Cancer Chemother Pharmacol (2009) 65:13–25
DOI 10.1007/s00280-009-0998-5

ORIGINAL ARTICLE

PNAS-4, a novel pro-apoptotic gene, can potentiate antineoplastic effects of cisplatin

Zhu Yuan · Fei Yan · Yong-sheng Wang · Huan-yi Liu · Lan-tu Gou · Xin-yu Zhao · Song-tao Lai · Hong-xin Deng · Jiong Li · Zhen-yu Ding · Shao-qun Xiong · Bing Kan · Yong-qiu Mao · Li-juan Chen · Yu-quan Wei · Xia Zhao

Received: 2 December 2008 / Accepted: 3 April 2009 / Published online: 22 April 2009
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Abstract
Purpose PNAS-4, a novel pro-apoptotic gene activated during the early response to DNA damage, can inhibit proliferation via apoptosis when overexpressed in some tumor cells. The objectives of this study were to determine whether *PNAS-4* could enhance apoptosis induced by cisplatin besides its induction of apoptosis, and to evaluate the usefulness of combined treatment with mouse *PNAS-4* (*mPNAS-4*) gene therapy and low-dose cisplatin chemotherapy in the inhibition of tumor growth in colon carcinoma (CT26) and Lewis lung carcinoma (LL/2) murine models.
Methods In this study, the in vitro growth-inhibitory and pro-apoptotic effects of *PNAS-4* and/or cisplatin on CT26, LL/2, and SKOV3 cancer cells were assessed by MTT assay, flow cytometric analysis, DNA fragmentation, and morphological analysis, respectively. The in vivo antitumor activity of combined treatment with *mPNAS-4* gene therapy and low-dose cisplatin were evaluated in the inhibition of tumor growth in colon carcinoma (CT26) and Lewis lung carcinoma (LL/2) murine models. Tumor volume and survival time were observed. Induction of apoptosis was also assessed in tumor tissues.
Results In vitro, *PNAS-4* inhibited proliferation of colon carcinoma (CT26), Lewis lung carcinoma (LL/2) and human ovarian cancer (SKOV3) cell lines via apoptosis, and significantly enhanced the apoptosis of CT26, LL/2, and SKOV3 cells induced by cisplatin. In vivo systemic administration of expression plasmid encoding *mPNAS*-4 (pcDNA3.1-mPS) and cisplatin, significantly decreased tumor growth through increased tumor cell apoptosis compared to treatment with mPNAS-4 or cisplatin alone.
Conclusions Our data suggests that the combined treatment with mPNAS-4 plus cisplatin may augment the induction of apoptosis in tumor cells in vitro and in vivo, and that the augmented antitumor activity in vivo may result from the increased induction of apoptosis. The present study may provide a novel way to augment the antitumor efficacy of cytotoxic chemotherapy.

Keywords PNAS-4 · Apoptosis · Gene therapy · Cisplatin · Chemotherapy

Abbreviations

HLE B-3	Human lens epithelial cells
pcDNA3.1-mPS	pcDNA3.1 Expression plasmid encoding mouse PNAS-4 gene
pcDNA3.1-hPS	pcDNA3.1 Expression plasmid encoding human PNAS-4 gene
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling

Z. Yuan and F. Yan contributed equally to this work.

Z. Yuan · F. Yan · Y. Wang · L. Gou · X. Zhao · S. Lai · H. Deng (✉) · J. Li · Z. Ding · S. Xiong · B. Kan · Y. Mao · L. Chen · Y. Wei
State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, 1# Keyuan Road 4, Gaopeng Street, High Technological Development Zone, 610041 Chengdu, China
e-mail: denghongx@yahoo.com.cn

X. Zhao (✉)
Department of Gynecology and Obstetrics, West China Second Hospital, West China Medical School, Sichuan University, 610041 Chengdu, China
e-mail: xia-zhao@126.com

H. Liu
Cancer Center, Chengdu Military General Hospital, 610083 Chengdu, China

Introduction

The traditional troika of cancer therapies is comprised of chemotherapy, surgical intervention, and radiation. Cytotoxic chemotherapy has been the mainstay of medical approaches to treat a wide variety of solid tumors. Cisplatin, which inhibits cell proliferation and induces cell-cycle arrest by forming inter- and intrastrand DNA cross-links [1], is one of the most widely used chemotherapeutic agents in the treatment of cancer, and has been proved to be effective against many tumors, such as cervical, lung, bladder, and prostate cancer [2–4]. However, the efficacy of cisplatin based on the treatment is limited in curing most tumors due to dose-dependent toxicity and development of cisplatin resistance [5–7]. To overcome the two major hurdles for successful use of cisplatin, in the oncology community, investigations have increasingly focused on the search for new therapeutic strategies, such as modulation of cellular chemosensitivity, reversing tumor resistance, and increasing therapeutic effects of chemotherapy [8, 9].

PNAS-4 was previously identified as a novel apoptosis-related protein in human acute promyelocytic leukemia cell line NB4. Recent studies show that *PNAS-4* is up-regulated in human papillomavirus-infected invasive cervical cancer [10], in primary prostate cancer after androgen ablation therapy [11], in human papillomavirus 16 E6-expressing U2OS cells (U2OSE64b) following mitomycin C treatment [12], and in peripheral blood mononuclear cells exposed to carcinogenic agent, such as benzene [13]. In addition, another report show that the transcript level of *PNAS-4* is greatly upregulated in glucocorticoid-treated HLE B-3 cells, suggesting that *PNAS-4* may be involved in perturbation of lens epithelial cell proliferation and differentiation [14].

Recently, *hPNAS-4* was identified by large-scale bioinformatic analysis and sequencing as a novel pro-apoptotic gene activated during the early response to DNA damage, and when overexpressed in osteosarcoma U2OS cells, it could induce significant apoptosis [15]. Similarly, a recent report show that porcine *PNAS-4* is widespread across many tissues and organs in pigs, with the highest levels in muscle tissue, and its expression tends to decrease gradually during the period for development of muscle fibers and determination of muscle fiber numbers, i.e., the muscle fiber number appears to increase as a result of the decrease of *PNAS-4* mRNA expression level during prenatal muscle development, suggesting that *PNAS-4* may be involved in apoptosis [16].

Some original investigations on the functions of PNAS-4 have been carried out in our laboratory. By using zebrafish as animal model, our group observed that overexpression of PNAS-4 led to the elongation of the antero-posterior (AP) body axis, while knocking down PNAS-4 caused gastrulation defects, indicating that *PNAS-4* may regulate convergence and extension during vertebrate gastrulation [17]. By searching mouse database with full-length amino acid sequence of hPNAS-4 (GenBank accession no. CAH70880), we found a mouse PNAS-4 (mPNAS-4) homolog and named it as mPNAS-4 (GenBank accession no. NM_024282). The mPNAS-4 cDNA sequence contains an ORF of 585 bp encoding a 194-amino-acid protein containing a putative DUF862 conserved domain and three hydrophobic motifs, with a calculated molecular weight of 21,434 Da. The amino-acid sequence identity between mPNAS-4 and hPNAS-4 was 96.9%. By transfecting EGFPN1-mPNAS-4 expression plasmid or EGFPN1 control plasmid into LL/2 cells, we found that the percentage of apoptotic cells in the LL/2 cells transfected with EGFPN1-mPNAS-4 expression plasmid was significantly higher than that of the LL/2 cells transfected with EGFPN1 control plasmid, suggesting that overexpression of mPNAS-4 in some tumor cells could induce significant apoptosis [18], which is similar to a previous report by Filippov et al. [15]. Furthermore, we found that overexpression of hPNAS-4 could efficiently inhibit growth of SKOV3 ovarian cancer cell line and prolonged survival in the ovarian cancer mouse model [19]. In addition, the polyclonal antibodies against mPNAS-4 protein serving as an effective tool to explore the function of mPNAS-4 have been produced in our laboratory [18].

