Lentiviral short hairpin RNA screen of genes associated with multidrug resistance identifies PRP-4 as a new regulator of chemoresistance in human ovarian cancer

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Abstract

Published reports implicate a variety of mechanisms that may contribute to drug resistance in ovarian cancer. The chief aim of this study is to understand the relationship between overexpression of drug resistance associated genes and multidrug resistance in ovarian cancer. Using lentiviral short hairpin RNA collections targeting 132 genes identified from transcriptional profiling of drug-resistant cancer cell lines, individual knockdown experiments were done in the presence of sublethal doses of paclitaxel. Specific genes whose knockdown was found to be associated with cellular toxicity included MDR1 (ABCB1), survivin, and pre-mRNA processing factor-4 (PRP-4). These genes, when repressed, can reverse paclitaxel resistance in the multidrug-resistant cell line SKOV-3$_{TR}$ and OVCAR8$_{TR}$. Both MDR1 and survivin have been reported previously to play a role in multidrug resistance and chemotherapy-induced apoptosis; however, the effect of PRP-4 expression on drug sensitivity is currently unrecognized. PRP-4 belongs to the serine/threonine protein kinase family, plays a role in pre-mRNA splicing and cell mitosis, and interacts with CLK1. Northern analysis shows that PRP-4 is overexpressed in several paclitaxel-resistant cell lines and confirms that PRP-4 expression could be significantly repressed by PRP-4 lentiviral short hairpin RNA. Both clonogenic and MTT assays confirm that transcriptional repression of PRP-4 could reverse paclitaxel resistance 5-10-fold in SKOV-3$_{TR}$. Finally, overexpression of PRP-4 in drug-sensitive cells could induce a modest level of drug resistance to paclitaxel, doxorubicin, and vincristine. [Mol Cancer Ther 2008;7(8):2377–85]

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Introduction

Standard chemotherapy for newly diagnosed and recurrent ovarian cancer includes a combination of paclitaxel and carboplatin. Although objective responses and survival benefits are seen, the efficacy of both of these agents is limited by the eventual development of multidrug resistance (1, 2). There is little understanding of how ovarian tumors, as well as other tumors, develop drug resistance. Published reports from several laboratories implicate several mechanisms that contribute to the drug resistance phenotype. Reversing drug resistance has been an important goal of clinical and investigational oncology (3–7).

Gene expression profile analysis is an efficient technology that allows screening for correlations between expression of many genes and acquisition of multidrug resistance. In an attempt to identify novel genes that are differentially expressed between paclitaxel-resistant and paclitaxel-sensitive cells, the paclitaxel-sensitive ovarian cancer cell lines, SKOV-3 and OVCAR8, and the paclitaxel-sensitive breast cancer cell line, MCF-7, were exposed to incrementally increasing concentrations of paclitaxel. This procedure resulted in the establishment of three paclitaxel-resistant daughter cell lines, SKOV-3$_{TR}$, OVCAR8$_{TR}$, and MCF-7$_{TR}$, respectively (4, 8). Gene expression profiles in these three paclitaxel-resistant cell lines and their corresponding paclitaxel-sensitive parental lines (SKOV-3 versus SKOV-3$_{TR}$, OVCAR8 versus OVCAR8$_{TR}$, and MCF-7 versus MCF-7$_{TR}$) were characterized using Affymetrix microarray technology. A large number of transcripts were identified as differentially expressed between pairs of sensitive and resistant cell lines; 790 (SKOV-3$_{TR}$), 689 (OVCAR8$_{TR}$), and 964 (MCF-7$_{TR}$) gene transcripts showed >2-fold overexpression in the paclitaxel-resistant lines relative to their expression in the parental lines (8). Although SKOV-3$_{TR}$, OVCAR8$_{TR}$, and MCF-7$_{TR}$ all show a paclitaxel-resistant phenotype, the transcripts identified with altered expression in each cell line pair were largely nonoverlapping and encoded proteins with a wide variety of biochemical functions. Establishing a strong correlation between drug sensitivity and expression of a particular gene has been challenging (9).

