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Identification and functional analysis of genes which confer resistance to cisplatin in tumor cells

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ABSTRACT

The efficacy of cisplatin during cancer chemotherapy is often impaired by the emergence of cancer cells which become resistant to chemotherapeutic agents. While various mechanisms have been proposed to explain resistance to cisplatin, the genes involved in this process still remain unclear. By using DNA microarrays, we performed a genome-wide analysis of cisplatin-resistant HeLa cells in order to identify genes involved in resistance. We identified nine genes (*NAPA*, *CITED2*, *CABIN1*, *ADM*, *HIST1H1A*, *EHD1*, *MARK2*, *PTPN21*, and *MVD*), which were consistently upregulated in two cisplatin-resistant HeLa cell lines. The upregulated genes, here referred to as cisplatin resistance genes (*CPR*), were further analyzed for their ability to modify the response of HEK293 cells to cisplatin. Short-hairpin RNA (shRNA) knockdown of *CPR* genes, individually or in combination, was shown to sensitize HEK293 cells to cisplatin, but not to vincristine or taxol, suggesting that *CPR* genes may be involved specifically in cisplatin resistance. Among the treatments performed, shRNA knockdown of *NAPA* was the most efficient treatment able to sensitize cells to cisplatin. Furthermore, shRNA knockdown of a single *CPR* gene was sufficient to partially reverse acquired cisplatin resistance in HeLa cells. Sensitization to cisplatin following knockdown of *CPR* genes was also observed in the tumorigenic cell lines Sk-ov-3, H1155, and CG-1. Based on these results, we propose that the *CPR* genes identified here may represent potential candidates for novel target therapies aimed at preventing resistance to cisplatin during chemotherapy.

1. Introduction

Despite the recent introduction of new platinum chemotherapeutic compounds, cisplatin remains widely used for the treatment of a variety of malignancies. Unfortunately, the efficacy of cisplatin treatment during chemotherapy is often impaired by the emergence of tumor cells which become resistant to the action of this drug [1,2]. Our main objectives are to study the mechanism of acquired resistance to cisplatin, and design novel and effective methods to prevent this phenomenon during chemotherapy. To do so, a thorough understanding of the mechanism of cisplatin resistance is necessary. The mechanism of drug resistance is complex and involves multiple factors. Resistance to cisplatin may be the result of an inadequate drug exposure and/or alterations in the cancer cell itself [3]. Several cellular alterations have been proposed to explain drug resistance, including decreased drug influx, increased drug efflux, activation of detoxification systems, alteration of the targets of the drug, increased DNA repair, impaired apoptosis and altered oncogene expression [1,2,4–7]. So far, however, the genes associated with cisplatin resistance are insufficient to accurately predict the status of cancer chemosensitivity during cancer treatment.

Although the mechanisms of drug resistance are complex, gene expression profiling provides a powerful tool for the identification of genes and pathways involved in this process. Genome-wide analysis using DNA microarrays allows for the rapid and quantitative analysis of gene expression in individual cancer specimens. As such, gene expression profiling of individual genomic tumor has been used with success in the past for the classification of histological classes of tumors. DNA microarrays also provide a platform for the development of powerful tools to predict tumor metastasis as well as disease relapse or prognosis of cancer patients [8]. In addition, genome-wide gene expression can be used to identify gene expression patterns that are predictive of response or resistance to chemotherapy [9]. From these observations, it appears that this approach may be helpful to study chemoresistance.

Abbreviations: ADR, adryamycin; CDDP, cisplatin; CPR, cisplatin resistance; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol tetra-acetic acid; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSF, N-ethylmaleimide-sensitive factor; PARP, poly-ADP ribose polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; RIPA, radio-immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short-hairpin RNA; VCR, vincristine.

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The main objective of this study was to identify genes involved in resistance to cisplatin. Therefore, we performed a genome-wide gene expression analysis of the parental human cervix cancer HeLa cell line and its cisplatin-resistant derivatives HeLa-R1 and HeLa-R3. The two latter cell lines were obtained earlier by repeatedly treating parental HeLa cells with increasing concentrations of cisplatin [10]. Using DNA microarrays, we observed several alterations in the gene expression profiles of cisplatin-resistant HeLa cells when compared with the control, parental cells. By using shRNA, we confirmed that nine of the genes identified were involved in cisplatin resistance.

2. Materials and methods

2.1. Cell lines and reagents

Human embryonic kidney cells (HEK293) and tumorigenic cell lines (cervix HeLa, ovarian Sk-ov-3, lung H1255 and H1299 cells; obtained from the American Tissue Type Collection (Manassas, VA), and nasopharyngeal CG-1 cells; a gift from Chang [11]) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Gaithersburg, MD) containing 10% (v/v) fetal bovine serum. The cell culture medium used throughout this study contained 100 mg/ml streptomycin and 100 units/ml penicillin. Each cell line was incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. The human cervix carcinoma HeLa cell line and its cisplatin-resistant derivatives (HeLa-R1 and HeLa-R3) were characterized and cultured as described earlier [10]. Human lymphoblastoma HOB cells as well as multidrug resistance (MDR)-variant cells, which were originally selected with vincristine (HOB/VCR), were maintained in cell culture conditions as described before [12]. Colon cancer SW620 cells as well as MDR-variant cells selected with adriamycin (SW620/ADR) were characterized earlier [13]. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or Cell Signaling Technology (Danvers, MA). Cisplatin, vincristine, and taxol (also known as paclitaxel) were purchased from Bristol-Myers Squibb (New York, NY). Unless indicated otherwise, chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All reagents were prepared and used according to the instructions provided by the manufacturer.

2.2. Western blot analysis

For whole protein extract, cells were washed twice with phosphate-buffered saline (PBS) and lysed with 1 ml of modified radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4 and 1 mM NaF) on ice for 30 min. The insoluble material was removed by centrifugation at 16,000 × *g* for 10 min at 4 °C. Protein concentration was determined by the Bradford assay [14] using the BioRad dye reagent (BioRad, Hercules, CA). Equivalent amounts of proteins from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [15], transferred onto PVDF membrane, and incubated with the antibodies according to the instructions of the manufacturer. The signal on the membrane was revealed using enhanced chemiluminescence according to the specifications of the supplier (Pierce, Rockford, IL).

2.3. RNA isolation and DNA microarray analysis

Total RNA were extracted by using the Trizol reagent (Life Technologies, Bethesda, MD). mRNA were isolated from total RNA with the Dynal MPC-s extraction kit (Dynal Biotech, Carlsbad, CA). mRNA were reverse-transcribed with the Superscript II RNase H-reverse transcriptase (Gibco) in the presence of Cy3 and Cy5 (Invitrogen, Carlsbad, CA) to generate labeled cDNA probes corresponding to the parental and resistant cell samples, respectively. Competitive hybridization of Cy3 and Cy5 probes was performed on a commercial 7500 human cDNA microarray chip (ABC Human UniversoChip 8K; Asia BioInnovations, Taipei, Taiwan) according to the information provided by the manufacturer (GEO platform: GPL1392, Egenomix Technology Corporation, Taipei, Taiwan). The chip contained 7597 elements representing 6577 genes based on the Stanford SOURCE database (http://source.stanford.edu; accessed January 2010). Fluorescence intensity of Cy3 and Cy5 targets were measured and scanned separately using the GenePix 4000B Array Scanner (Axon Instruments, Union City, CA). Data analysis was performed using the GenePix Pro 3.0.5.56 software (Axon Instruments). The microarray results were normalized for labeling and detection efficiencies of the two fluorescence dyes by using the LOWESS method [16]. The data obtained were then used to determine differential gene expression between parental and resistant cells. The Cy5/Cy3 ratios of each gene duplicate were averaged and normalized based on total fluorescence of Cy5/Cy3 readings across the slide. Normalized ratios were transformed to base-2 logarithms. Genes with a 2-fold upregulation in both resistant cells (HeLa-R1 and HeLa-R3) were selected and considered as candidates for cisplatin resistance genes (*CPR*). Gene classification was performed with the gene ontology tool of the DAVID Bioinformatic Resources (http://david.abcc.ncifcrf.gov/; accessed January 2010).

2.4. Quantitative real-time reverse transcription-PCR (qRT-PCR)

qRT-PCR, or in short qPCR, was performed on total cellular RNA extracted as described above. Reverse transcription was performed with oligo$(dT)_{16}$ on total DNase I-treated RNA (10 μg) with the Ominiscript Reverse Transcriptase (Qiagen, Valencia, CA). Real-time PCR was performed on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA) and SYBR green I master mix (Applied Biosystems) using the software SDS 1.0.1 (Applied Biosystems). PCR primers (Supplementary Table S1) were designed using Primer Express 2.0.0 (Applied Biosystems). The primers were used at 100 nM and validated against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following cycles were applied to all PCR assays: 2 min at 50 °C; 15 min at 95 °C; followed by 40 PCR cycles, each consisting of denaturation 15 s at 95 °C, annealing 60 s at 60 °C, and extension 60 s at 60 °C. Specific amplification was verified for each sample by establishing independent melting curves. All unknown samples and controls were done in triplicate on the same plate. Relative quantification was calculated by the ΔΔCt method and normalized based on GAPDH. Namely, the ΔCt for each candidate was calculated as ΔCt (candidate) = [Ct (candidate) − Ct (GAPDH)]. The relative abundance of the candidate gene X was shown as $2^{\Delta Ct(X) - \Delta Ct(GAPDH)}$.

2.5. Analysis of cell viability and apoptosis

Cells were treated with cisplatin, vincristine, or taxol in serum-free medium for 2 h and were subsequently cultured in drug- and serum-containing medium for 3 days unless indicated otherwise. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described previously [10]. Percentage of cell viability was calculated as the ratio of $OD_{570\ nm}$ values for treated cells divided by the $OD_{570\ nm}$ values for control cells in quadruplicates. The trypan blue dye exclusion assay was also performed to monitor cell death. After incubation, cells treated with 0.2% trypan blue were counted using a hemocytometer. Percentage of cell death was calculated as the ratio of stained cells divided by the total number of cells counted in triplicates. To study apoptosis, the sub-G1

population of cells was measured as described earlier [17]. Briefly, the cells were washed in ice-cold PBS and fixed in 70% (v/v) ethanol at 4 °C overnight. They were then resuspended in 0.5 ml of PBS and incubated in 0.5 ml of DNA extraction buffer (40 mM Na_2HPO_4, 20 mM citric acid, pH 7.8) for 5 min at room temperature. The cells were washed once with PBS, followed by incubation in PBS containing 20 μg/ml propidium iodine and 200 μg/ml RNase A (Sigma–Aldrich) for 30 min at room temperature in the dark. Stained nuclei were then analyzed using the BD FACScan Flow Cytometer (Becton & Dickinson, San Jose, CA) with 10,000 events/determination. The LYSYS II software was used to assess cell cycle distribution. Three independent experiments were performed unless indicated otherwise. The data were reported as mean values ± standard deviation (SD). Statistical significance (p value) was calculated with a two-tailed Student's t-test for single comparison. The symbols * and ** denote $p < 0.05$ and $p < 0.01$, respectively.