On the basis of the observations that PNAS-4 can cause a significant increase in cell death via apoptosis when overexpressed in some tumor cells, indicating that PNAS-4 may be involved in the apoptotic response to DNA damage, and the known result that the cytotoxic action of cisplatin against tumor cells is mediated by DNA damage, we decided to investigate whether *PNAS-4* could augment the apoptosis of tumor cells induced by cisplatin in vitro and in vivo. The present study demonstrate that in vitro *PNAS-4* can induce the tumor cells to apoptosis and also enhance the apoptosis of tumor cells induced by cisplatin. The results also show that in vivo when gene therapy of mPNAS-4 is combined with low-dose cisplatin, a significant reduction in mouse xenograft tumor volumes and a remarkable increase of life span are observed without an apparent increase in toxicity. Taken together, our results may provide a novel way to enhance the antitumor efficacy of chemotherapeutic drugs.

Materials and methods

Plasmid construction

Cultured LL/2 murine Lewis lung carcinoma cells were harvested and total RNA was isolated using Trizol reagent

(Invitrogen) according to manufacturer's protocol. Based on the cDNA sequence of mPNAS-4, the RNA sample was then subjected to RT-PCR for amplification of the encoding region of mPNAS-4, using a One Step RNA PCR Kit (AMV) (TaKaRa) with upstream primer 5′-*GC***GGATCC** GCCACC ATGGCCAACCAGCCCATCATC-3′ and downstream primer 5′-*CCG***CTCGAG**CTATAGTTTTGTGTGGCGC CCAGG-3′. The incorporated 5′BamHI and 3′XhoI restriction sites are shown in bold while protective base in italics. The amplified cDNA fragment (608 bp) was then cloned into the expression plasmid pcDNA3.1 (Invitrogen). The resulting recombinant plasmid was named as pcDNA3.1-mPS. In addition, pcDNA3.1-hPS plasmid was constructed as previously described [19]. pcDNA3.1 plasmid was used as a control. Both pcDNA3.1-mPS and pcDNA3.1 were purified by two rounds of passage over EndoFree columns (Qiagen, Chatsworth, CA), as reported previously [20]. The purified plasmids, which were solubilized in endotoxin-free normal saline solution, were mixed with liposome to form DNA–liposome complex before they were used in animal experiments.

Cell culture, transfection

LL/2, Colon carcinoma (CT26), and human ovarian cancer SKOV3 cells were purchased from the American Type Culture Collection (Manassas, VA) and were grown in DMEM (GIBCO) and RPMI 1640 (GIBCO) containing 10% fetal bovine serum, respectively, at 37°C in a humidified atmosphere containing 5% CO_2. Transfection was performed with Lipofectamine^{TM} 2000 according to the manufacturer's instruction. Briefly, aliquots of $2 \times 10^5/2 \times 10^3$ cells were grown in each well of 6/96-well plates in triplicate and incubated overnight to 70% confluence. DNA (pcDNA3.1, pcDNA3.1-mPS, or pcDNA3.1-hPS, 2 μg/ml)/lipofectamine^{TM} 2000 (5 μl/ml) were complexed in DMEM/RPMI 1640 medium, and left at room temperature for 30 min. LL/2, CT26, and SKOV3 cells were incubated for 6 h with the above complexes, followed by rinsing 3 times, and then 1.5 ml/100 μl of DMEM/RPMI 1640 supplemented with fetal calf serum were added to each well of 6/96-well plates and incubated for further 48 h.

Treatments of cells in the in vitro experiments

LL/2 and CT26 cells were classified into the following five groups, and the treatments of the cells were 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, then transfected with pcDNA3.1 plasmid. About 48 h after transfection, cells were harvested for subsequent experiments. Group 3, cisplatin alone, when the cells were cultured for 48 h, cisplatin was added at a concentration of 5 μg/ml. About 24 h later, cells were harvested for subsequent experiments. Group 4, mPNAS-4 alone, the cells were first incubated for 24 h, then transfected with pcDNA3.1-mPS plasmid. About 48 h after transfection, cells were harvested for subsequent experiments. Group 5, mPNAS-4 plus cisplatin (combination), the cells were first incubated for 24 h, then transfected with pcDNA3.1-mPS plasmid. About 24 h posttransfection, cisplatin was added at a concentration of 5 μg/ml. About 48 h after transfection, cells were harvested for subsequent experiments.

The harvested cells above were used for the following in vitro experiments including MTT assay, flow cytometric analysis, agarose gel DNA electrophoresis, and morphological analysis. In the experiments of morphological analysis, different group cells were treated as described above. However, in order to identify transfected cells from non-transfected cells upon green fluorescence, the cells were cotransfected with pcDNA3.1 or pcDNA3.1-mPS and pEGFP-N1 plasmids. The concentration of pEGFP-N1 vector was one-fifth of that of the pcDNA3.1 or pcDNA3.1-mPS vector.

In addition, SKOV3 cells were also classified into five groups, i.e., control, pcDNA3.1, cisplatin alone, hPNAS-4 alone, and hPNAS-4 plus cisplatin (combination). The treatments of different group cells were the same as those described above, and the SKOV3 cells after treatments were used for the in vitro experiments including MTT assay and flow cytometric analysis.

Detection of mPNAS-4 expression

Overexpression of mPNAS-4 in vitro was confirmed in the transfected cells by RT-PCR and Western blot analysis while, in vivo, it was confirmed by RT-PCR. On one hand, for detection of mPNAS-4 expression in vitro, LL/2 cells were treated according to the schedules as described above. After the cells were harvested, one part of it was used to isolate RNA and then subjected to RT-PCR for amplification of the encoding region of mPNAS-4, and other samples were used for the detection of protein expression by Western blot analysis [21]. Western blot analysis was performed with total protein from cell homogenates. Briefly, the total protein was separated on 12% SDS-PAGE which were electroblotted with Sartoblot onto a PVDF membrane. The membrane blots were blocked at 4°C in 5% non-fat dry milk, washed, and incubated with anti-mPNAS-4 polyclonal antibodies isolated from rabbit serum by our group [18], and probed with goat anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:2000, v/v), followed by transfer to Vectastain ABC (Vector Laboratories) [22]. On the other hand, when mice were sacrificed at the end of the experiment, the tumor tissues were collected for detection

of mPNAS-4 expression in vivo by RT-PCR. The primers used for RT-PCR amplification of mPNAS-4 (608 bp) were the same as those described in "Plasmid construction", while the primers used for RT-PCR amplification of GAPDH (187 bp) were designed according to the method reported previously [23].

MTT assay for cell growth and proliferation after treatment with PNAS-4 and cisplatin

LL/2, CT26, and SKOV3 cells were treated according to the schedules as described above. Survival of cells after treatments was quantified using MTT assay [24]. Data represent the average of six wells, and the experiment was repeated 3 times.

Flow cytometric analysis

Flow cytometric analysis was performed to identify sub-G1 cells/apoptotic cells and to measure the percentage of sub-G1 cells after PI staining in hypotonic buffer as described previously [25]. Briefly, cells were suspended in 1 ml hypotonic fluorochrome solution containing 50 μg 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). Apoptotic cells appeared in the cell-cycle distribution as cells with a DNA content less than that of G1 cells and were estimated with Listmode software.

Agarose gel DNA electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described previously [25]. Briefly, cells (3×10^6) were lysed with 0.5 ml lysis buffer containing 5 mM Tris/HCL (pH 8.0), 0.25% Nonidet P-40, and 1 mM EDTA, followed by the addition of RNase A (Sigma) at a final concentration of 200 μg/ml, and incubated for 1 h at 37°C. Cells were then treated with 300 μg proteinase K/ml for an additional hour at 37°C. After addition of 4 μl loading buffer, 15 μl samples in each lane were subjected to electrophoresis on 1.5% agarose at 50 V for 3 h. DNA was stained with ethidium bromide.