The ability to use RNA interference as a tool for functional gene silencing in tumor cells has enabled us to

perform genetic loss-of-function studies in tissue culture systems (10, 11). However, although small interfering RNA has been shown to be effective for short-term gene inhibition in mammalian cell lines, there is a clear problem in its use for stable transcript knockdown (12). Recently, short hairpin RNA (shRNA) libraries in lentiviral vectors have been described and used in stable cell lines and in transgenic mice (13, 14). Our aim was to identify genes essential for drug resistance by screening for shRNAs that selectively reverse drug resistance in cancer cell lines. This type of screen holds promise for the discovery of novel targets in reversing drug resistance in cancer therapy and for genetically validating combination therapies.

In the present study, we used the above-mentioned collection of 132-lentiviral shRNA constructs targeting the expression of drug resistance associated genes to address whether loss of these transcripts impact paclitaxel sensitivity *in vitro*. This preselected shRNA library was drawn from our previous cDNA array studies; the screen has been carried out in the well-characterized ovarian cancer paclitaxel-resistant cell line SKOV-3$_{TR}$ (4, 7, 8). We show that inhibition of several target genes could sensitize SKOV-3$_{TR}$ to paclitaxel. Specifically, we observe that knockdown of human pre-mRNA processing factor-4 (PRP-4) kinase in ovarian cancer multidrug-resistant cells is associated with increased sensitivity to paclitaxel. Additionally, we observe that PRP-4 kinase is overexpressed in multidrug-resistant ovarian cancer cell lines. Furthermore, we have identified a set of drug resistance associated genes that, when silenced, cause cell sensitivity to paclitaxel, several of which [such as ABCB1/MDR1, murine double minute 2 (MDM2), and survivin] have been shown previously to be involved in multidrug resistance (11, 15–17). This observation suggests that overexpression of PRP-4 is a new mechanism for the acquisition of drug resistance and therefore a potential target for the development of therapies treating drug resistance in ovarian cancer.

Materials and Methods

Cell Culture

The human ovarian cancer cell line SKOV-3 used in this study was obtained from the American Type Culture Collection. Dr. Patricia Donahoe (Massachusetts General Hospital) provided the OVCAR8 human ovarian cancer cell line. The paclitaxel-resistant SKOV-3$_{TR}$ and OVCAR8$_{TR}$ cell lines were established as reported previously (4, 7, 18) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (all obtained from Invitrogen). Resistant subclones were continuously cultured in paclitaxel. Paclitaxel, doxorubicin, vincristine, and cisplatin were obtained as unused residual clinical material at the Massachusetts General Hospital.

Drug Resistance Associated Genes Lentiviral shRNA Library

The genes were chosen based on their relevance to multidrug resistance in human ovarian cancer from our

previous studies (4, 8). Based on 291 gene transcripts that showed overexpression in at least two resistant cell lines, we constructed a lentiviral shRNA library composed of 132 genes at Sigma-Aldrich Research Biotechnology. Details of the library production methods can be found at the Sigma Web site.[5] In particular, these genes were preselected because they also included full-length open reading frames. These open reading frames will be useful for confirmatory functional studies in which full-length transcripts would be transfected back into sensitive cell lines.
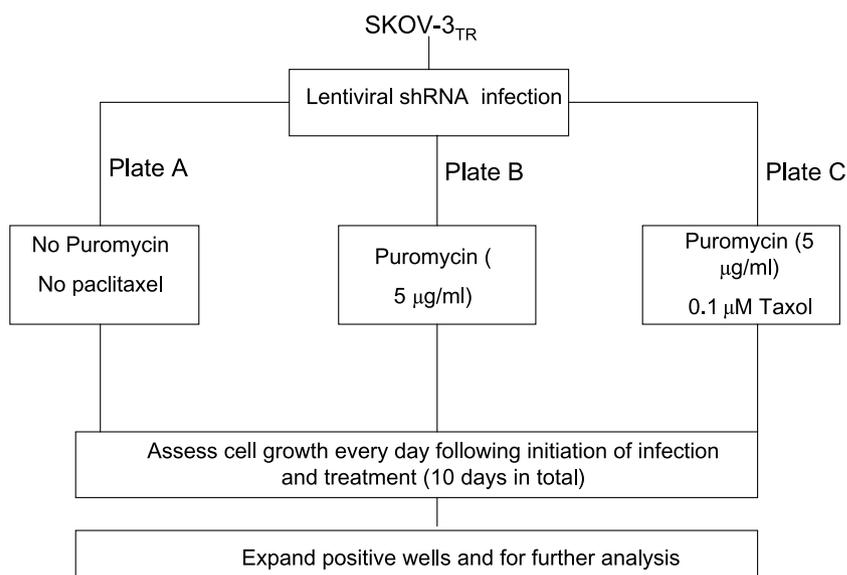
Lentiviral Infection