2.6. Knockdown of candidate genes by shRNA

pLKO.1 plasmids expressing shRNA were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luciferase shRNA (TRCN0000072244) was used as a negative control. Five plasmid clones for each gene were tested for gene knockdown efficiency in HEK293 cells. Transient transfection was performed by adding 2 μg/well (unless indicated otherwise) of shRNA plasmids along with 3 μl/well of Lipofectamine (Invitrogen) into cells suspensions kept in 6-well plates (1.5×10^4 cells/well). The plasmids encoding *CPR* shRNA that were selected and used in this study included *NAPA* (TRCN0000029169), *CITED2* (TRCN0000015653), *CABIN1* (TRCN0000142076), *ADM* (TRCN0000083201), *HIST1H1A* (TRCN0000106824), *EHD1* (TRCN0000053763), *MARK2* (TRCN0000001581), *PTPN21* (TRCN0000220135), and *MVD* (TRCN0000078480). The stable clones selected expressing the shRNA plasmids via lentivirus as vector were established in cisplatin-resistant cells or in tumorigenic cells. Recombinant lentivirus preparation and cell infection were performed according to the methods described by the supplier (National RNAi Core Facility, Academia Sinica).

2.7. Inhibition of p53 activity

p53-null H1299 cells were transfected with wild-type p53 expression plasmid, pcep4-p53 (a generous gift from Dr. Y. S. Lin from Academia Sinica), using 2 μg/well (unless indicated otherwise) of the plasmid along with 3 μl/well of Lipofectamine for 48 h before treating the cells with cisplatin. To inhibit p53, HEK293 cells were pre-treated with 30 μM of PFT-α (Biomol Research Laboratories, Plymouth Meeting, PA), a reversible inhibitor of p53 activity [18], for 8 h before exposure to cisplatin.

2.8. Statistical analysis

The data were reported as mean values ± standard deviation (SD). Three independent experiments were performed and the treatment shRNA was compared to shLuc control unless indicated otherwise. Statistical significance (p value) was calculated with a two-tailed Student's t-test for single comparison.

3. Results

3.1. Identification of several genes implicated in cisplatin resistance by DNA microarray analysis

We used DNA microarrays to determine the gene expression profile associated with resistance to cisplatin in HeLa cells. On a total of 6577 genes examined, 253 showed at least a 2-fold increase or a 0.5-fold decrease of mRNA level in cisplatin-resistant HeLa cells when compared to control, parental cells. These genes were classified into 96 different categories, including cell cycle (14 genes), apoptosis (9 genes), DNA repair (3 genes) (Supplementary Table S2) whereas 101 genes showed no clear classification. Table 1 shows a selection of nine genes which showed the highest level of upregulation in HeLa-R1 and HeLa-R3 resistant cell lines when compared with the control, parental HeLa cells. These nine genes, referred to as cisplatin resistance (*CPR*) genes in the present study, were upregulated at least 2-fold in HeLa-R1 or at least 4-fold in HeLa-R3, and were therefore chosen for further functional analysis. Their original names and major functions are also given in Table 1.

3.2. Confirmation of CPR gene upregulation in cisplatin-resistant cells by qPCR

To confirm that the *CPR* genes were upregulated in cisplatin-resistant cells, we verified their gene expression by qPCR. When compared with the expression of control, parental cells, the level of mRNA corresponding to *CPR* genes was indeed upregulated in cisplatin-resistant cells (Fig. 1A). The level of upregulation of *CPR* varied between 2-fold for *EHD1* in HeLa-R1 cells up to 10-fold for *PTPN21* in HeLa-R3 cells (Fig. 1A). The concentrations of cisplatin, which produced a 50% decrease of cell viability (IC_{50}), were shown as an indication of the level of cisplatin resistance in the cell lines used. Variation of the level of CPR gene expression was noted between DNA microarray and qPCR experiments. For instance, while the level of *PTPN21* gene expression reached a 10-fold increase in HeLa-R3 cells when examined by qPCR (Fig. 1A), a more modest 4.07-fold increase was observed by DNA microarray (Table 1). These variations were attributed to the sensitivity and specificity of the respective techniques used.

In order to verify whether *CPR* genes were altered specifically in cisplatin-resistant cells, *CPR* gene expression was also examined in vincristine (VCR) and adriamycin (ADR)-resistant cells using qPCR

Table 1
Level of upregulation of *CPR* genes in cisplatin-resistant HeLa cells assessed by DNA microarray analysis.

Symbol NCBI (NM_ID)	Function	R1/HeLa	R3/HeLa
NAPA (NM_003827)	Intracellular transport	2.02 ± 0.33*	7.18 ± 3.11*
CITED2 (NM_006079)	Regulation of transcription	2.58 ± 0.11*	4.38 ± 0.53*
CABIN1 (NM_012295)	Calcineurin-binding protein	3.75 ± 0.24*	4.66 ± 0.27*
ADM (NM_001124)	Biosynthesis of C21-steroid hormone	4.81 ± 0.02*	4.16 ± 0.06*
HIST1H1A (NM_005325)	Histone cluster	4.57 ± 2.93	7.21 ± 6.26
EHD1 (NM_006795)	EH domain-containing protein	2.25 ± 0.13*	4.48 ± 0.87**
MARK2 (NM_004945)	Kinase phosphorylation	2.27 ± 0.52	4.02 ± 1.28*
PTPN21 (NM_007039)	Tyrosine phosphatase	2.32 ± 0.01*	4.07 ± 1.06**
MVD (NM_002461)	Cholesterol metabolism	2.72 ± 0.21*	4.20 ± 0.84**

* Significance at $p < 0.05$.
** Significance at $p < 0.01$.

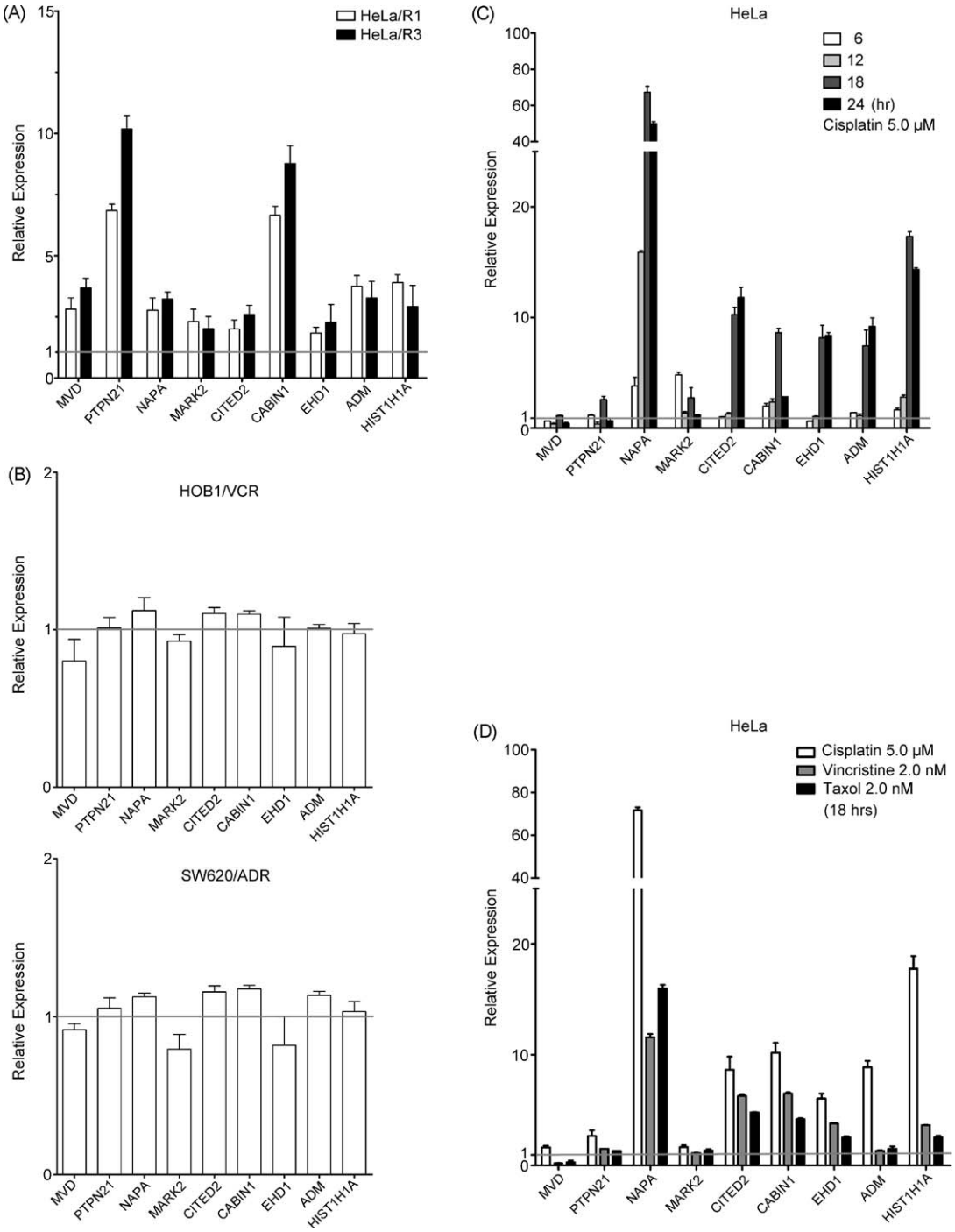

Fig. 1. Upregulation of *CPR* genes in cisplatin-resistant cells and activation of CPR genes by cisplatin. (A) The level of mRNA corresponding to *CPR* genes was determined by qPCR. Total RNA from parental human cervical carcinoma HeLa cells and cisplatin-resistant HeLa-R1 and HeLa-R3 cells was used for the assay. The dose of cisplatin which resulted in 50% decrease in cell viability (IC_{50}) was shown for each cell line. (B) CPR gene expression in parental lymphoblastoma HOB1 cells and vincristine (VCR)-resistant cells was monitored by qPCR (upper panel). CPR gene expression was also determined in colon carcinoma SW620 and adriamycin (ADR)-resistant cells (lower panel). (C) Induction of *CPR* gene expression in HeLa cells following treatment with cisplatin. mRNA level was determined by qPCR in HeLa cells treated with cisplatin (CDDP; 5 μM) for the time indicated. (D) Induction of *CPR* gene expression in HeLa cells treated with either cisplatin, vincristine, or taxol. The cells were treated with the chemotherapeutic drugs at the indicated dose for 18 h. The results represent mean values ± standard deviation (SD) of experiments performed in triplicate. The expression level of the control parental cells was illustrated by the horizontal line at a value of 1 expression unit.