Morphological analysis

LL/2 cells were treated according to the schedules above. According to the methods as reported previously [26, 27], the pcDNA3.1-mPS or pcDNA3.1 expression vector was cotransfected with EGFP expression vector, pEGFP-N1 (Clontech Laboratories), into LL/2 cells. The treated cells were cultured for an indicated period of time and then used for apoptotic morphology analysis. The apoptotic morphology of tumor cells was determined at 48 h posttransfection and the percentage of apoptotic cells was determined by the number of green cells with apoptotic morphology divided by the total number of green cells. The images of EGFP expression were captured by a ZEISS fluorescence microscope (Jena, Inc.).

Animal tumor models and treatment

The following studies were approved by the Institute's Animal Care and Use Committee. CT26 colon carcinoma or LL/2 Lewis lung carcinoma cells (2×10^5 cells) were injected into the right flank of BALB/c and C57BL/6 mice, respectively, 6–8 weeks of age. To explore the therapeutic efficacy of mPNAS-4 plus cisplatin, we treated the mice on day 10 after the implantation of tumor cells, when the tumors were 5–8 mm in diameter. The mice were randomly divided into the following 5 groups (10 mice per group). Group 1, combination of mPNAS-4 and low-dose cisplatin, mice received pcDNA3.1-mPS plasmid–liposome complex plus low-dose cisplatin as follows: day 0 (10 days after tumor cell injection), mice were treated with pcDNA3.1-mPS plasmid by intravenous (i.v.) administration via the tail vein at a dose of 100 μg/mouse twice a week for 4 weeks. At day 4 (14 days after tumor cell injection), 5 mg/kg of cisplatin was injected by intraperitoneal (i.p.) route at an interval of every 7 days for a total of 4 doses. Group 2, mPNAS-4 alone, mice received pcDNA3.1-mPS plasmid in a scheme as that in group 1, but not cisplatin. Group 3, low-dose cisplatin alone, mice received the same dose of cisplatin in a scheme as that in group 1, but not pcDNA3.1-mPS plasmid. Group 4, pcDNA3.1 (empty vector) alone, mice received pcDNA3.1 plasmid in a scheme as that in group 1. Group 5, untreated group, mice received sterile PBS i.v. as the scheme of pcDNA3.1-mPS plasmid in the group 1 and i.p. as the scheme of cisplatin in the group 1, respectively. Tumor size was monitored by measuring the longest (length) and shortest dimension (width) in a 3-day interval with a dial caliper, and tumor volume was calculated by the following formula: tumor volume (mm^3) = 0.52 × length (mm) × width (mm) × width (mm) [28]. At the end of the experiment, mice were sacrificed and the tumor tissues were collected for subsequent histologic experiments (see below).

TUNEL detection of apoptotic tumor cells

Terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (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 the nuclei from 20 randomly chosen fields/section at 200× magnification. Slides of the apoptosis studies

were quantified in a blind manner by two independent reviewers at two different times.

Histological analysis

Dissected tumors were weighed and each was divided in half, one-half for paraffin sections fixed in 10% neutral buffered formalin and embedded in paraffin and the other half frozen at −80°C. The paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E) as described previously [29], and observed by two pathologists in a blind manner. For observation of potential side effects in the treated mice, the organs, such as heart, liver, spleen, lung, kidney etc., were also isolated for histology.

Synergistic index calculation

Relative ratio of tumor volume (RRTV) was used for calculation of corresponding synergistic indices using the methods described previously [30]. Briefly, RRTV in each treatment group was obtained by dividing the mean value by that in the untreated control group. The expected relative ratio of the combination treatment group was obtained by timing the observed relative ratio of the cisplatin treatment group to that of the mPNAS-4 treatment group. Then, the synergistic index of tumor volume (RRTV) was obtained by dividing the expected relative ratio by the observed relative ratio. Calculated synergistic indices of tumor volume are given in Table 1. An index of >1 indicates a synergistic effect; an index of <1 indicates a less than additive effect.

Data analysis and statistics

For comparison of individual time points, ANOVA and an unpaired Student's *t*-test were used [28]. Survival curves were constructed according to the Kaplan–Meier method [31]. Statistical significance was determined by the log-rank test [32]. *P*-value <0.05 was considered significant. Error bars represent the SEM unless otherwise indicated.

Results

Overexpression of mPNAS-4 in vitro and in vivo

In vitro expression of plasmid DNA in transfected LL/2 cells was confirmed by RT-PCR, as shown in Fig. 1a, the mPNAS-4 expression level of the LL/2 cells treated with pcDNA3.1-mPS was significantly increased in contrast to that of the LL/2 cells untreated or treated with pcDNA3.1 empty vector. The production of mPNAS-4 protein was further confirmed by Western blot analysis (Fig. 1b). We tried to detect the overexpression of mPNAS-4 in tumor tissues by immunohistochemistry analysis using the polyclonal antibodies against mPNAS-4 produced in our laboratory; however, there were some strong non-specific staining that would prevent us from judging the expression level of exogenous mPNAS-4. To exactly investigate whether intravenous injections of pcDNA3.1-mPS expression plasmid led to an apparently improved expression level of exogenous mPNAS-4 within the tumor tissues, we decided

Table 1 Synergistic indices of combination treatment calculated by relative ratio of tumor volume (RRTV)[a]

Day[b]	Cisplatin	mPNAS-4	Combination treatment		
			Expected[c]	Observed	Ratio[d]
LL/2 Lewis lung cancer model					
22	0.73	0.31	0.23	0.17	1.35
25	0.57	0.25	0.14	0.13	1.08
28	0.61	0.30	0.18	0.16	1.13
31	0.78	0.47	0.37	0.20	1.85
34	0.76	0.45	0.34	0.22	1.55
CT26 Colon adenocarcinoma model					
22	0.46	0.38	0.17	0.18	0.94
25	0.48	0.36	0.17	0.16	1.06
28	0.46	0.35	0.16	0.13	1.23
31	0.43	0.35	0.15	0.11	1.36
34	0.54	0.36	0.19	0.14	1.36

[a] *RRTV*, relative ratio of tumor volume, RRTV = mean tumor volume experimental/mean tumor volume control

[b] Day after tumor cell transplantation

[c] Mean RRTV of mPNAS-4 × mean RRTV of chemotherapy group

[d] Obtained by dividing the expected RRTV by the observed RRTV. An index of >1 indicates a synergistic effect; an index of <1 indicates a less than additive effect

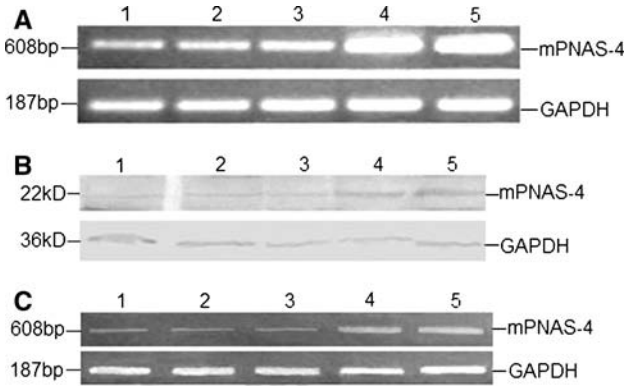

Fig. 1 In vitro and in vivo expression of recombinant mPNAS-4. **a** RT-PCR analysis of expression of mPNAS-4 in vitro. Results from LL/2 cells untreated, cells transfected with pcDNA3.1, cells treated with cisplatin, cells transfected with pcDNA3.1-mPS, and cells treated with pcDNA3.1-mPS plus cisplatin are shown in *lanes 1–5*, respectively. GAPDH was used as an internal control, DNA mass molecular markers are indicated on the left. **b** Western blotting analysis of mPNAS-4 protein in vitro after transfection of LL/2 cells. Total protein samples were obtained from IL/2 cells untreated (*lane 1*), cells transfected with pcDNA3.1 (*lane 2*), cells treated with cisplatin (*lane 3*), cells transfected with pcDNA3.1-mPS (*lane 4*), and cells treated with pcDNA3.1-mPS plus cisplatin (*lane 5*). Protein mass molecular markers are indicated on the left. **c** RT-PCR analysis of expression of exogenous mPNAS-4 in vivo. Total RNA samples were isolated from tumor tissues in untreated group (*lane 1*), pcDNA3.1 treatment group (*lane 2*), cisplatin treatment group (*lane 3*), pcDNA3.1-mPS treatment group (*lane 4*), and pcDNA3.1-mPS plus cisplatin treatment group (*lane 5*), respectively

to detect it by RT-PCR analysis. As expected, in vivo overexpression of recombinant mPNAS-4 was further confirmed by RT-PCR (Fig. 1c).