Infection conditions in SKOV-3$_{TR}$ were optimized with MISSON TurboGFP Control Transduction particles (SHC003V) and Nontarget shRNA Control Transduction particles (SHC002V; Sigma) in 96-well plates for optimal growth conditions, viral dosage, puromycin selection concentration, and assay times before screening. On day 1, SKOV-3$_{TR}$ cells were seeded at 1.6×10^4 per well in triplicate on 96-well plates and labeled with plate A, B, or C and incubated for 24 h at 37°C (Fig. 1). On day 2, 8 µg/mL hexadimethrine bromide were added to each well and infected using 8 µL lentiviral particles encoding shRNA against different drug resistance associated genes to appropriate wells and incubated for 24 h at 37°C. On day 3, plate A was kept as the control without the addition of puromycin or paclitaxel, the shRNA viral particle-containing medium was removed and replaced it with fresh medium. For plate B, viral particle-containing medium was removed and replaced with fresh medium containing 5 µg/mL puromycin for selection of transduced cells. For plate C, viral particle-containing medium was removed and replaced with fresh medium containing 5 µg/mL puromycin and 0.1 µmol/L paclitaxel. From days 4 to 10, fresh medium was replaced with necessity as described above and evaluated for cytotoxicity in any wells under the microscope. On day 10, the number of viable cells was determined via CellTiter 96 AQ$_{ueous}$ One Solution Cell Cytotoxicity Assay (Promega).

Determination of the Results and Analysis

The general format was designed to include evaluation of each target gene through shRNA lentiviral-mediated gene knockdown. The experiments were conducted in two control plates and one experimental plate to permit plate-to-plate comparisons (Fig. 1). The control plates (plates A and B) were used to evaluate transduction efficiency and to exclude target genes that were lethal during knockdown in the absence of chemotherapy. The latter control (plate B) was necessary because the goal of this study was to identify genes that are important in acquired drug resistance and not cell survival. Therefore, one plate (plate A) was given only lentivirus to confirm that the shRNA is not lethal in the absence of puromycin and paclitaxel (a positive result is cell survival); a second plate (plate B) was exposed to puromycin and lentivirus to confirm efficiency of infection

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[5] http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Functional_Genomics_and_RNAi/shRNA

Figure 1. Flowchart of the functional lentiviral shRNA screen measuring the effect of drug-resistant gene knockdown on paclitaxel sensitivity. The drug resistance associated 132-lentiviral shRNA library was established. For the screen, 1.6×10^4 SKOV-3$_{TR}$ cells per well in 96-well plates were infected with lentiviral particles encoding shRNA against different drug resistance associated genes. The infected cells were subsequently selected with puromycin following a protocol provided by the manufacturer. The number of viable cells was analyzed using the CellTiter 96 AQ$_{ueous}$ One Solution Cell Cytotoxicity Assay and visualized under the microscope.

SKOV-3$_{TR}$

Lentiviral shRNA infection

Plate A — No Puromycin / No paclitaxel

Plate B — Puromycin (5 μg/ml)

Plate C — Puromycin (5 μg/ml) / 0.1 μM Taxol

Assess cell growth every day following initiation of infection and treatment (10 days in total)

Expand positive wells and for further analysis

(once again a positive result is cell survival). The third plate (plate C) was given both lentiviral shRNA and 0.1 μmol/L paclitaxel. This is typically a sublethal dose of paclitaxel for the SKOV-3$_{TR}$ cell line and a positive result would be cell death at 4 to 10 days. shRNA targeted genes that are associated with cells surviving in plates A and B and dying in plate C were identified as "hits" and selected for further study. Each of the 132 genes is represented by three to five different shRNA lentiviral particle constructs targeting different sites in each gene. Because these shRNAs are complimentary to different regions of the mRNA, it reduces the risk of "off-target effects." To further minimize the possibility of "off-target" hits, we only focused on genes identified as functional drug resistance genes by two or more shRNA targeting the same gene. All experiments used cells infected with a MISSION nontarget vector control virus (Sigma) as a negative control for expression changes. Once hits were identified, we validated that the targeted genes indeed have been knocked down by reverse transcription-PCR (RT-PCR) and Northern analysis.