(Fig. 1B, upper and lower panels, respectively). Similar *CPR* gene expression was noted for both vincristine-resistant cells and control, parental lymphoblastoma HOB1 cells (Fig. 1B, upper panel). In addition, *CPR* gene expression was obtained for both adriamycin-resistant cells and control, parental colon carcinoma SW620 cells (Fig. 1B, lower panel). These observations suggest that *CPR* genes may be involved specifically in cisplatin resistance.

3.3. Cisplatin induces the expression of CPR genes in parental HeLa cells

The relatively high level of constitutive *CRP* gene expression observed in cisplatin-resistant cells may represent a sensitive response to cisplatin in HeLa cells. To examine this possibility, we treated the parental HeLa cells with a toxic dose of cisplatin (5 μM)

and observed the expression of *CPR* genes by qPCR (Fig. 1C). Exposure of the parental HeLa cells to cisplatin resulted in a 5- to 10-fold upregulation of most *CPR* genes (Fig. 1C). In contrast, the expression of *MVD* and *PTPN21* remained relatively constant despite cisplatin treatment (Fig. 1C). Notably, we observed that *NAPA* was induced more than 60-fold following cisplatin treatment for 18 h (Fig. 1C). This observation led us to study the role of *NAPA* in more details as described below. We also examined the expression of *CPR* genes in parental HeLa cells following treatment with vincristine or taxol (Fig. 1D). Some *CPR* genes appeared to be responsive to vincristine or taxol to some extent (Fig. 1D). For example, *NAPA* could be induced more than 10-fold by either vincristine or taxol (Fig. 1D). Nonetheless, cisplatin consistently induced *CPR* genes to higher levels than the two other drugs used (Fig. 1D).

3.4. NAPA gene knockdown sensitizes HEK293 cells to cisplatin

To evaluate the effect of *CPR* genes on cisplatin resistance, we performed knockdown of *CRP* genes using shRNA. The non-tumorigenic cell line HEK293 was chosen due to its high transfection efficiency, and these experiments were followed with further testing in tumorigenic cancer cells. Results obtained for *NAPA* knockdown are shown here as the first example of *CPR* genes. NAPA-shRNA reduced *NAPA* mRNA level by 85% and considerably decreased NAPA protein (Fig. 2A). We examined the effect of several chemotherapeutic drugs on these knockdown cells by using the MTT cell viability assay. Remarkably, knockdown of *NAPA* sensitized the cells to cisplatin when compared to controls (Fig. 2B, left panel). To quantify the level of sensitization, we used a sensitization factor (SF) defined as the dose of cisplatin which decreased cell viability by 50% in the control Luc-shRNA divided by the dose of cisplatin that decreased cell viability by 50% in cells expressing the treatment shRNA. In the case of *NAPA* knockdown, the level of sensitization to cisplatin observed was relatively high (Fig. 2B, left panel; SF = 5.34). In opposition, cells expressing NAPA-shRNA were not sensitized to either vincristine (Fig. 2B, middle panel) or taxol (Fig. 2B, right panel).

To further assess the effect of *NAPA* knockdown following cisplatin treatment, cells expressing NAPA-shRNA were treated with cisplatin and subjected to flow cytometry to monitor the level of apoptotic, sub-G1 cells (Fig. 2C). Following cisplatin treatment, accumulation of apoptotic cells was increased in NAPA-shRNA-expressing cells compared to Luc-shRNA control (Fig. 2C). On the other hand, treatments with either vincristine or taxol produced levels of sub-G1 cells similar for NAPA-shRNA and control cells (Fig. 2C). In addition, cells were exposed to near equitoxic concentrations of the three chemotherapeutic drugs for 24 h, before analysis by Western blot to verify markers of apoptosis (Fig. 2D). Enhanced cleavage of caspase-3 following cisplatin treatment was detected in NAPA-shRNA-expressing cells compared to control (Fig. 2D, compare lane 8 with lane 5). Treatment of NAPA-shRNA-expressing cells with cisplatin resulted in increased caspase-3 activation compared to the same cells treated with either taxol or vincristine (Fig. 2D, compare lane 8 with lanes 6 and 7). We observed that knockdown of *NAPA* alone also caused caspase-3 activation to some extent (Fig. 2D, compare lane 5 with lane 1).

Given that anti-cancer drugs can affect the cell cycle, the role of *CPR* genes in regulating this process was also studied. A low dose of cisplatin (0.5 μM) was applied to HEK293 cells, followed by incubation for 24–72 h, prior to monitoring of the cell cycle by flow cytometry. A representative cell cycle distribution of NAPA-shRNA-treated cells displayed a reduced S-phase blockage and an increased sub-G1 cell population (Fig. 2E). A statistically significant increase of the sub-G1 cell population for NAPA-shRNA cells was observed 48 and 72 h following cisplatin treatment (Fig. 2F). Taken together, these results indicate that *NAPA* gene knockdown sensitized HEK293 cells to cisplatin.

3.5. CPR gene knockdown sensitizes HEK293 cells to cisplatin

To assess the biological role of other *CPR* genes, their expression was reduced in HEK293 using shRNAs. As before, cell viability of the shRNA-expressing cells was examined following drug treatment by using the MTT assay. We noticed a sensitization response to cisplatin following knockdown of either *CITED2* (MF = 2.42) or *CABIN1* (SF = 1.60) (Fig. 3A, upper left panel). On the other hand, knockdown of *CITED2* or *CABIN1* did not sensitize the cells to either vinscritine or taxol (Fig. 3A, middle and lower left panel, respectively). The efficiency of the mRNA knockdowns reached more than 50% for both genes, and the corresponding protein levels were also considerably reduced under these conditions (Fig. 3A, see the two upper right panels for *CITED2*-shRNA and the two lower right panels for *CABIN1*-shRNA). Consistent with the cell viability assays shown in Fig. 3A, knockdown of either *CITED2* or *CABIN1* in cisplatin-treated HEK293 cells induced the accumulation of apoptotic, sub-G1 cells (Fig. 3B).

Sensitization to cisplatin following knockdown of *ADM* (SF = 1.67) and *HIST1H1A* (SF = 2.05) was also observed (Fig. 3C, upper left panel). Sensitization was not found when these cells were treated with either vincristine or taxol (Fig. 3C, middle and lower left panels). Examination of *ADM* and *HIST1H1A* mRNA/protein levels following shRNA transfection confirmed efficient knockdown in both cases (Fig. 3B, right panels). Knockdown of *ADM* and *HIST1H1A* increased the sub-G1 population of HEK293 cells treated with cisplatin (Fig. 3D). Knockdown of the other *CPR* genes studied in HEK293 cells also consistently produced a higher degree of sensitization to cisplatin when compared to either vincristine or taxol treatments (Table 2). When examined by Western blot, the level of the other *CPR* proteins was also considerably decreased in cells expressing the corresponding shRNA (data not shown).

3.6. Sensitization of HEK293 cells to cisplatin is dependent on the extent of CPR knockdown

In order to verify that the sensitization to cisplatin described above was due specifically to the knockdown of *CPR* genes, HEK293 cells were exposed to different concentrations of shRNA, prior to cell viability analysis. Knockdown of NAPA produced a dose-dependent sensitization effect to cisplatin when compared to controls (Fig. 4A). A similar shRNA dose-dependent sensitization effect to cisplatin was noticed following knockdown of either CITED2 or CABIN1 (Fig. 4B and C, respectively). Decreased mRNA and protein levels following each knockdown were confirmed by qPCR and Western blot, respectively (Fig. 4A–C, right panels). Similar dose-dependent sensitization effects to cisplatin were also observed following knockdown of *ADM* and *HIST1H1A* (data not shown).

Table 2
Sensitization factor (SF) of CPR genes knockdown in HEK293 cells.

Symbol NCBI (NM_ID)	Cisplatin	Vincristine	Taxol
NAPA (NM_003827)	$5.24 \pm 0.23^{**}$	1.16 ± 0.24	1.08 ± 0.21
CITED2 (NM_006079)	$2.42 \pm 0.19^{**}$	1.02 ± 0.12	1.01 ± 0.13
CABIN1 (NM_012295)	$1.60 \pm 0.14^{*}$	1.01 ± 0.11	1.01 ± 0.17
ADM (NM_001124)	$1.67 \pm 0.02^{*}$	1.03 ± 0.08	1.01 ± 0.16
HIST1H1A (NM_005325)	$2.05 \pm 0.03^{*}$	1.02 ± 0.06	1.04 ± 0.06
EHD1 (NM_006795)	$1.60 \pm 0.02^{*}$	1.02 ± 0.07	1.02 ± 0.07
MARK2 (NM_004945)	1.12 ± 0.12	1.00 ± 0.08	0.97 ± 0.18
PTPN21 (NM_007039)	$2.34 \pm 0.01^{**}$	1.01 ± 0.06	1.00 ± 0.06
MVD (NM_002461)	1.02 ± 0.01	1.02 ± 0.04	1.01 ± 0.14

* Significance at $p < 0.05$.
** Significance at $p < 0.01$.