Inhibition of mouse tumor cell proliferation after treatment with mPNAS-4 and cisplatin

The observations by other authors suggested that overexpression of PNAS-4 in some carcinoma cell lines could result in their proliferation inhibition [15, 19]. To assess whether mPNAS-4 can augment the antiproliferative effect of cisplatin, we first treated LL/2 cells with cisplatin at indicated concentrations, with a 24- or 48-h interval, and found that the dose of IC_{50} of cisplatin ranged from 5 to 10 μg/ml (Fig. 2a). Then, in the following in vitro experiments, we treated tumor cells with cisplatin at a suboptimal dose (5 μg/ml), with a 24-h interval, according to the various schedules as described in "Materials and methods". After treatment, viability of cells was determined by MTT assay. As shown in Fig. 2b, the treatment of mPNAS-4 in combination with cisplatin reduced CT26 cell viability by 74%, whereas the treatment of mPNAS-4 alone or cisplatin alone reduced CT26 cell viability by 59% and 51%, respectively. The treatment of mPNAS-4 in combination with cisplatin reduced LL/2 cell viability by 57%, whereas the treatment of mPNAS-4 alone or cisplatin alone reduced LL/2 cell viability by <25% (Fig. 2c). These results indicate that the treatment of mPNAS-4 in combination with cisplatin shows additive cytotoxicity to CT26 and LL/2 cells.

Induction of apoptosis of mouse tumor cells in vitro by mPNAS-4 and cisplatin

In the next set of experiments, we tried to identify the type of cell death induced by mPNAS-4 plus cisplatin. The quantitative assessment of sub-G1 cells by flow cytometry was used to estimate the number of apoptotic cells. As shown in Fig. 3a, the combined treatment of LL/2 cells with mPNAS-4 plus cisplatin increased sub-G1 cells (apoptotic cells) compared with the two single treatment groups or the two control groups. Furthermore, agarose gel electrophoresis of mPNAS-4 plus cisplatin group demonstrated, compared with the two single treatment groups showed a more apparent ladder-like pattern of DNA fragments consisting of multiples of approximately 180–200 base pairs, consistent with internucleosomal DNA fragmentation (Fig. 3b). To further assess apoptosis by morphologic changes, LL/2 cells were transfected with pcDNA3.1-mPS or pcDNA3.1 empty vector. These transfections also included a pEGFP-N1 expression vector at one-fifth the concentration of the above vectors to identify transfected cells from non-transfected cells upon green fluorescence. After transfection cisplatin was added into the LL/2 cells in the mPNAS-4 plus cisplatin treatment group. As shown in Fig. 3c, the morphologic changes of cells at 48 h posttransfection monitored by fluorescence microscope showed characteristic for apoptosis (rounded or floating). In contrast, the untreated cells showed green normal shape. The percentage of apoptotic cells was determined by the number of green cells with apoptotic morphology (rounded or floating) divided by the total number of green cells. As shown in Fig. 3d, the percentage of apoptotic cells in combination treatment group is higher than that of other groups. Although the ratio of the scoring floating cells is not an accurate assay for quantifying apoptosis, this method can be used to preliminarily estimate the rate of apoptosis. As expected, results obtained with apoptotic cell counting in the morphological analysis strongly correlated with those obtained with flow cytometry.

Inhibition of human tumor cell proliferation via apoptosis in vitro by hPNAS-4 and cisplatin

Our observation suggested that overexpression of human PNAS-4 (hPNAS-4) in human ovarian cancer SKOV3 cells could result in their proliferation inhibition [19]. To evaluate

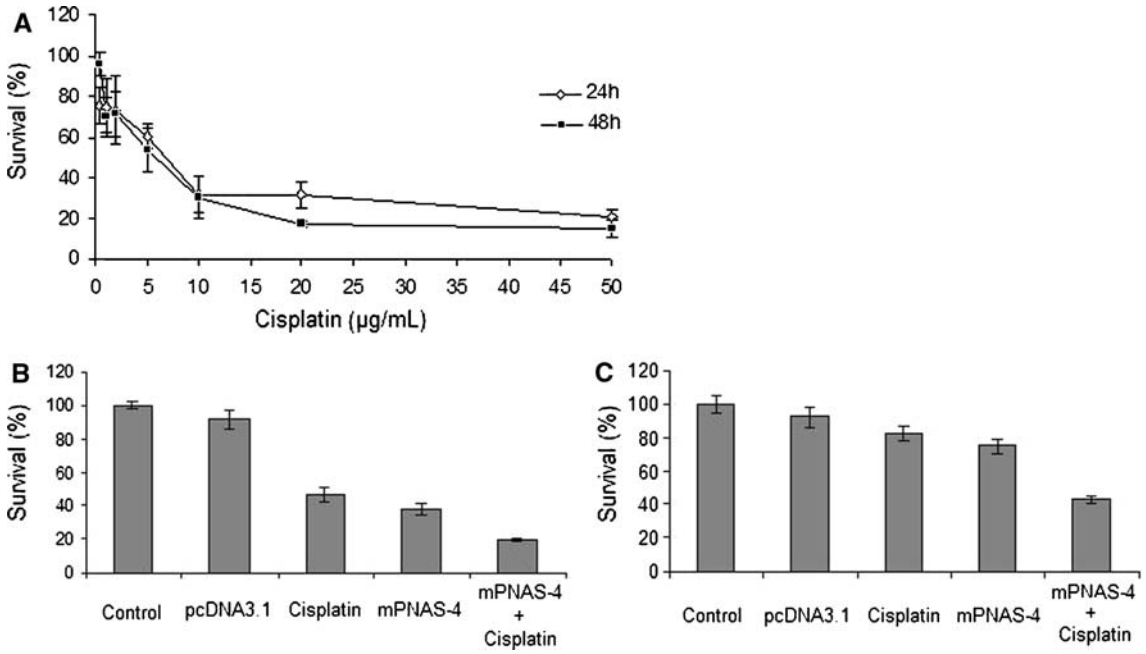

Fig. 2 Inhibition of mouse tumor cell proliferation in vitro by mPNAS-4 plus cisplatin. The MTT assay was performed as described in "Materials and methods". **a** The treatment of cisplatin at indicated concentrations and periods reduced LL/2 cell viability, showing that the dose of IC_{50} ranged from 5 to 10 μg/ml. **b** The treatment of mPNAS-4 plus cisplatin reduced CT26 cell viability more significantly than the treatment of mPNAS-4 alone or cisplatin alone did. **c** The treatment of mPNAS-4 plus cisplatin reduced LL/2 cell viability more significantly than the treatment of mPNAS-4 alone or cisplatin alone did. Percentage of survival was calculated. Results are shown as mean ± SD of six wells and triplicate experiments. In each experiment, the medium-only treatment (untreated) indicates 100% cell viability

whether hPNAS-4 could also augment the antiproliferative effect of cisplatin, we first treated SKOV3 cells with cisplatin at indicated concentrations, with a 24- or 48-h interval. As shown in Fig. 4a, we found that the dose of IC_{50} of cisplatin ranged from 5 to 15 μg/ml. Then, we treated SKOV3 cells with cisplatin at a suboptimal dose (5 μg/ml), with a 24-h interval, according to the various schedules as described in "Materials and methods". After treatment, viability of cells was determined by MTT assay. As shown in Fig. 4b, the treatment of hPNAS-4 in combination with cisplatin reduced SKOV3 cell viability by 64%, whereas the treatment of hPNAS-4 alone or cisplatin alone reduced SKOV3 cell viability by 43% and 40%, respectively. The results of flow cytometric analysis also showed that the combined treatment of SKOV3 cells with hPNAS-4 plus cisplatin increased sub-G1 cells (apoptotic cells) compared with the two single treatment groups or the two control groups (Fig. 4c). These results indicate that hPNAS-4 could also enhance apoptosis of SKOV3 cells induced by cisplatin besides its induction of apoptosis.