RNA Extraction

RNA was collected from SKOV-3 and SKOV-3$_{TR}$. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Additionally, RNA quality was determined via ethidium bromide staining following agarose/formaldehyde gel electrophoresis.

Reverse Transcription-PCR

For the PRP-4 gene, the results from the shRNA screening were verified using RT-PCR and Northern blot analysis. RT-PCR was done using the sense and antisense primers to human PRP-4 sense primer (5′-ATAAGAAT*GCGG-CCCGCGGAAGTTCAAGATGGCCGCCG*-3′) to introduce a *Not*I site as italicized and antisense primer (5′-GGT-*GGATCC*CACACACTCAAACCCTTGGAG-3′; GenBank no. NM_003913) to introduce a *Bam*HI site as italicized. The introduced restriction enzyme sites were designed for following confirmatory functional studies. TRIzol-extracted total RNA was DNase treated to remove contaminating genomic DNA according to the manufacturer's protocol (Invitrogen). RT-PCRs were done using the Titan One Tube RT-PCR systems (Roche) following the manufacturer's protocol.

Northern Analysis

PRP-4 RT-PCR products were cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen) and the cloned fragment was confirmed by sequencing. The cDNA inserts were cut using *Not*I and *Bam*HI (Promega) and purified using the QIAEXII Gel Extraction Kit (Qiagen). PRP-4 probes were labeled with [^{32}P]dCTP using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). Total RNA was extracted using the TRIzol reagents as described above. RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels (~5 μg total RNA/lane), transferred to Hybond N-plus nylon membranes (Amersham Biosciences), and UV cross-linked. A 1 h prehybridization step was done in Rapid-hyb buffer (Amersham Biosciences) followed by a 2 h hybridization of the ^{32}P-labeled PRP-4 probe in the same buffer. The blots were washed twice at room temperature with 2× SSC-0.1% SDS for 15 min and twice at 65°C with 0.2× SSC-0.1% SDS for 15 min. Blots were exposed to autoradiography X-ray film with an intensifying screen. Finally, to confirm the amounts of RNA loaded in each lane, blots were hybridized with a β-actin probe. Relative quantification was done using densitometry.

Cytotoxicity Assay

Chemotherapy drug cytotoxicity was assessed *in vitro* using the MTT assay as described previously (19). Briefly, 2×10^3 cells per well were plated in 96-well plates in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin) containing increasing concentrations of chemotherapy drug such as paclitaxel. After 7 days of culture, 10 μL MTT (5 mg/mL in PBS, obtained from Sigma) was added to each well and the

plates were incubated for 4 h. The resulting formazan product was dissolved with acid isopropanol and the absorbance at a wavelength of 490 nm (A_{490}) was read on a SPECTRAmax Microplate Spectrophotometer (Molecular Devices). The absorbance values were normalized by assigning the value of the control line in the medium without drug to 1.0 and the value of the no cell control to 0. Experiments were done in triplicate. Dose-response curves were fitted with use of GraphPad PRISM 4 software (GraphPad Software).

$pIRES_{PRP-4}$ Expression Vector Construction

Clontech mammalian expression vector pIRESneo was used for functional expression study. The expression cassette of pIRESneo contains the human cytomegalovirus major immediate early promoter/enhancer (pCMV) followed by a multiple cloning site and a synthetic intron known to enhance the stability of the mRNA. A 3,094-bp cDNA fragment containing the full open reading frame of human PRP-4 was amplified by RT-PCR from the RNA of SKOV-3$_{TR}$, a paclitaxel-resistant cell line that highly overexpresses PRP-4. The resulting PRP-4 RT-PCR product was cloned into pCR2.1 vector using the Invitrogen Original TA Cloning Kit. After sequence confirmation, PRP-4 was cut from the pCR2.1 vector, purified, subcloned into the multiple cloning site of expression vector pIRESneo, and subsequently sequenced to confirm the correct open reading frame. Expression of PRP-4 cDNA was under the control of the pCMV.