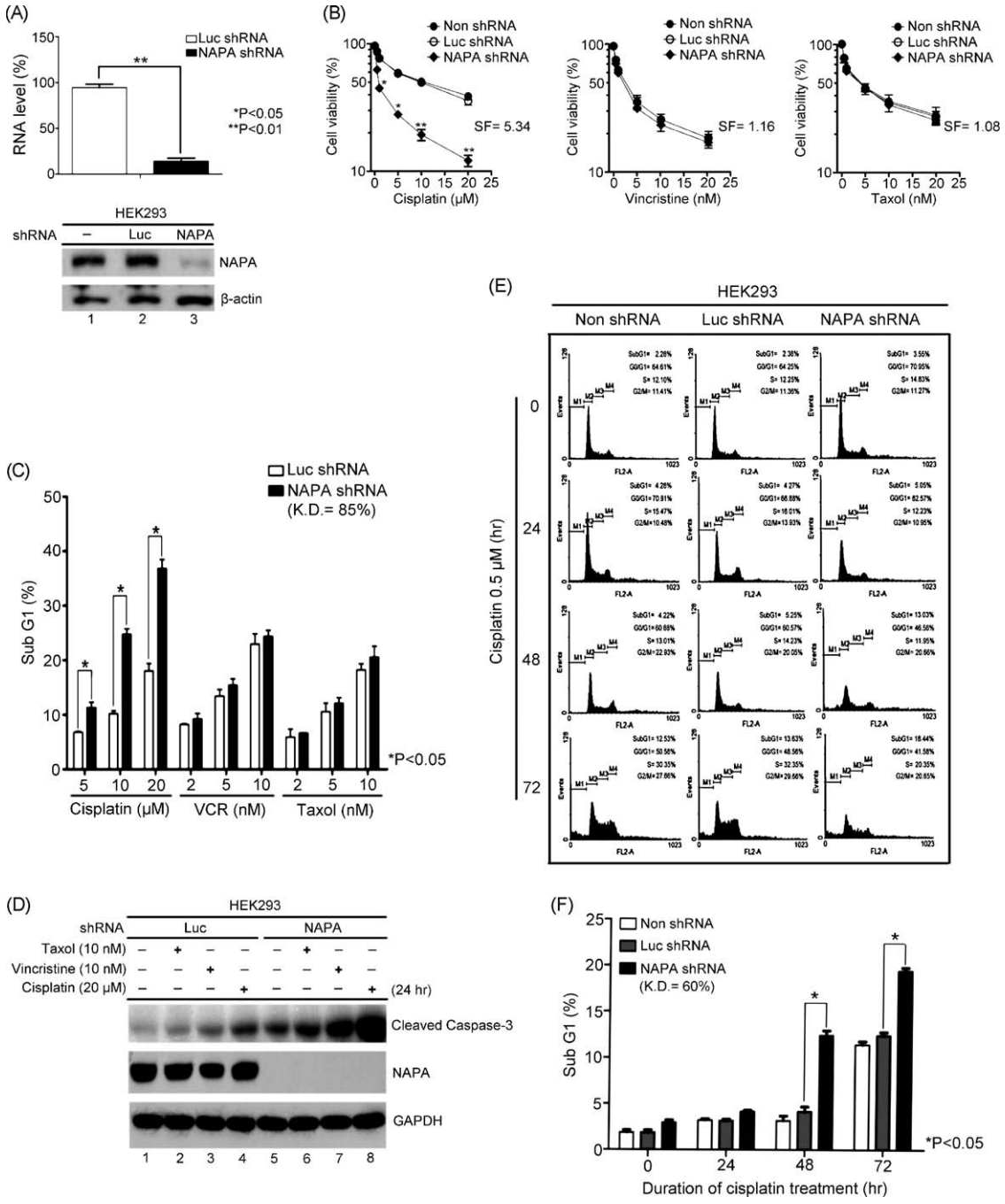

Fig. 2. Knockdown of *NAPA* sensitizes HEK293 cells to cisplatin. (A) The level of NAPA mRNA following transfection of NAPA-shRNA or luciferase (Luc)-shRNA in HEK293 cells was monitored by qPCR while the level of NAPA protein was examined by Western blot. (B) Sensitization of HEK293 cells to cisplatin following knockdown of *NAPA*. HEK293 cells expressing NAPA-shRNA were more susceptible to cisplatin when compared to controls (left panel), while no sensitization effect was noted for vincristine (middle panel) or taxol (right panel). Cell viability was assessed by the MTT assay. The sensitization factor (SF), which represents the ratio of IC_{50} for Luc-shRNA divided by IC_{50} for NAPA-shRNA, is shown for each panel. (C) Sensitization of HEK293 cells to cisplatin following knockdown of *NAPA*. Expression of NAPA-shRNA resulted in a dose-dependent effect on sub-G1 cell accumulation following treatment with cisplatin, but not with vincristine (VCR) or taxol. Sub-G1 cell accumulation was monitored by flow cytometry. The level of *NAPA* knockdown (K.D.) in this case reached 85% when assessed by qPCR. (D) Increased level of cleaved caspase-3 following treatment with cisplatin in NAPA-shRNA-expressing cells. Cells were exposed to near equitoxic concentrations of the indicated drugs for 24 h and the cleavage of caspase-3 and protein level of NAPA were verified by Western blot. Actin was added as a control for protein loading. (E) Cell cycle distribution of NAPA-shRNA-expressing HEK293 cells following treatment with cisplatin. Knockdown of *NAPA* resulted in a reduced S-phase blockage and accumulation of sub-G1 cells following treatment with cisplatin (0.5 μM) for the indicated period of time. (F) Knockdown of *NAPA* sensitizes HEK293 cells to cisplatin. In this case, NAPA-shRNA resulted in a 60% gene knockdown. The results are expressed as mean values ± SD for experiments performed in triplicate. The degree of significance (p value), relative to Luc-shRNA, was indicated in (C) and (F). The symbols * and ** represents $p < 0.05$ and $p < 0.01$, respectively.

3.7. Sensitization of HEK293 cells to cisplatin following combined knockdowns of CPR genes

As shown earlier, individual knockdown of *CPR* genes was sufficient to sensitize HEK293 cells to cisplatin. In order to verify whether *CPR* genes can have an additive effect in regulating drug sensitivity, we performed combined knockdowns of *CPR* genes. The combination of NAPA-shRNA (SF = 3.92) and CITED2-shRNA (SF = 1.67) produced an additive sensitization effect to cisplatin (SF = 6.67) when assessed by the cell viability assay (Fig. 5A).

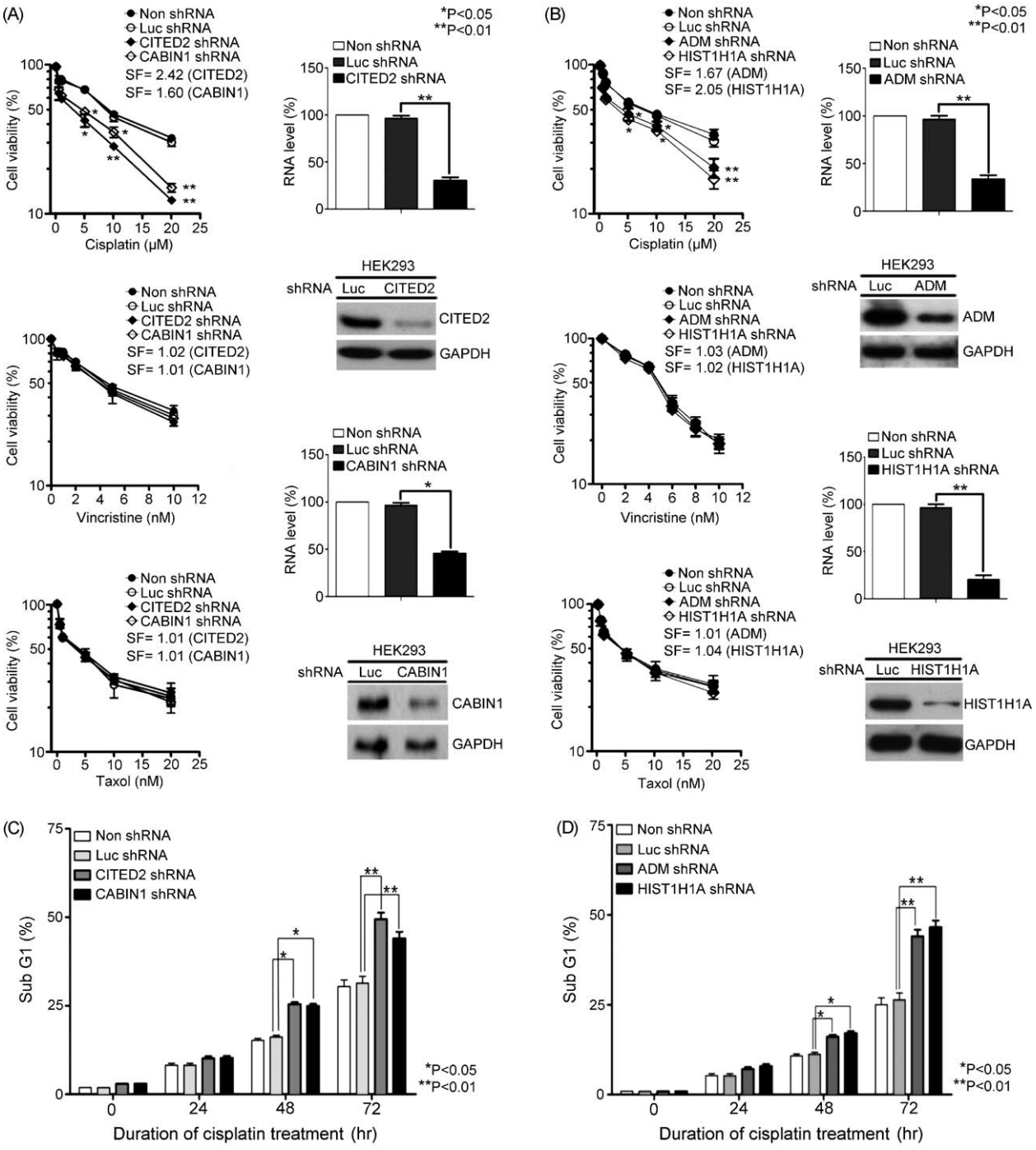

Fig. 3. *CPR* gene knockdown enhances cisplatin-induced decrease viability of HEK293 cells. (A) *CITED2* or *CABIN1* gene knockdown resulted in an increased of cisplatin-induced decrease cell viability following treatment with cisplatin (upper left panel), but not with vincristine (middle left panel) or taxol (lower left panel). Knockdown efficiency was monitored by qPCR and Western blot as shown on the right panels. GADPH was used as a loading control. (B) Knockdown of *CITED2* or *CABIN1* resulted in an increased sub-G1 cell population following treatment with cisplatin as assessed by flow cytometry. (C) Increased sensitization to cisplatin was noted following knockdown of *ADM* and *HIST1H1A* in HEK293 cells (upper left panel). No sensitization effect was noted when the cells expressing ADM-shRNA or HIST1H1A-shRNA were treated with vincristine (middle left panel) or taxol (lower left panel). The efficiency of gene knockdown is shown on the right. (D) Increase in cisplatin-induced sub-G1 accumulation in ADM- or HIST1H1A-shRNA-expressing HEK293 cells. The results are shown as mean values ± SD for experiments done in triplicate. The degree of significance (*p* value) was indicated in (B) and (D).

Similarly, the combination of CITED2-shRNA (SF = 1.67) and CABIN1-shRNA (SF = 1.54) also resulted in an additive sensitization effect to cisplatin (Fig. 5A, SF = 5.00). The combination of NAPA-shRNA and CABIN1-shRNA resulted in a MF of 4.10, suggesting that these two *CPR* genes may also have an additive effect in sensitizing HEK293 cells to cisplatin (Fig. 5A). To confirm these results, cisplatin-induced apoptotic, sub-G1 cell accumulation was also examined following combined knockdown of *CPR* genes (Fig. 5B). The various knockdown combinations also seemed to produce additive sensitization effects in this case (Fig. 5B).