Enhanced antitumor efficacy of the combination regime of *mPNAS-4* gene therapy plus low-dose cisplatin

Based on the in vitro growth-inhibitory and pro-apoptotic effects of *PNAS*-4 and cisplatin, we examined the combined effect of mPNAS-4 and cisplatin on the growth of CT26 colon carcinoma in BALB/c mice and on Lewis lung carcinoma in C57BL/6 mice. Tumor volume and life span of mice assay showed that either mPNAS-4 or cisplatin individually resulted in effective suppression of tumor growth, compared with PBS or empty vector control. Combined treatment had a superior antitumor effect, resulting in about 20-day (cisplatin 8.0 and 4.0 days) delay of tumor growth to reach a volume of 900 mm^3 compared with sterile PBS treatments in CT26 and LL/2 carcinomas (Fig. 5a). In the two tumor models, control animals that received PBS treatment survived 35.6 and 36.8 days on average, respectively. In addition, animals that received pcDNA3.1 empty vector treatment survived 40.2 and 40.8 days on average, respectively. In contrast, the combination of *mPNAS-4* gene therapy with low-dose cisplatin resulted in a significant increase in life span ($P < 0.01$, by log-rank test; Fig. 5b). Systemic therapy with mPNAS-4 plus cisplatin resulted in apparent tumor inhibition versus PBS ($P < 0.01$) or pcDNA3.1 controls ($P < 0.01$), and cisplatin ($P < 0.01$) or mPNAS-4 alone ($P < 0.05$).

The mice treated with mPNAS-4 alone, cisplatin alone or in combination have been, in particular, investigated for potential side effects during the course of the experiment. No adverse consequences were detected in gross measures, such as weight loss, ruffling of fur, life span, behavior, and feeding. Furthermore, no pathologic changes in heart, liver, spleen, lung, and kidney were found by microscopic

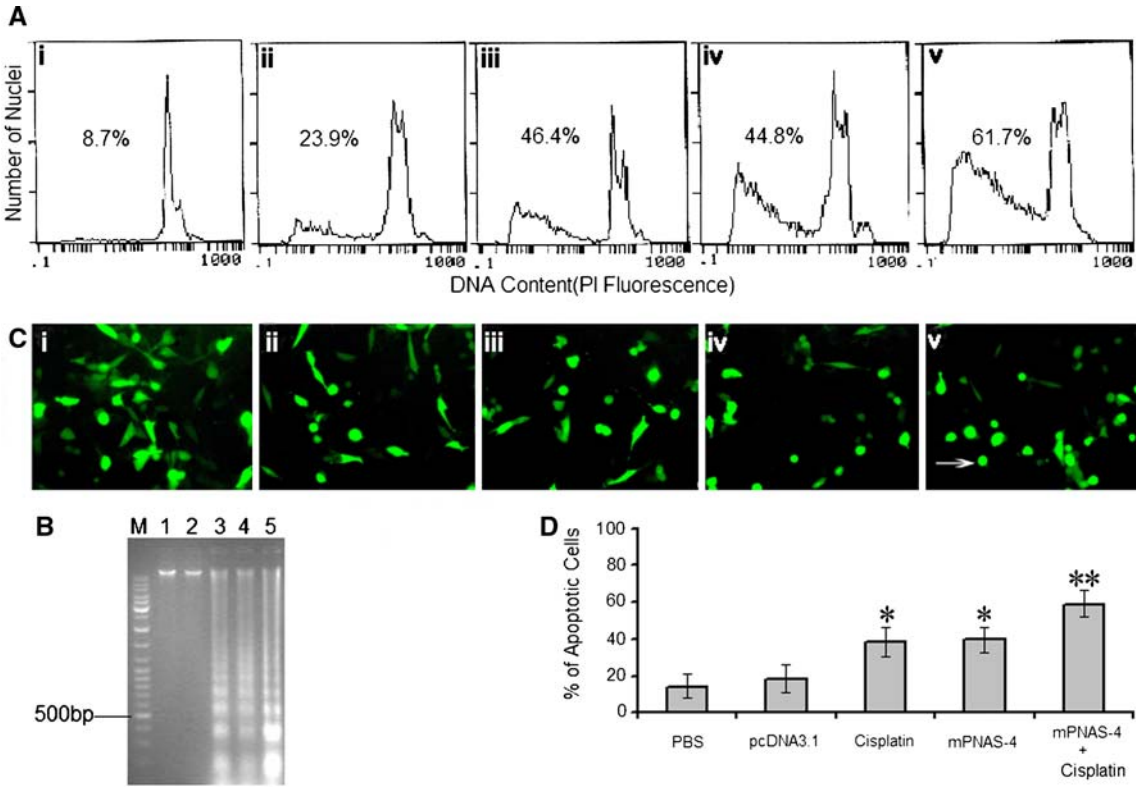

Fig. 3 Induction of apoptosis of mouse tumor cells by the treatment with mPNAS-4 and cisplatin in vitro. **a** Representative DNA fluorescence histograms of PI-stained cells. LL/2 cells were treated with mPNAS-4 for 24 h, then with 5 μg/ml cisplatin for an additional 24 h. LL/2 cells were untreated (*i*), treated with empty vector (*ii*) or treated with cisplatin (*iii*), mPNAS-4 alone (*iv*) or mPNAS-4 plus cisplatin (*v*) and groups *i*, *ii*, *iii*, *iv*, and *v* correspond to these five treatments (the same as shown in the subsequent panels), with 8.7% (*i*), 23.9% (*ii*), 46.4% (*iii*), 44.8% (*iv*), and 61.7% (*v*) sub-G1 cells (apoptotic cells), respectively, as assessed by flow cytometry. **b** Agarose gel electrophoretic patterns of DNA. LL/2 cells were treated with the same condition as mentioned in flow cytometric analysis. *Lane M*, DNA marker; *lane 1*, untreated LL/2; *lane 2*, treated with empty vector; *lane 3*, treated with cisplatin alone; *lane 4*, treated with mPNAS-4 alone; *lane 5*, treated with mPNAS-4 plus cisplatin. **c** Morphology of *green* normal cells and *green* apoptotic cells (rounded or floating). LL/2 cells were treated with the same condition as mentioned above. *Arrow* indicates an example of apoptotic cells. **d** The percentage of apoptotic cells. Sequential analysis showed that the percentage of apoptotic cells in the mPNAS-4 plus cisplatin treatment group was significantly higher than that in the two controls (**$P < 0.01$), and higher than that in the mPNAS-4 or Cisplatin alone treatment group (*$P < 0.05$). *Columns*, mean of percentage of apoptotic cells; *bars*, ±SD. The percentage was determined by the number of *green* cells with apoptotic morphology divided by the total number of green cells (average of three individual experiments)

examination. In addition, we found a synergistic interaction between mPNAS-4 and cisplatin. The RRTV of the combination group showed an additive effect about 25 days after tumor cell transplantation and a synergistic effect about 28 days after tumor cell transplantation in both tumor models (Table 1).