Transfection and Production of Stable Cell Lines

Transfections were done using LipofectAMINE Plus reagents (Invitrogen) as follows: ~5 × 10^5 SKOV-3 cells were plated into 90 mm tissue culture dishes and cultured overnight. Before transfection, the growth medium was replaced with serum-free RPMI 1640 and cultured for 3 h. LipofectAMINE reagent containing 5 μg pIRES$_{empty}$, pIRES$_{PRP-4}$, was combined with Plus reagent and applied to the cells. After culture for 4 h, the medium was replaced with RPMI 1640 containing 10% fetal bovine serum. G418 sulfate (Invitrogen) selection (300 μg/mL) was started at 24 h after transfection. The selection medium was changed every 2 days.

Western Blotting

The human PRP-4 antibody was generously provided by Dr. Regis Giet (University Rennes; ref. 20). The P-glycoprotein antibody C219 was purchased from Signet. The mouse monoclonal antibody to human actin was purchased from Sigma-Aldrich. PRP-4 and P-glycoprotein proteins were analyzed in total cell lysates. Total cell lysates were prepared, and Western blot analysis was done as described previously. Briefly, the cells were lysed in 1× radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology) and protein concentration was determined by the DC Protein Assay (Bio-Rad). Total protein (25 μg) was resolved on NuPage 4% to 12% Bis-Tris gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween 20 with gentle agitation overnight at 4°C. Horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) were incubated in TBS (pH 7.4) with 5% nonfat milk (Bio-Rad) and 0.1% Tween 20 at a 1:2,000 dilution for 1 h at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Results

Lentiviral shRNA Screen for Mediators of Paclitaxel Sensitivity in Ovarian Cancer Cells

To investigate the potential functional role of previously identified drug resistance associated genes in ovarian cancer, we identified 291 human gene transcripts listed in the National Center for Biotechnology Information database that have been found to be overexpressed in at least two of three resistant cell lines (SKOV-3$_{TR}$, OVCAR8$_{TR}$, and MCF-7$_{TR}$). Based on the list of 291 genes, we constructed a 132-gene lentiviral shRNA library. This was submitted as part of the Sigma MISSION shRNA collection. This library contains 504 small interfering RNA templates targeting 132 drug resistance associated genes. The library was cloned into a lentiviral-based pLKO.1-puro shRNA expression vector. Up to five shRNA sequences were individually cloned into pLKO.1-puro for broad coverage of each target gene. The hairpin structure includes an intramolecular 20 to 21 bp stem and 6-base loop that is recognized and cleaved by Dicer on expression via the U6 (pol III) promoter in the host cell. The resulting small interfering RNA duplex then continues in the RNA interference pathway by association with RISC. The puromycin resistance marker is present to allow for stable selection in mammalian cells.

Initial screening conditions used the paclitaxel-resistant cell line SKOV-3$_{TR}$ (Fig. 1). We found a 5-day incubation

Table 1.   List of positive hits from the lentiviral shRNA screen in SKOV-3$_{TR}$

Gene name	Symbol	Functions	Positive hits
Pre-mRNA processing factor 4	PRP-4	Pre-mRNA splicing, interacts with Clk1 COOH terminus	3 of 5
Survivin	EPR-1	Inhibit apoptosis, regulate mitosis	2 of 4
Multidrug resistance 1	MDR1	Efflux pump responsible for decreased drug accumulation	2 of 5
Nuclear matrix protein p84	THOC1	Protein transcription and RNA export	2 of 5
Oncoprotein mdm2	MDM2	Inhibit cell cycle arrest and apoptosis	2 of 4
Krev interaction trapped 1	Krit1	Unknown	2 of 5

