR, et al. (1998) Bcl-xL is expressed in ovarian carcinoma and modulates chemotherapy-induced apoptosis. *Gynecol. Oncol.* 70:398–403.
  48. Marone M, et al. (1998) bcl-2, bax, bcl-XL, and bcl-XS expression in normal and neoplastic ovarian tissues. *Clin. Cancer Res.* 4:517–24.
  49. Kassim SK, et al. (1999) Increased bcl-2 expression is associated with primary resistance to chemotherapy in human epithelial ovarian cancer. *Clin. Biochem.* 32:333–8.
  50. Dodier P, Piché A. (2006) Bcl-X(L) is functionally non-equivalent for the regulation of growth and survival in human ovarian cancer cells. *Gynecol. Oncol.* 100:254–63.
  51. Beale PJ, Rogers P, Boxall F, Sharp SY, Kelland LR. (2000) BCL-2 family protein expression and platinum drug resistance in ovarian carcinoma. *Br. J. Cancer.* 82:436–40.
  52. Simonin K, et al. (2009) Mcl-1 is an important determinant of the apoptotic response to the BH3-mimetic molecule HA14-1 in cisplatin-resistant ovarian carcinoma cells. *Mol. Cancer Ther.* 8:3162–70.
  53. Herrant M, et al. (2004) Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. *Oncogene.* 23:7863–73.
  54. Weng C, Li Y, Xu D, Shi Y, Tang H. (2005) Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J. Biol. Chem.* 280:10491–500.
  55. Maurer U, et al. (2006) Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol. Cell.* 21:749–60.
  56. Adams KW, Cooper GM. (2007) Rapid turnover of Mcl-1 couples translation to cell survival and apoptosis. *J. Biol. Chem.* 282:6192–200.
  57. Agarwal R, Linch M, Kaye SB. (2006) Novel therapeutic agents in ovarian cancer. *Eur. J. Surg. Oncol.* 32:875–86.
  58. Kojima H, et al. (1998) Abrogation of mitochondrial cytochrome c release and caspase-3 activation in acquired multidrug resistance. *J. Biol. Chem.* 273:16647–50.
  59. Yang Z, et al. (2006) Cisplatin preferentially binds mitochondrial DNA and voltage-dependent anion channel protein in the mitochondrial membrane of head and neck squamous cell carcinoma: possible role in apoptosis. *Clin. Cancer Res.* 12:5817–25.
  60. Li P, et al. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 91:479–89.
  61. Asselin E, Mills GB, Tsang BK. (2001) XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res.* 61:1862–8.
  62. Henkels KM, Turchi JJ. (1999) Cisplatin-induced apoptosis proceeds by caspase-3-dependent and -independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. *Cancer Res.* 59:3077–83.
  63. Blanc C, et al. (2000) Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. *Cancer Res.* 60:4386–90.
  64. Yang X, et al. (2006) Akt-mediated cisplatin resistance in ovarian cancer: modulation of p53 action on caspase-dependent mitochondrial death pathway. *Cancer Res.* 66:3126–36.
  65. Fricker M, O'Prey J, Tolkovsky AM, Ryan KM. (2010) Phosphorylation of Puma modulates its apoptotic function by regulating protein stability. *Cell Death Dis.* 1:e59.
  66. Callus BA, et al. (2008) Triggering of apoptosis by puma is determined by the threshold set by pro-survival Bcl-2 family proteins. *J. Mol. Biol.* 384:313–23.
  67. Corina L, Pilar J, Ana B, Jesús E, Alberto O. (2005) Role of Bcl-xL in paracetamol-induced tubular epithelial cell death. *Kidney Int.* 67:592–601.
  68. Ji LL, Chen Y, Liu TY, Wang ZT. (2008) Involvement of Bcl-xL degradation and mitochondrial-mediated apoptotic pathway in pyrrolizidine alkaloids-induced apoptosis in hepatocytes. *Toxicol. Appl. Pharmacol.* 231:393–400.
  69. Droga-Mazovec G, et al. (2008) Cysteine cathepsins trigger caspase-dependent cell death through cleavage of Bid and antiapoptotic Bcl-2 homologues. *J. Biol. Chem.* 283:19140–50.