Induction of apoptosis in tumor tissues

To further investigate the antitumor mechanism of the combined treatment with mPNAS-4 plus cisplatin, we applied the TUNEL assay to detect apoptosis of tumor cells. Cell nuclei were observed to stain dark green, as viewed by fluorescence microscopy (high magnification, ×200), indicating apoptosis, and were recorded as TUNEL positive nuclei. Our data suggested that treatment with mPNAS-4 or Cisplatin alone (Fig. 6c and d) affected the apoptotic rate of tumor cells when compared with empty vector (Fig. 6b) or PBS (Fig. 6a), however, the density of apoptotic cancer cells increased after the combined therapy (Fig. 6e). The apoptotic index, calculated as a ratio of the apoptotic cell number to the total cell number in each field, further showed a significant augmented effect in the combined treatment group (Fig. 6f). These results suggest that the increased apoptosis of the tumor cells may be mainly responsible for the enhanced antitumor activity of combination therapy.

Histologic analysis

Histologically, those mPNAS-4-treated tumors showed visible responses with large necrosis/apoptosis regions (Fig. 7d) that was similar in size and morphology of cisplatin-responsive tumors (Fig. 7c). Tumors from the two

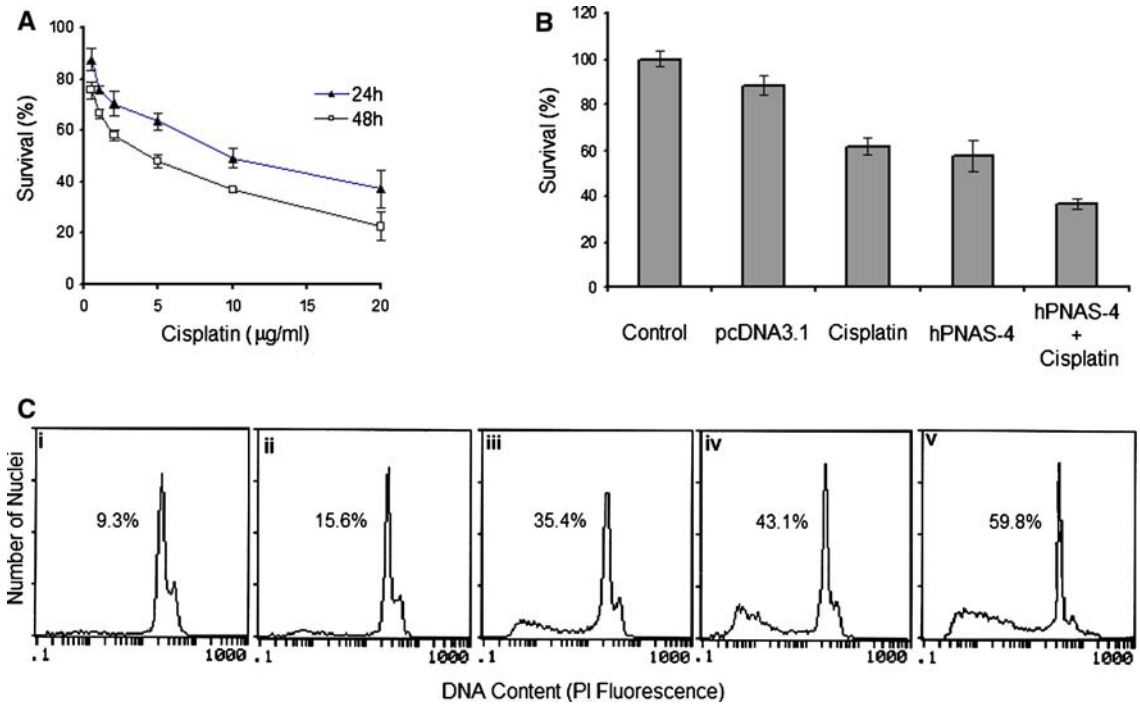

Fig. 4 Inhibition of human ovarian cancer SKOV3 cells proliferation via apoptosis in vitro by hPNAS-4 plus cisplatin. The MTT assay and flow cytometric analysis were performed as described in "Materials and methods". **a** The treatment of cisplatin at indicated concentrations and periods reduced human ovarian cancer SKOV3 cell viability, showing that the dose of IC_{50} ranged from 5 to 15 μg/ml. **b** The treatment of hPNAS-4 plus cisplatin reduced SKOV3 cell viability more significantly than the treatment of hPNAS-4 alone or cisplatin alone did. Percentage of survival was calculated. Results are shown as mean ± SD of six wells and triplicate experiments. In each experiment, the medium-only treatment (untreated) indicates 100% cell viability. **c** Representative DNA fluorescence histograms of PI-stained cells. SKOV3 cells were treated with hPNAS-4 for 24 h, then with 5 μg/ml cisplatin for an additional 24 h. SKOV3 cells were untreated (*i*), treated with empty vector (*ii*) or treated with cisplatin (*iii*), hPNAS-4 alone (*iv*) or hPNAS-4 plus cisplatin (*v*) and groups *i*, *ii*, *iii*, *iv*, and *v* correspond to these five treatments, with 9.3% (*i*) 15.6% (*ii*), 35.4% (*iii*), 43.1% (*iv*), and 59.8% (*v*) sub-G1 cells (apoptotic cells), respectively, as assessed by flow cytometry

control groups, i.e., PBS-treated group and pcDNA3.1 empty vector group, displayed little or no tumor tissue necrosis/apoptosis (Fig. 7a and b). Analysis of the extent of tumor necrosis or apoptosis revealed that the coadministration of mPNAS-4 plus cisplatin was clearly more effective, eliciting a significant increase in tumor necrosis or apoptosis relative to single element treatment (Fig. 7e). Representative sections of different treatment groups from CT26 neoplasm tissue were depicted.

Discussion

Cisplatin has been proved to be effective against many tumors, including lung, bladder, breast cancer, head and neck carcinoma, and prostate cancer [2–4, 33]. However, dose-dependent toxicity, such as inhibition of T-cell proliferation [34] and decrease of NK cell activity [35], and development of cisplatin resistance [5–7] greatly limit its therapeutic efficacy. Therefore, it would be highly desirable to develop new approaches that would overcome the drug resistance and lower the doses administered to the patient. Recently, attempts have been made in many laboratories to target chemotherapy specifically to tumor cells by using efficient gene delivery tools, such as adenovirus [36, 37], and to attack cellular functions that influence drug resistance, such as apoptosis, thereby improving therapeutic effects of chemotherapy.

PNAS-4 was previously identified as a putative apoptosis-related protein in human acute promyelocytic leukemia cell line NB4. Previous studies showed that PNAS-4 was up-regulated in human papillomavirus-infected invasive cervical cancer [10], in primary prostate cancer after androgen ablation therapy [11], in human papillomavirus 16 E6-expressing U2OS cells (U2OSE64b) following mitomycin C treatment [12], and in peripheral blood mononuclear cells exposed to carcinogenic agent, such as benzene [13], indicating that PNAS-4 may play a role in regulating the process of some cancers. Recently, human PNAS-4 was identified as a novel pro-apoptotic gene activated during the early response to DNA damage, and it can induce significant apoptosis when overexpressed in osteosarcoma U2OS cells [15]. As a mPNAS-4 homolog, mPNAS-4 is expressed in some normal tissues, such as liver and heart, and extensively