Table 2. MISSION TRC PRP-4 shRNA target set

TRC no.	Sequence	Target region	Reverse resistance
TRCN0000000719 (TRC 719)	CCGGCCCTATCAACTGTCTTATGTACTCGAGTACATAAGACAGTTGATAGGGTTTTT	3UTR	Yes
TRCN0000000720 (TRC 720)	CCGGGCTGCTGATGTTAAAGAGTATCTCGAGATACTCTTTAACATCAGCAGCTTTTT	CDS	No
TRCN0000000721 (TRC 721)	CCGGCTCAAGATCAAGCAAGGAAATCTCGAGATTTCCTTGCTTGATCTTGAGTTTTT	CDS	No
TRCN0000000722 (TRC 722)	CCGGGAATGAAAGTTGAGCAGGAATCTCGAGATTCCTGCTCAACTTTCATTCTTTTT	CDS	Yes
TRCN0000000723 (TRC 723)	CCGGAGCAAGTCAAAGGAGAGGAAACTCGAGTTTCCTCTCCTTTGACTTGCTTTTT	CDS	Yes

with 5 μg/mL puromycin was effective for generating complete cell death of untransfected cells. Infection of SKOV-3$_{TR}$ cells with MISSON TurboGFP Control lentivirus and subsequent selection with puromycin confirmed high transduction efficiency. Likewise, transduction with a MISSON Nontarget lentivirus containing a "nontarget shRNA" control that fails to target any known genes was nontoxic to SKOV-3$_{TR}$. After optimizing the transduction efficiency and puromycin selection concentration, we infected SKOV-3$_{TR}$ cells with a preselected lentiviral shRNA library that targeted each of the 132 drug resistance associated genes. After 4 to 10 days, the specific gene knockdown associated with 0.1 μmol/L paclitaxel-induced cell death was identified. Overall, 6 gene "hits" were identified where knockdown could reverse paclitaxel resistance; of these, MDR1, survivin, and MDM2 were already known to be associated with drug resistance or apoptosis resistance while PRP-4, THOC1, and Krit1 are

novel genes with unknown function in drug resistance (Table 1). Significantly, three of five PRP-4 knockdown constructs resulted in the reversal of paclitaxel sensitivity in SKOV-3$_{TR}$ (Table 2; Fig. 2). Levels of knockdown ranged from ~60% to 90%. These data suggest that a shRNA screen is a practical platform for genome-scale screening of drug-resistant genes in drug-resistant cancer cells and show that shRNA knockdown of specific genes can synergize drug sensitivities. These results also show the feasibility of screening with large collections of lentiviral vectors of drug resistance associated genes after genome-wide microarray studies to identify drug enhancers and suppressors.

Confirmation of Identified PRP-4 Knockdown and Reversal of Paclitaxel Resistance

The insertion of viral DNA into a host chromosome often leads to disruption of host genes. Therefore, to validate that the reversal of paclitaxel-resistant phenotype of the positive
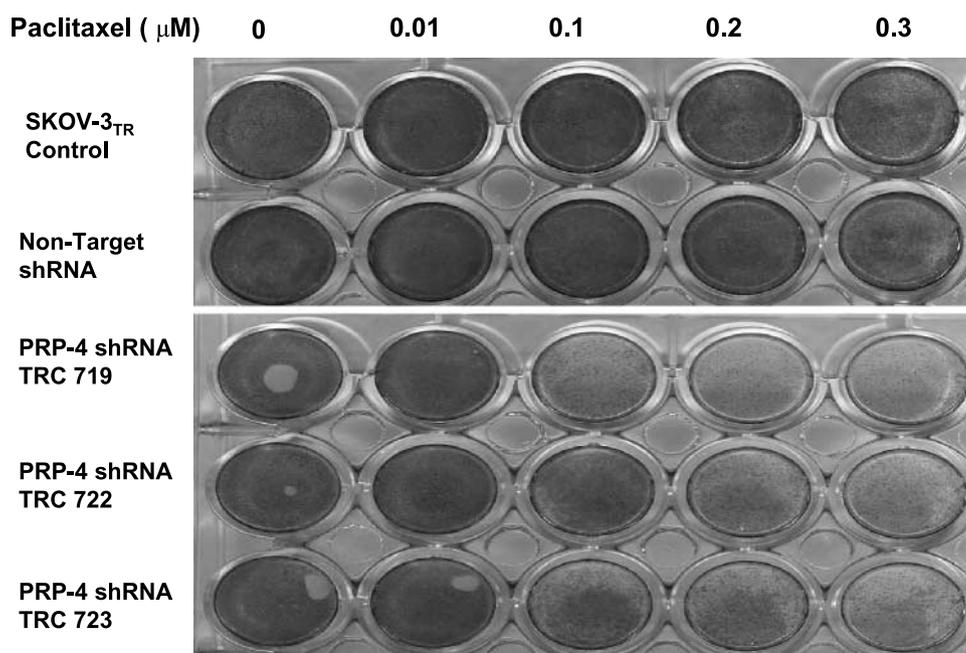