Combined shRNA treatments appeared to produce an additive effect on sensitization to cisplatin when verified by caspase-3 activation (Fig. 5C). For example, a combination of NAPA-shRNA and CITED2-shRNA appeared to cause more intense PARP cleavage

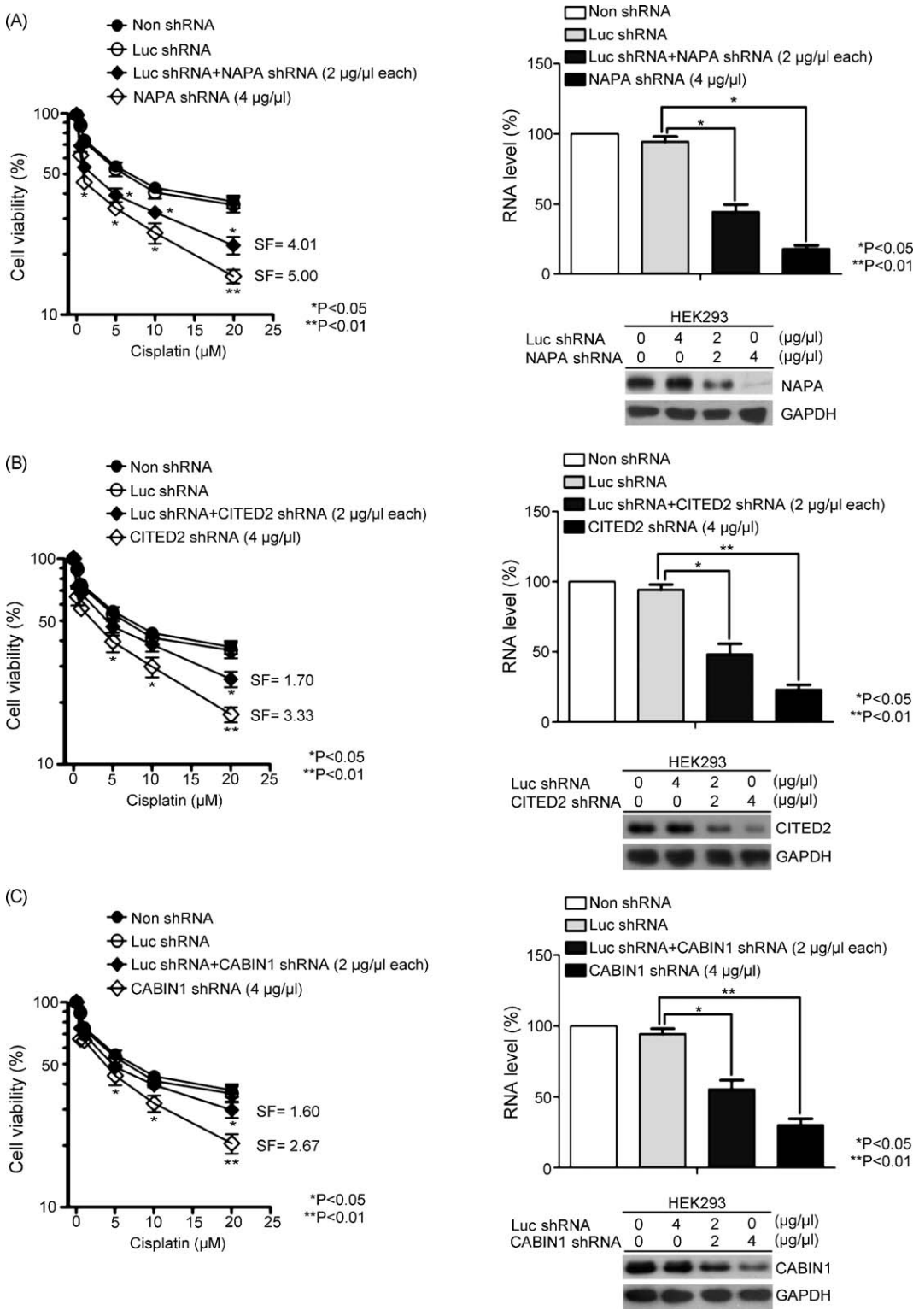

Fig. 4. shRNA dose-dependent sensitization effect to cisplatin following knockdown of *CPR* genes in HEK293 cells. (A) Sensitization to cisplatin was observed after knockdown of *NAPA* (2 μg/μl) when compared to Luc-shRNA control. Sensitization to cisplatin was increased by using a higher concentration of NAPA-shRNA (4 μg/μl). The cells were examined by the MTT cell viability assay. Knockdown of *CITED2* (B) and *CABIN1* (C) resulted in a similar shRNA dose-dependent sensitization effect to cisplatin in HEK293 cells. The concentrations of shRNA used for the knockdowns are indicated in each case. The effect of each knockdown on mRNA and protein levels is shown to the right of each panel (A–C). The results represent mean values ± SD for experiments performed in triplicate.

than the individual shRNA treatments (Fig. 5C, compare lane 14 with lanes 4 and 6). Surprisingly, the double knockdown of NAPA and CITED2 induced the cleavage of caspase-3 even without cisplatin treatment, suggesting that the simultaneous attenuation of these two components may somehow activate apoptosis. Similarly, a combination of CITED2-shRNA and CABIN1-shRNA also seemed to produce a more pronounced PARP cleavage when compared to each individual treatment (Fig. 5C, compare lane 12

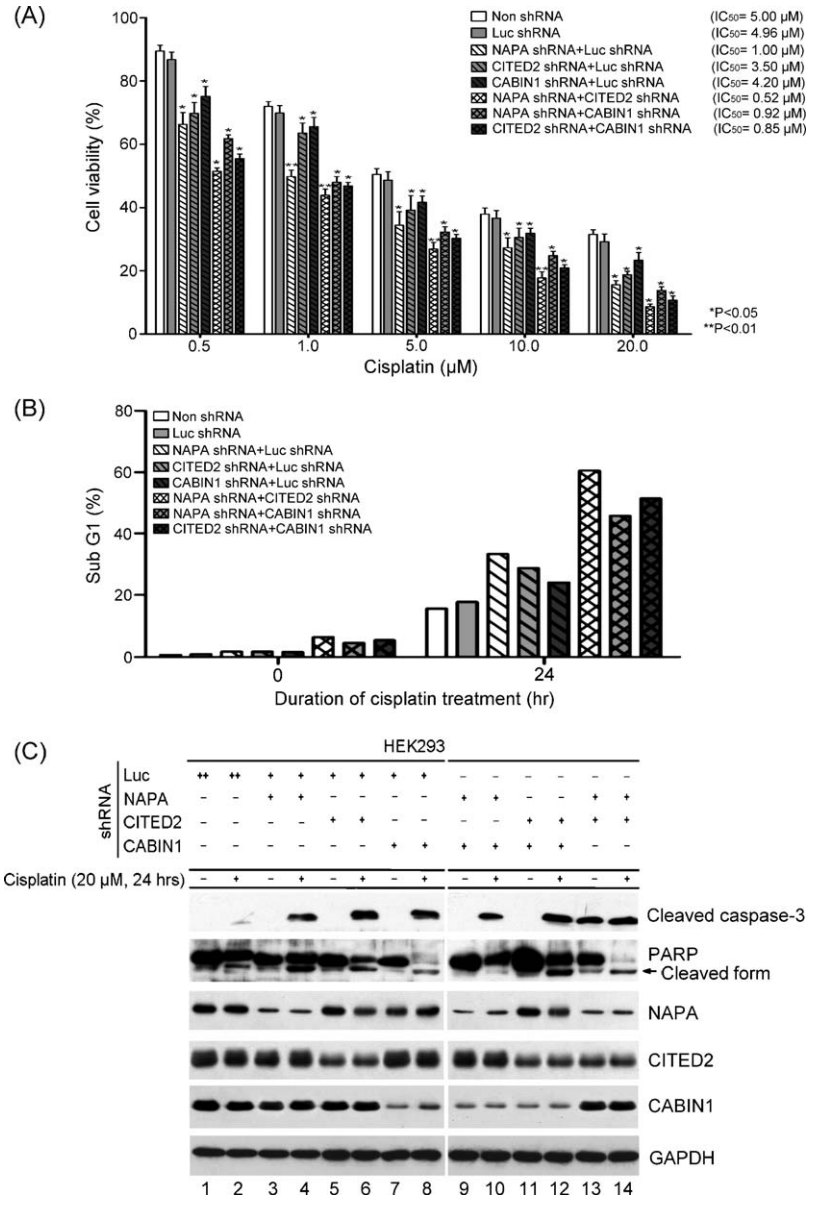

Fig. 5. Effect of combined *CPR* gene knockdowns in HEK293 cells. (A) Enhancement of cisplatin-induced decrease cell viability by combined shRNA knockdowns of *CPR* genes. Results of the MTT cell viability assays are expressed as means ± SD of triplicate experiment. The IC_{50} of each treatment was indicated. (B) Increase in cisplatin-induced sub-G1 accumulation following combined knockdowns of *CPR* genes. This graph is representative of the two flow cytometry experiments performed. (C) Caspase-3 and PARP activation following knockdown of *CPR* genes and cisplatin treatment. Protein levels were determined by Western blot.

with lanes 6 and 8). A corresponding increase of caspase-3 cleavage was less evident in this case (Fig. 5C). However, a combination of NAPA-shRNA and CABIN1-shRNA did not result in a greater activation of caspase-3 or PARP (Fig. 5C, compare lane 10 with lanes 4 and 8). Taken together, these results suggest that the *CPR* genes may act on different pathways in regulating cell sensitivity to cisplatin.