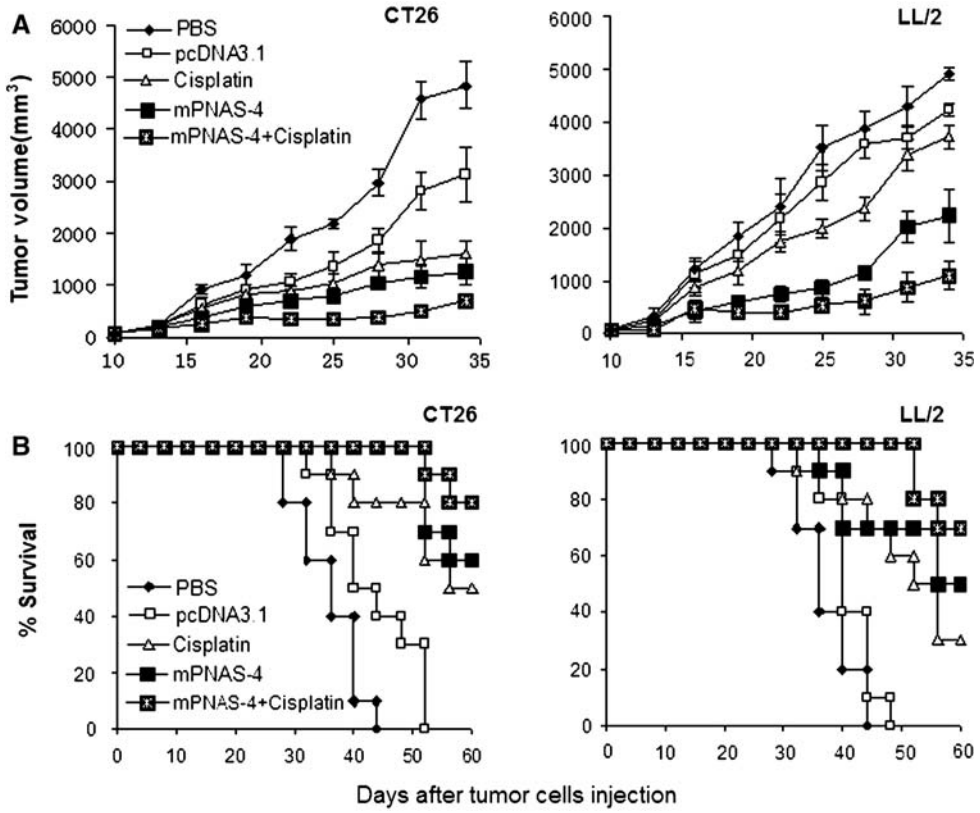

Fig. 5 Antitumor efficacy of the combination regime. Female mice at 6–8 weeks of age were transplanted subcutaneously with 2×10^5 CT26 cells or with 2×10^5 LL/2 cells. After 10 days tumor cells were transplanted, the mice were randomly divided into five groups and treated with pcDNA3.1-mPS + cisplatin (mPNAS-4 + cisplatin), pcDNA3.1-mPS (mPNAS-4), cisplatin, pcDNA3.1, and sterile phosphate-buffered saline (control) for 28 days and groups 1, 2, 3, 4, and 5 correspond to these five treatments (the same as shown in all subsequent figures). **a** Suppression of tumor growth in mice. Graph of systemic therapy with mPNAS-4 + cisplatin resulting in significant tumor growth inhibition versus PBS control ($P < 0.01$), pcDNA3.1 ($P < 0.01$), cisplatin alone ($P < 0.01$), mPNAS-4 alone ($P < 0.05$) from day 22 after initiation of mPNAS-4 administration. *Points*, average tumor volume; *bars*, ±SD. **b** A significant increase in survival of mice treated with the combination of mPNAS-4 and cisplatin, compared with the control groups ($P < 0.01$, by log-rank test), was found in CT26. Similar result was also found in LL/2 tumor model

expressed in a variety of tumor cells, such as LL/2 murine Lewis lung carcinoma, colon carcinoma (CT26), MethA fibrosarcoma, and B16 melanoma cells (data not shown). In addition, we found that both overexpression of mPNAS-4 in LL/2 cells and the increased expression level of hPNAS-4 in SKOV3 ovarian cancer cell line resulted in reduced cell viability via apoptosis [18, 19], while overexpression of mPNAS-4 in HEK293 cells had no significant effect on their proliferation (data not shown). On the basis of these findings, we speculate that the functions of PNAS-4 are different in normal tissues and cancers. In normal tissues PNAS-4 may play a role in protecting cells from some accidental stresses, such as DNA damage, and it has no significant effect on cell proliferation even when overexpressed, while in cancers PNAS-4 may be involved in eliminating carcinoma cells through induction of apoptosis. However, this speculation needs to be elucidated in the future.

Based on the observations that PNAS-4 can cause a significant increase in cell death via apoptosis when overexpressed in some tumor cells, indicating that PNAS-4 may be involved in the apoptotic response to DNA damage, and the known result that the cytotoxic action of cisplatin against tumor cells is mediated by DNA damage, the present study was designed to investigate whether PNAS-4 could inhibit proliferation of the cells via apoptosis when overexpressed in tumor cells and enhance the apoptosis of tumor cells induced by cisplatin in vitro and in vivo.

Several observations have been made in the current study concerning the induction of apoptosis and the combined treatment with gene therapy and low-dose cisplatin chemotherapy in vitro and in vivo. The in vitro experiments showed that mPNAS-4 or cisplatin alone was able to inhibit carcinoma cells proliferation via apoptosis, and mPNAS-4 plus cisplatin could augment the induction of apoptosis, as evidenced by MTT assay (Fig. 2), flow cytometry analysis (Fig. 3a), DNA fragmentation (Fig. 3b), and apoptotic morphology analysis (Fig. 3c and d) compared to treatment with mPNAS-4 or cisplatin alone. Similarly, hPNAS-4

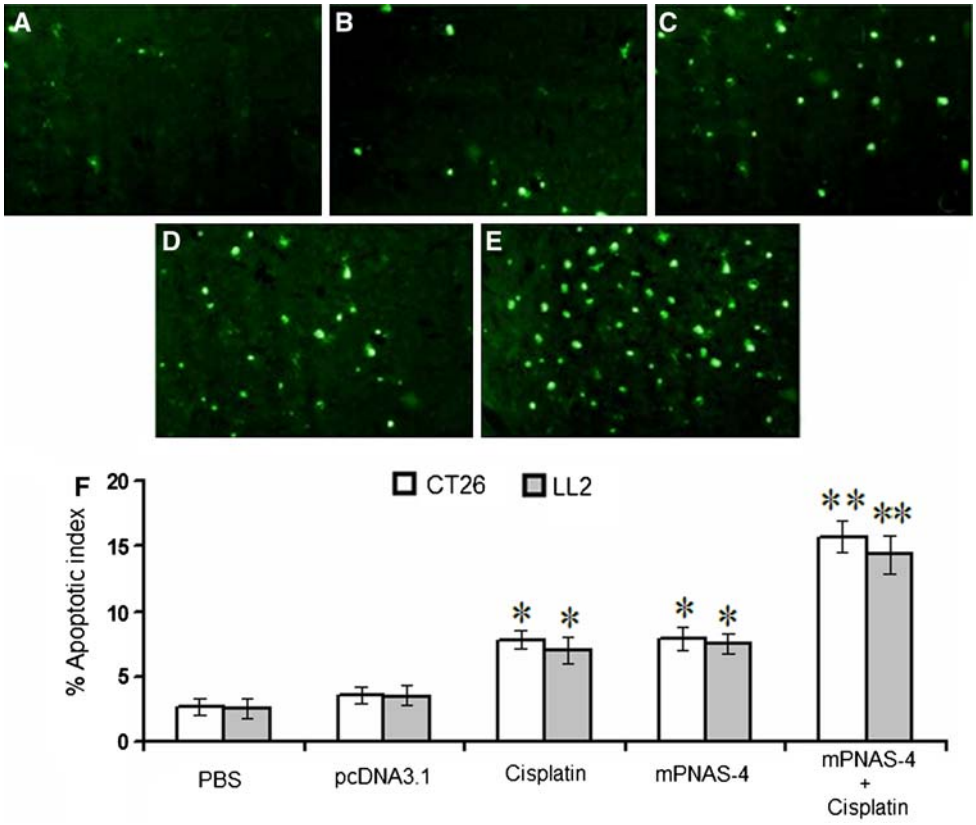

Fig. 6 Induction of tumor cells apoptosis in vivo. Apoptosis was estimated with the TUNEL assay, as described in "Materials and methods". Representative sections from CT26 tumor tissue: **a** PBS-treated group, **b** pcDNA3.1, **c** cisplatin alone, **d** mPNAS-4 alone, **e** mPNAS-4 + cisplatin, **f** apoptotic index within tissue from CT26 (*open columns*) and LL/2 (*solid columns*) from 10 mice. Sequential analysis showed systemic therapy with mPNAS-4 + cisplatin that resulted in a significant increase of apoptotic index versus the two controls (**$P < 0.01$), mPNAS-4 or Cisplatin alone versus the two controls (*$P < 0.05$). *Columns*, mean apoptotic index of cancer cells; *bars*, ±SD. The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each field