Figure 2. Effect of PRP-4 knockdown on paclitaxel sensitivity in SKOV-3$_{TR}$ cells. Clonogenic assay transduction of PRP-4 lentiviral shRNA vector confers paclitaxel sensitivity to SKOV-3$_{TR}$ cells. After transfection with the PRP-4 lentiviral shRNA, puromycin selected clones were plated and exposed to varying concentrations of paclitaxel for 6 d. The cells were fixed and stained with crystal violet to visualize the viable cells, with darker staining representing the more viable cells.

cells is indeed caused directly by the shRNA knockdown of the PRP-4 gene instead of nonspecific defects induced by random viral DNA insertion, we reinfected the SKOV-3$_{TR}$ cells with each positive lentivirus and assayed for cell death with paclitaxel. We used a clonogenic assay in our initial assay to determine paclitaxel sensitivity (Fig. 2). For confirmation, we used an alternative liquid culture cell MTT cytotoxic assay to show that the reversal phenotypes we detected were not assay specific (Fig. 3A). Using our established viral infection protocol, we found if viral infection and knockdown of the target gene in SKOV-3$_{TR}$ cells does not alter the paclitaxel sensitivities, the infected cell will grow well in 0.1 μmol/L paclitaxel-containing medium. On the other hand, if viral infection and knockdown of specific genes (such as MDR1, survivin, or PRP-4) does lead to drug sensitivity, the SKOV-3$_{TR}$ cells will die in medium containing 0.1 μmol/L paclitaxel. We confirmed that three of five PRP-4 shRNA target knockdowns significantly reversed paclitaxel resistance in SKOV-3$_{TR}$ cells (Figs. 2 and 3A). The two of five constructs that did not reduce paclitaxel resistance could not significantly block PRP-4 expression (Table 2). These results suggest that by treating the SKOV-3$_{TR}$ cells with low doses of paclitaxel (0.1 μmol/L) combined with PRP-4 shRNA, apoptosis or cell killing induced by paclitaxel could be enhanced. Northern blot analysis showed that PRP-4 was significantly depleted in the PRP-4 shRNA infected and puromycin-selected cells. Both RT-PCR (data not shown)
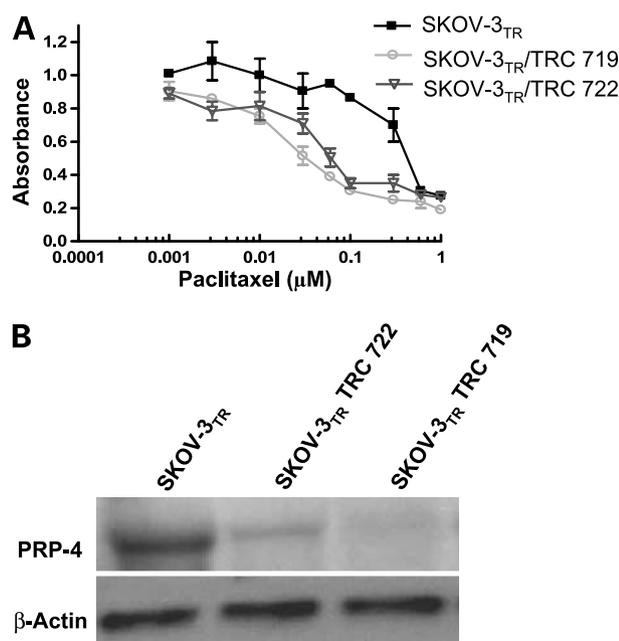
and Northern blot analysis showed that PRP-4 gene expression was indeed knocked down in shRNA-infected clones (Fig. 3B).