3.8. Knockdown of CPR genes reverses acquired resistance to cisplatin in HeLa cells

To verify the role of *CPR* genes in acquired cisplatin resistance, the effect of *NAPA* knockdown was studied in cisplatin-resistant HeLa cells. Knockdown of *NAPA* in HeLa and HeLa-R3 cells increased the sensitivity of these cells to cisplatin as monitored by the MTT cell viability assay (Fig. 6A and B, middle panels). NAPA protein levels were considerably reduced following shRNA treatments (Fig. 6A and B, left panels). The resistant cells displayed approximately a 10-fold resistance to cisplatin when compared to HeLa cells. Compared to the parental cells (Fig. 6A, middle panel, SF = 2.85), resistance of HeLa-R3 cells to cisplatin appeared to be reversed in these conditions (Fig. 6B, middle panel, SF = 13.33). Knockdown of *NAPA* in cisplatin-treated HeLa or HeLa-R3 cell lines also induced increased accumulation of sub-G1 cells compared to control (right panels of Fig. 6A and B, respectively; gene knockdown reached 90% in both cases). The increase of sub-G1 cells appeared to be more intense in the cisplatin-resistant cells when compared to the parental cells (Fig. 6A and B, right panels). Knockdown of the other *CPR* genes also increased sensitivity to cisplatin (upper panels of Fig. 6C for HeLa cells and Fig. 6D for HeLa-R3). CPR protein levels were considerably decreased following treatments with the corresponding shRNA (Fig. 6C and D, lower panels). Notably, knockdown of *CPR* genes consistently sensitized cells to cisplatin to a greater extent in HeLa-R3 compared to HeLa

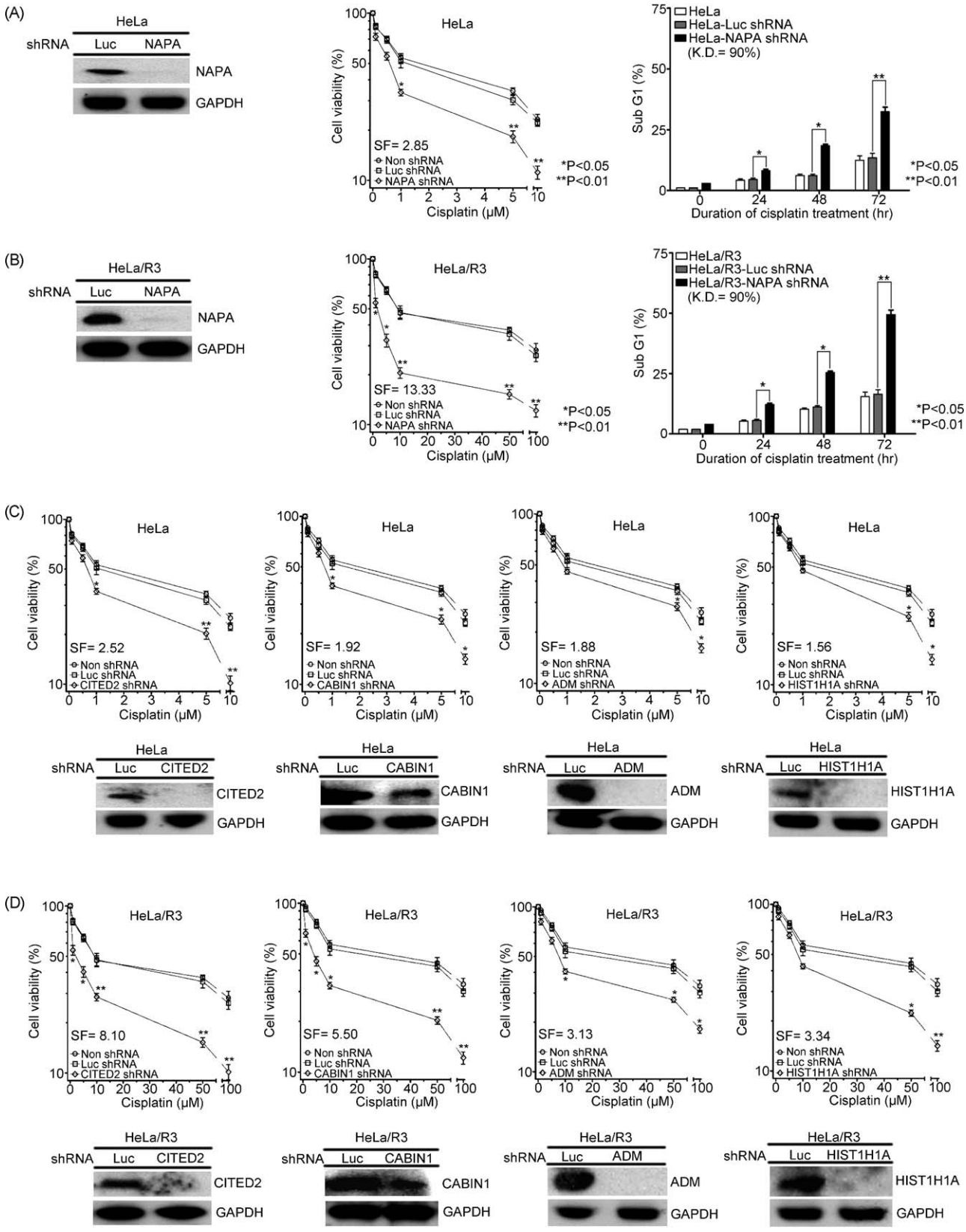

Fig. 6. Reversal of acquired cisplatin resistance following knockdown of *CPR* genes in cisplatin-resistant HeLa cells. (A) Knockdown of *NAPA* gene expression in HeLa cells (left panel, assessed by Western blot) sensitized these cells to cisplatin when assessed by the MTT assay (middle panel). Cisplatin-induced apoptosis was increased following knockdown of *NAPA* as shown by the accumulation of sub-G1 cells monitored by flow cytometry (right panel). (B) Knockdown of *NAPA* gene expression (left panel) also sensitized HeLa-R3 cells to cisplatin (middle panel). Knockdown of *NAPA* in resistant cells also resulted in a larger population of the sub-G1 phase when assessed by flow cytometry (right panel). Knockdown efficiency for these experiments shown in A and B reached 90% when verified by qPCR. (C) Knockdown of *CPR* genes sensitizes HeLa cells to cisplatin. (D) Knockdown of *CPR* genes sensitizes HeLa-R3 cells to cisplatin. Western blots showing reduction of *CPR* proteins in HeLa cells (C) and HeLa-R3 cells (D) are shown in the bottom panels. The results shown represent mean values ± SD of MTT cell viability experiments performed in triplicate.

cells. For instance, *CITED2* knockdown produced a SF of 8.10 in HeLa-R3 cells and a SF of 2.52 for HeLa cells. *HIST1H1A* knockdown caused a less pronounced effect, with an SF of 3.34 in HeLa-R3 cells and an SF of 1.56 in HeLa cells. These results indicate that *NAPA* may play a major role in acquired cisplatin resistance in the HeLa cell model used. Our results also suggest that the other CPR genes are involved in the establishment of cisplatin resistance.

Since the MTT cell viability assay may reflect both cytotoxic and cytostatic effects, we examined whether similar sensitization effects could be observed using assays which monitor more closely cell death. At low cisplatin dose, cytostatic effects may predominate while high doses may produce mainly cytotoxic effects. We treated HeLa and HeLa-R3 cells expressing various CPR shRNA with a low and a high dose of cisplatin, and measured cell death with the trypan blue exclusion assay (Supplementary Fig. S1). Knockdown of *CPR* genes enhanced cisplatin-induced cell death in both cell lines. We observed an increase of cell death following knockdown of NAPA, CITED, or CABIN1 in cells treated with a low dose of cisplatin compared to controls (Supplementary Fig. S1, $p < 0.05$). Higher cell death values were also observed for ADM and HIST1H1A knockdown when compared to control, but with significant difference between low and high doses of cisplatin (Supplementary Fig. S1, $p < 0.01$ vs. $p < 0.05$, respectively).

3.9. Knockdown of CPR genes sensitizes tumorigenic cells to cisplatin

In order to study the relevance of *CPR* gene during chemotherapy, we verified the effect of *CPR* gene knockdown on cells from tissues where cisplatin is mainly used during cancer treatment. The effect of *CPR* gene knockdown was investigated in the ovarian Sk-ov-3, lung H1155, and nasopharyngeal carcinoma (NPC) CG-1 cell lines, which are known to be tumorigenic. When the expression of *CPR* genes was monitored by qPCR, all of them, except *MVD*, were shown to be upregulated in Sk-ov-3 and H1255 cells when compared to HeLa cells (Fig. 7A). Knockdown of *NAPA* or *CITED2*, the two genes which produced the highest level of cisplatin

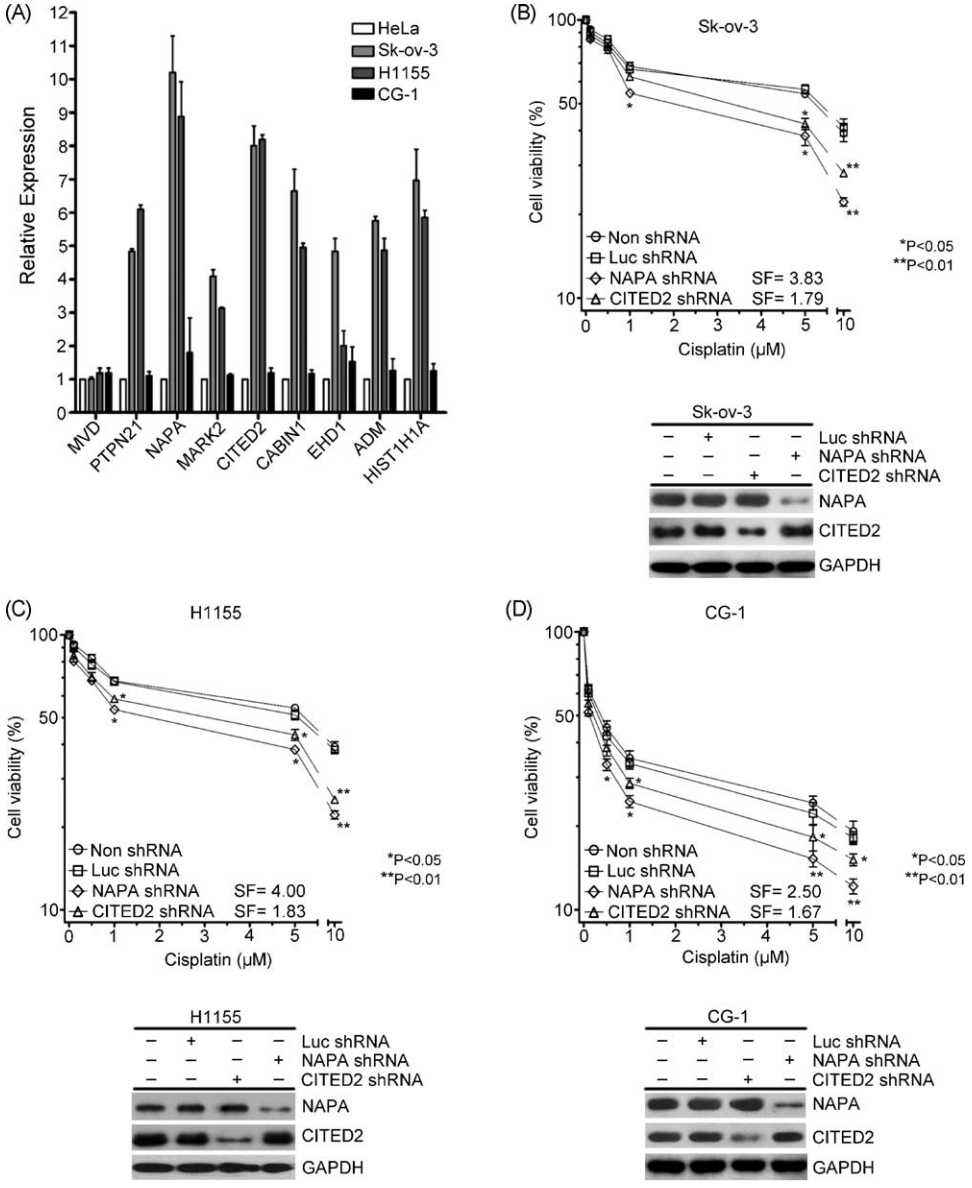

Fig. 7. Upregulation of *CPR* genes and sensitization to cisplatin following knockdown of *CPR* genes in tumorigenic cell lines. (A) Upregulation of *CPR* gene expression in tumorigenic cell lines. The level of mRNA in each cell line was estimated by qPCR. Sensitization to cisplatin after knockdown of *NAPA* or *CITED2* in ovarian Sk-ov-3 cells (B), lung H1155 cells (C), and nasopharyngeal CG-1 cells (D). Knockdown of protein levels in each cell line as assessed by Western blot. The results shown represent mean values ± SD of MTT cell viability experiments done in triplicate. MF values are indicated for each gene knockdown.