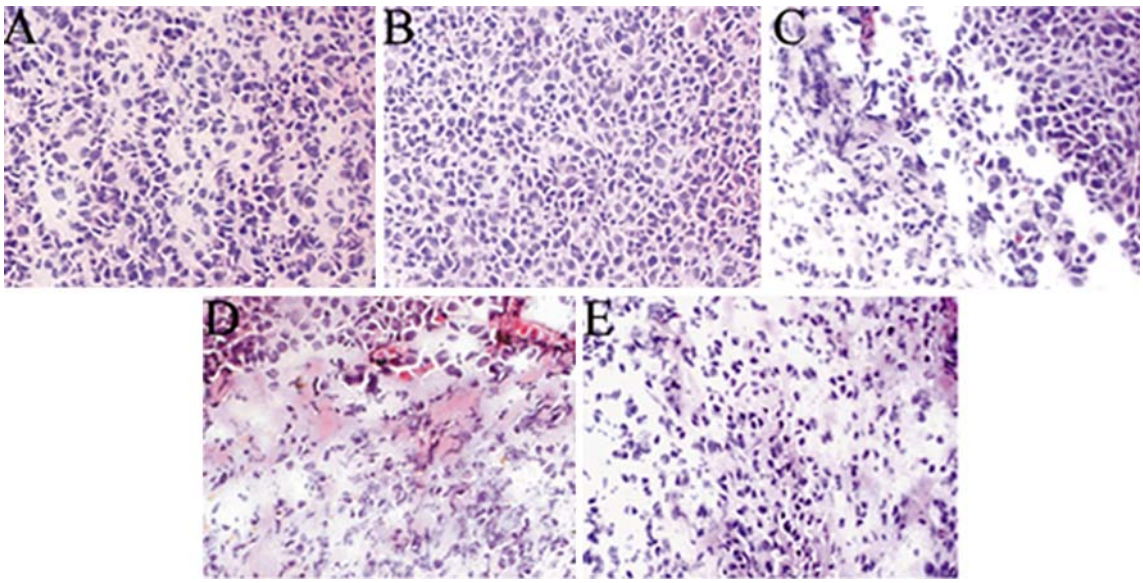

Fig. 7 Histochemical analysis of tumors. Tumor species were prepared as described in "Materials and methods". Sections of paraffin embedded from each group were stained with H&E. Tumor tissues from PBS-treated control (**a**) and pcDNA3.1 empty vector groups (**b**) had large areas of confluent tumor cells with little or no tumor tissue necrosis. Tumors with distinct necrosis were shown in Cisplatin (**c**), mPNAS-4 (**d**), and Cisplatin in combination with mPNAS-4 treated group (**e**), respectively. Representative sections of CT26 neoplasm tissue in each treatment group were photomicrographed at ×200 magnification

could also enhance the apoptosis induced by cisplatin besides its induction of apoptosis (Fig. 4). Apoptosis has been described as multiple pathways converging from numerous different initiating events and insults [36–38]. Although the exact mechanism of PNAS-4 for induction of apoptosis in cancer cells is not fully clear, it can be postulated

that *PNAS-4*, as a novel pro-apoptotic gene activated during the early response to DNA damage [15], may inhibit tumor cell proliferation via apoptosis through similar DNA damage-induced apoptotic pathway. Previous reports have demonstrated that cisplatin, as a classical DNA-damaging agent, has been effective in inducing apoptosis in a variety of tumor cell lines and its apoptosis-inducing effects are thought to be mediated by DNA damage via the formation of mono-, inter-, and intrastrand cisplatin-DNA adducts and DNA strand breaks, which can ultimately result in cell cycle arrests at G1, S, or G2-M phase and induction of apoptosis [1, 39–41].

Furthermore, the in vitro experimental enhanced interaction between mPNAS-4 and cisplatin on the proliferative and pro-apoptotic activity of carcinoma cells correlates well with the in vivo data. The enhanced antitumor efficacy in vivo may result from the increased induction of apoptosis in the combined treatment. This suggestion is supported by the present findings. The more apparent apoptotic cells in the tumors treated with mPNAS-4 plus cisplatin were found in fluorescent in situ TUNEL assay compared to the treatment with mPNAS-4 or cisplatin alone or empty vector or no-treatment groups (Fig. 6). Although, the mechanism for the increased induction of apoptosis in the tumors treated with mPNAS-4 plus cisplatin is not known, the enhanced antitumor effect may be the result of the following three factors. First, there may be a commonly synergistic apoptotic pathway between mPNAS-4 and cisplatin, which may be the principal reason for the improvement of antitumor effectiveness. Second, the enhanced interaction between mPNAS-4 and cisplatin may partly result from the enhancing chemosensitivity of the tumor cells to cisplatin by mPNAS-4. Third, due to the involvement of mPNAS-4 in the response to DNA damage, the expression of endogenous mPNAS-4 in tumor cells may be increased in the presence of cisplatin, which in turn can amplify their interaction of inducing apoptosis. Studies are currently underway in our laboratory to elucidate the precise mechanism by which mPNAS-4 plus cisplatin causes an additive induction of apoptosis in the cancer cells.

Theoretically, necrosis could also account for increased cell killing. In the current study, analysis of the extent of tumor necrosis revealed that the killing efficacy of coadministration of mPNAS-4 plus cisplatin was clearly more significant, eliciting an apparent increase in tumor necrosis relative to single treatment of mPNAS-4 or cisplatin (Fig. 7). Apart from induction of apoptosis, cisplatin also seems to kill target cells in a caspase-independent manner [42]. A recent article show that the ability of cisplatin to kill transformed fibroblasts and MCF7 cells is directly proportional to cell density, perhaps due to the establishment of intercellular contacts through gap junctions [43]. Therefore we cannot rule out the possibility that the increased killing efficacy, which may lead to the increased necrosis, may also involve the promoting penetration of cisplatin by mPNAS-4 into the gap junctions of the solid tumor.

In summary, our data suggest that *PNAS-4*, a pro-apoptotic gene, can inhibit tumor cell proliferation via apoptosis and augment the apoptosis of tumor cells induced by cisplatin when overexpressed in cancer cells in vitro, and that in vivo the combination of *mPNAS-4* gene therapy with low-dose cisplatin can enhance the antitumor activity, significantly prolonging the lifespan of tumor-bearing mice without an apparent increase in toxicity. To our knowledge, the present study may provide a novel way to augment the antitumor efficacy of chemotherapeutic drugs.

Acknowledgments The authors thank Dr. Ning Xu for her advice, discussion, and critical review of the manuscript, and Dr. Yang Wan for their technical support. This work was supported by National Key Basic Research Program of China (2004CB518800, 2005CB522506).

Conflicts of interest statement No potential conflicts of interest were disclosed.

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