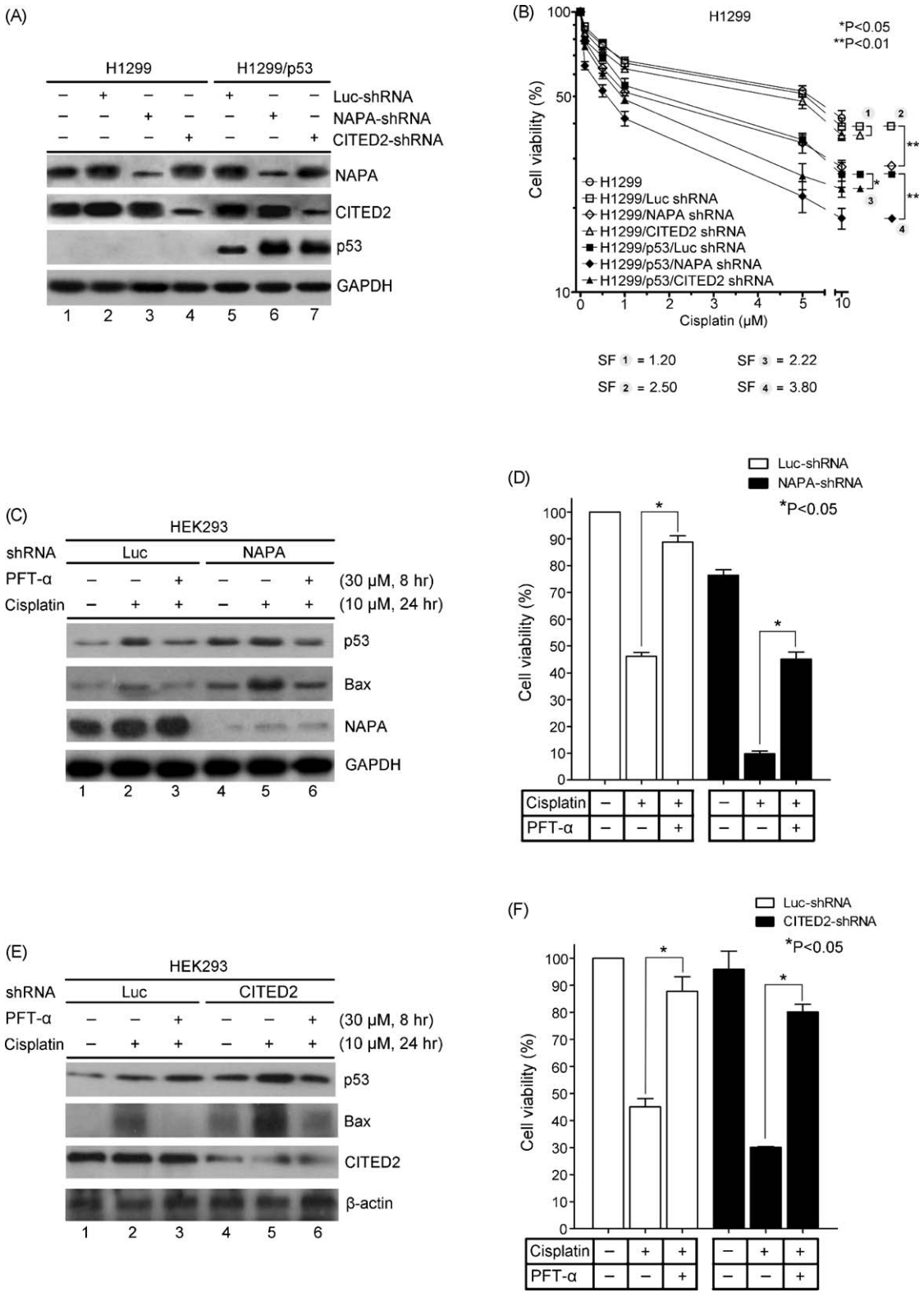

Fig. 8. Sensitization to cisplatin in tumorigenic cells following knockdown of *CPR* genes depends on p53. (A) Reduction of cisplatin-induced p53 activity in H1299 cells. The cells were transfected with the indicated shRNA and protein levels were assessed by Western blot. H1299 cells which are p53-null were used along with H1299 cells which overexpress functional p53. (B) Sensitization to cisplatin following *CPR* gene knockdown is enhanced by exogenous expression of p53 in H1299 cells. Cells were treated as for (A) and cell viability was assessed by the MTT assay. The MF values for the various paired treatments are shown. (C) Effect of cisplatin and *NAPA* gene knockdown on p53 and Bax protein levels. HEK293 cells were transfected with control Luc-shRNA (lanes 1–3) or *NAPA*-shRNA plasmids (lanes 4–6) for 72 h, followed by treatments with PFT-α and cisplatin as indicated. Western blot showed increased p53 and Bax following cisplatin treatment and reduced p53 and Bax in cells treated with PFT-α. (D) Influence of p53 inhibition on the viability of cisplatin-treated cells expressing NAPA-shRNA. Cells were treated as for (A) and monitored by the MTT assay. (E) Effect of cisplatin and *CITED2* gene knockdown on p53 and Bax protein levels by Western blot. (F) Influence of p53 inhibition on the viability of cisplatin-treated cells that express CITED2-shRNA. Cells were treated as for (A) and were evaluated by the MTT assay.

sensitization following knockdown in HeLa and HEK293 cells, was also tested in the three tumorigenic cell lines. Interestingly, knockdown *NAPA* or *CITED2* sensitized these cells to cisplatin in a manner similar to what we described earlier for HEK293 cells (Fig. 7B–D). The protein levels following each knockdown were shown for reference (Fig. 7B–D). While knockdown of *NAPA* in HeLa cells produced an SF of 2.85 (Fig. 6A), knockdown of *NAPA* in tumorigenic cells produced an even higher SF in both Sk-ov-3 (SF = 3.83) and H1155 cells (SF = 4.00) whereas an SF slightly lower was obtained for CG-1 cells (SF = 2.50). As such, we conclude that sensitization to cisplatin following *CPR* gene knockdown was also demonstrated in tumorigenic cells.

3.10. Sensitization to cisplatin by knockdown of CPR genes and p53 expression in tumorigenic cells

Since most tumorigenic cells harbor mutations in the p53 gene, this gene may influence the regulation of cisplatin sensitivity by CPR genes. To assess this possibility, H1299 lung cancer cells, which harbor p53-null alleles, were treated with shRNA to knockdown *NAPA* or *CITED2*, following by treatment with cisplatin. For comparison, p53 was re-introduced in these cells by transfection of a functional p53 allele. Protein amounts following gene knockdown were evaluated by Western blot (Fig. 8A). While knockdown of *NAPA* (SF2 = 2.50) or *CITED2* (SF1 = 1.20) increased sensitivity to cisplatin in H1299 cells, knockdown of *NAPA* or *CITED2* in H1299 cells overexpressing p53 produced higher SF values (Fig. 8B, *NAPA*, SF4 = 3.80; *CITED2*, SF3 = 2.22). As such, it appears that CPR genes may regulate cisplatin sensitivity in a p53-dependent manner. To further test this hypothesis, HEK293 cells with *NAPA* or *CITED2* gene knockdown were treated with cisplatin in the presence of PFT-α, an inhibitor of p53. A representative cisplatin treatment (10 μM, 24 h) led to the accumulation of p53 protein and its target gene product Bax (Fig. 8C, compare lanes 2 and 1). This effect was partially inhibited when the cells were pre-treated with PFT-α (Fig. 8C, compare lanes 3 and 2). More pronounced effects on p53 and Bax accumulation following cisplatin treatment was found in HEK293 cells expressing NAPA-shRNA (Fig. 8C, lanes 4–6). Notably, knockdown of NAPA alone induced the accumulation of p53 and Bax proteins (Fig. 8C, compare lanes 4 and 1).

The sensitivity of these cells to cisplatin was then analyzed using the MTT cell viability assay (Fig. 8D). Cisplatin treatment reduced cell viability by more than 50%, but this process was almost completely rescued by PFT-α (Fig. 8D). While knockdown of *NAPA* alone decreased cell viability by more than 20%, cisplatin produced a 90% decrease in cell viability, a phenomenon which could be partially prevented by inhibition of p53 with PFT-α (Fig. 8D). Similar changes of p53 and Bax protein levels and cell response to cisplatin were obtained following *CITED2* knockdown (Fig. 8E and F). Taken together, these observations suggest that sensitization to cisplatin following knockdown of *NAPA* or *CITED2* may be mediated by p53.

4. Discussion

In the present study, we observed that the expression of several genes was upregulated in cisplatin-resistant HeLa cancer cells when examined by DNA microarrays and qPCR. Several of the upregulated CPR genes identified were implicated in apoptosis while others were classified in categories such as regulation of cell cycle and DNA repair (Supplementary Table 2). This finding was not surprising since cisplatin-induced cell death is often associated with non-classical apoptosis pathways [19]. Notably, we observed that down-regulation of CPR genes sensitized several types of human cancer cells to cisplatin. Moreover, down-regulation of these genes was also shown to reverse acquired cisplatin resistance in HeLa cells. Therefore, the results presented in this study support the concept that the CPR genes identified may be involved in resistance to cisplatin.

Although the mechanism of sensitization to cisplatin by *CPR* gene knockdown remains to be determined, our preliminary observations suggest that this effect is unlikely to be due to increased cisplatin uptake in knockdown cells. For instance, we observed no significant difference in intracellular cisplatin between control and *NAPA*-shRNA-expressing HEK293 cells using mass spectrophotometry (Supplementary Fig. S2A). In addition, we observed that MRP2 protein, which is thought to correlate positively with cisplatin resistance in various cell lines, was slightly decreased in NAPA knockdown cells (Supplementary Fig. S2B). From another perspective, it is possible that the sensitization to cisplatin following NAPA knockdown may be associated with reduced DNA repair since we found that repair gene products such as XRCC1 and ERCC1 appeared to recover more slowly in NAPA knockdown cells than in controls after removal of cisplatin (Supplementary Fig. S2C). Similarly, the level of γ-H2AX, which reflects repair of DNA adducts, appeared to recover more slowly following knockdown of NAPA when compared to control HEK293 cells (Supplementary Fig. S2C). Further experiments would be needed to determine the mechanism of action of *CPR* genes in sensitizing cancer cells to cisplatin.

Among the *CPR* genes identified, *NAPA* and *CITED2* are especially interesting since they provide a link with the tumor suppressor p53. Knockdown of *NAPA* and *CITED2* genes produced an additive effect in increasing sensitivity to cisplatin. Yet, these two gene products are unlikely to interact in cancer cells since they are located in different cellular compartments. That is, *NAPA* encodes a protein which is found in the endoplasmic reticulum (ER) [20] whereas *CITED2* encodes a member of the CITED family of transcriptional regulatory proteins found in the nucleus [21]. For one, *NAPA* represents an adaptor protein that serves as a link between the chaperone ATPase N-ethylmaleimide-sensitive factor (NSF) and several SNAREs. In addition, it has been shown that the cytoplasmic degradation of p53 is mediated by the ER-resident ubiquitin ligase synoviolin [22]. Together, these observations may be linked to our results that knockdown of *NAPA* resulted in a p53-dependent sensitization to cisplatin (Fig. 8).

We also observed that knockdown of *CITED2* sensitized HEK293 cells to cisplatin. Notably, a recent study showed that the knockdown of *CITED2* using siRNA in platinum complex-resistant ovarian cancer cells improved the sensitivity of the cells to platinum compounds [23]. DNA-damage-induced phosphorylation of p53 enhances the association of this protein with the CBP/p300 transcriptional coactivators which results in the increased acetylation and stability of p53 [24–26]. Accordingly, our preliminary data indicate that cisplatin treatment readily resulted in p53 acetylation at lysine 373 in the cell system used (unpublished observations). Taken together, these observations suggest that the sensitization of cells to cisplatin by *CITED2* knockdown may be due to increased p53 acetylation, which in turn may prevent the degradation of this protein. In this case, both *CITED2* and *NAPA* may represent targets of cisplatin and may regulate drug sensitivity by influencing the stability of p53. This model could represent a new molecular mechanism to explain cisplatin resistance in cancer cells.

On the other hand, it is possible that *CPR* genes also regulate drug resistance independently of p53 since we observed that knockdown of *CPR* genes also sensitized tumorigenic lung H1299 cells and ovarian SK-ov-3 cells to cisplatin even though these cells harbor mutant p53 alleles. Besides, it has been reported that BNIP1 is a component of a complex comprising syntaxin 18, a SNARE

protein consisting of a receptor for NSF attachment protein (SNAP), which is located in the ER. BNIP1 plays an important role not only in the induction of apoptosis, but also in the binding of NAPA (also called α-SNAP) [27]. Instead of influencing p53, it is possible that NAPA may suppress apoptosis alternatively by competing with anti-apoptotic proteins for BNIP1.

Our findings indicate that *CPR* genes are inducible by cisplatin treatment. The induction of *CPR* genes may be explained by the activation of upstream kinase pathways following treatment with cisplatin. JNK in the MAP kinase pathways is activated in cells following cisplatin treatment. Activation of AP-1, which is downstream of JNK, and subsequent trans-activation of several target genes is responsible for cell response to cisplatin. Notably, our analysis of the 5′-end upstream sequence of *CPR* genes (10 kbp) using Match 1.0 Public (BIOBASE Corporation, Beverly) suggests that at least some of them may be regulated by the transcription factors AP-1, HNF-1, or USF (data not shown). It has been demonstrated that the induction of cellular genes by cisplatin occurs at least partially via activation of AP-1, thus leading to gene expression dominated mainly by DNA repair genes [28]. Indeed, the gene showing the highest degree of alteration in gene expression was a member of the AP-1 transcription complex family of proteins, which is not only implicated in the regulation of expression of many genes, but may be critical in determining cell response to external stimuli [29]. Based on these observations, we suggest that the members of the AP-1 transcription factor family may contribute to produce differential gene regulation and cell responses during chemoresistance. Further studies would be required to explore the prevalence of these changes in primary cancers.

Other *CPR* genes identified in the present study have been shown to have anti-apoptotic properties. For example, upregulation of CABIN1, which represents an endogenous inhibitor of calcineurin, is associated with inhibition of apoptosis induced by ER stress [30]. Given that the functions of the other *CPR* genes on the regulation of apoptosis or cell growth are less understood, this topic could be explored further in future studies.

We observed that the sensitization effect to cisplatin following knockdown of *CPR* genes was not detected in cells treated with mitotic damaging agents such as vincristine or taxol, suggesting that resistance to these compounds may proceed through different mechanisms. Consistent with this idea, phosphatidylinositol 3-kinase/AKT was found to be associated with taxol resistance based on the results of a Connectivity Map, whereas this association was not found for cisplatin resistance [31]. Alternatively, a molecule or pathway may be involved in cell resistance to both DNA-damage and mitotic damage agents. For example, the Bcl-2 pathway is associated with cisplatin resistance and the AKT pathway is enriched in both cisplatin- and taxol-resistant lung cancer cell lines [31].

In conclusion, the present study identified several *CPR* genes which may play a role in establishing chemoresistance to cisplatin. These results suggest that down-regulation of *CPR* gene could be used to limit cisplatin resistance during cancer therapy. In addition, our work supports the idea that genome-wide analysis of gene expression patterns in cells resistant to chemotherapeutic drugs can lead to the identification of genes involved in resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.03.029.

References

[1] Chao CC. Molecular basis of cis-diamminedichloroplatinum(II) resistance: a review. J Formos Med Assoc 1996;95:893–900.
[2] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
[3] Baird RD, Kaye SB. Drug resistance reversal—are we getting closer? Eur J Cancer 2003;39:2450–61.
[4] Dempke W, Voigt W, Grothey A, Hill BT, Schmoll HJ. Cisplatin resistance and oncogenes—a review. Anticancer Drugs 2000;11:225–36.
[5] Niedner H, Christen R, Lin X, Kondo A, Howell SB. Identification of genes that mediate sensitivity to cisplatin. Mol Pharmacol 2001;60:1153–60.
[6] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 2005;4:307–20.
[7] Stewart DJ. Mechanisms of resistance to cisplatin and carboplatin. Crit Rev Oncol Hematol 2007;63:12–31.
[8] Wulfkuhle J, Espina V, Liotta L, Petricoin E. Genomic and proteomic technologies for individualisation and improvement of cancer treatment. Eur J Cancer 2004;40:2623–32.
[9] Potti A, Dressman HK, Bild A, Riedel RF, Chan G, Sayer R, et al. Genomic signatures to guide the use of chemotherapeutics. Nat Med 2006;12:1294–300.
[10] Chao CC, Lee YL, Cheng PW, Lin-Chao S. Enhanced host cell reactivation of damaged plasmid DNA in HeLa cells resistant to cis-diamminedichloroplatinum(II). Cancer Res 1991;51:601–5.
[11] Chang YS, Lin SY, Lee PF, Durff T, Chung HC, Tsai MS. Establishment and characterization of a tumor cell line from human nasopharyngeal carcinoma tissue. Cancer Res 1989;49:6752–7.
[12] Chao CC. Selective drug efflux in multidrug-resistant immunoblastic B lymphoma cells with overexpressed P-glycoprotein. Eur J Pharmacol-Environ Toxicol Pharmacol 1996;1:63–72.
[13] Chao CC, Ma CM, Cheng PW, Lin-Chao S. Increased mdr gene expression and decreased drug accumulation in a human colonic cancer cell line resistant to hydrophobic drugs. Biochem Biophys Res Commun 1990;172:842–9.
[14] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[15] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[16] Yang YH, Dudoit S, Luu P. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002;30:e15.
[17] Darzynkiewicz Z, Bruno S, Del BG, Traganos F. The cell cycle effects of camptothecin. Ann NY Acad Sci 1996;803:93–100.
[18] Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285:1733–7.
[19] Gonzalez VM, Fuertes MA, Alonso C, Perez JM. Is cisplatin-induced cell death always produced by apoptosis? Mol Pharmacol 2001;59:657–63.
[20] Clary DO, Griff IC, Rothman JE. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell 1990;61:709–21.
[21] Martinez-Barbera JP, Rodriguez TA, Greene NDE, Weninger WJ, Simeone A, Copp AJ, et al. Folic acid prevents exencephaly in Cited2 deficient mice. Hum Mol Genet 2002;11:283–93.
[22] Yamasaki S, Yagishita N, Nishioka K, Nakajima T. The roles of synoviolin in crosstalk between endoplasmic reticulum stress-induced apoptosis and p53 pathway. Cell Cycle 2007;11:1319–23.
[23] Yanagie H, Hisa T, Ogata A, Miyazaki A, Nonaka Y, Nishihira T, et al. Improvement of sensitivity to platinum compound with siRNA knockdown of upregulated genes in platinum complex-resistant ovarian cancer cells in vitro. Biomed Pharmacother 2009;63:553–60.
[24] Vassilev A, Yamauchi J, Kotani T, Prives C, Avantaggiati ML, Qin J, et al. The 400lkDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. Mol Cell 1998;2:869–75.
[25] Ito A, Lai CH, Zhao X, Saito S, Hamilton MH, Appella E, et al. p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J 2001;20:1331–40.
[26] Shi Y, Venkataraman SL, Dodson GE, Mabb AM, LeBlanc S, Tibbetts RS. Direct regulation of CREB transcriptional activity by ATM in response to genotoxic stress. Proc Natl Acad Sci USA 2004;101:5898–903.
[27] Nakajima K, Hirose H, Taniguchi M, Kurashina H, Arasaki K, Nagahama M, et al. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. EMBO J 2004;23:3216–26.

[28] Hayakawa J, Mittal S, Wang Y, Korkmaz KS, Adamson E, English C, et al. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. Mol Cell 2004;16:521–35.
[29] Macleod K, Mullen P, Sewell J, Rabiasz G, Lawrie S, Miller E, et al. Altered erbB receptor signaling and gene expression in cisplatin-resistant ovarian cancer. Cancer Res 2005;65:6789–800.
[30] Kim M-J, Jo D-G, Hong G-S, Kim BJ, Lai M, Cho D-H, et al. Calpain-dependent cleavage of cain_cabin1 activates calcineurin to mediate calcium-triggered cell death. Proc Natl Acad Sci USA 2002;99:9870–5.
[31] Riedel RF, Porrello A, Pontzer E, Chenette EJ, Hsu DS, Balakumaran B, et al. A genomic approach to identify molecular pathways associated with chemotherapy resistance. Mol Cancer Ther 2008;10:3141–9.