Evaluation of PRP-4 Knockdown and Drug Sensitivities in OVCAR8$_{TR}$ Cells

After identification of PRP-4 as a new regulator of chemoresistance in SKOV-3$_{TR}$, the effect of PRP-4 inhibition on drug sensitivities was further examined in human ovarian cancer cell drug-resistant cell line OVCAR8$_{TR}$. Inhibition of PRP-4 expression by shRNA in OVCAR8$_{TR}$ combined with sublethal doses of paclitaxel (0.1 μmol/L), doxorubicin (0.5 μmol/L), vincristine (0.1 μmol/L), or cisplatin (1 μmol/L) showed increase drug sensitivity to paclitaxel, doxorubicin, and vincristine but not to cisplatin (Supplementary Fig. S1).[6] It is noteworthy that both SKOV-3$_{TR}$ and OVCAR8$_{TR}$ were showed not resistance to cisplatin as previously reported.

### Effect of *PRP-4* Transfection on *In vitro* Growth of Transfected Cells

To further confirm the role of PRP-4 in drug resistance of ovarian cancer, we evaluated the effect of transfected overexpression of PRP-4 in drug-sensitive SKOV-3. Transfection of PRP-4 into SKOV-3 with subsequent cloning selection of transfectants showed overexpression of the respective gene and protein (Fig. 4A and *B*). Analysis of these same transfectants shows no change in MDR1 gene or P-glycoprotein protein expression compared with the parental line or empty vector transfectant control (Fig. 4B). MTT assay shows a modest increase in paclitaxel resistance in transfected cells. Cloned SKOV-3pIRES$_{PRP-4}$ is relatively resistant to paclitaxel, doxorubicin, and vincristine but not to cisplatin (Fig. 4C).

Discussion

Multidrug resistance often leads to eventual chemotherapy failure in patients with advanced ovarian cancer. There are many putative mechanisms for this process of inducing chemoresistance. MDR1 has been implicated in many examples of multidrug resistance. Additionally, prior studies have reported that non-MDR1 transporter proteins cause multidrug resistance in human cancer cell lines (21–23). We have reported previously that paclitaxel-resistant cell lines are associated with overexpression of a broad range of transcriptional changes, including gene families involved in cell growth/maintenance, cell structure, signal transduction, and inflammatory response (4, 8). Furthermore, transfection of HER-2/*neu*, c-H-ras, Bcl-2, interleukin-6, or MAGE into drug-sensitive cell lines has conferred drug resistance *in vitro* (24–28). These findings illustrate that multiple mechanisms can be selected to cause drug resistance in ovarian cancer cell lines, and they may all contribute partially to drug resistance in ovarian cancer.

Figure 3. Confirmation of PRP-4 depletion in clones and effect on paclitaxel sensitivity. **A,** MTT assay shows validation of PRP-4 knockdown in the reversal of paclitaxel resistance. Two of three positive target sites of PRP-4 by shRNA. **B,** duplicate sample sets of total RNA isolated from the SKOV-3 $_{TR}$, SKOV-3$_{TR}$/TRC719, and SKOV-3$_{TR}$/TRC722 cell lines analyzed in the MTT assay were subjected to Northern blot analysis using cDNA probe directed against PRP-4 (*top*) and β-actin (*bottom*).

Figure 4. Exogenous expression of PRP-4 confers paclitaxel resistance to the ovarian cancer cell line SKOV-3. **A,** confirmation of PRP-4 gene knockdown by Northern blot. Duplicate sample sets of total RNA isolated from the SKOV-3, SKOV-3pIRES, and SKOV-3pIRES$_{PRP-4}$ cell lines analyzed in the MTT assay were subjected to Northern blot analysis using cDNA probe directed against PRP-4 (*top*) and β-actin (*bottom*). **B,** confirmation of PRP-4 protein knockdown by Western blot. Total protein was isolated from SKOV-3, SKOV-3pIRES, and SKOV-3pIRES$_{PRP-4}$ cell lines and analyzed by Western blotting with anti PRP-4, P-glycoprotein (*Pgp*), or anti-actin antibodies. **C,** relative cytotoxicity of paclitaxel, doxorubicin, vincristine, and cisplatin in SKOV-3-derived cell lines (SKOV-3pIRES$_{PRP-4}$) stably transfected with a pIRES$_{PRP-4}$ expression vector and in the parental (SKOV-3) and empty vector (SKOV-3pIRES) controls were assessed using the MTT assay. All samples were analyzed in triplicate.
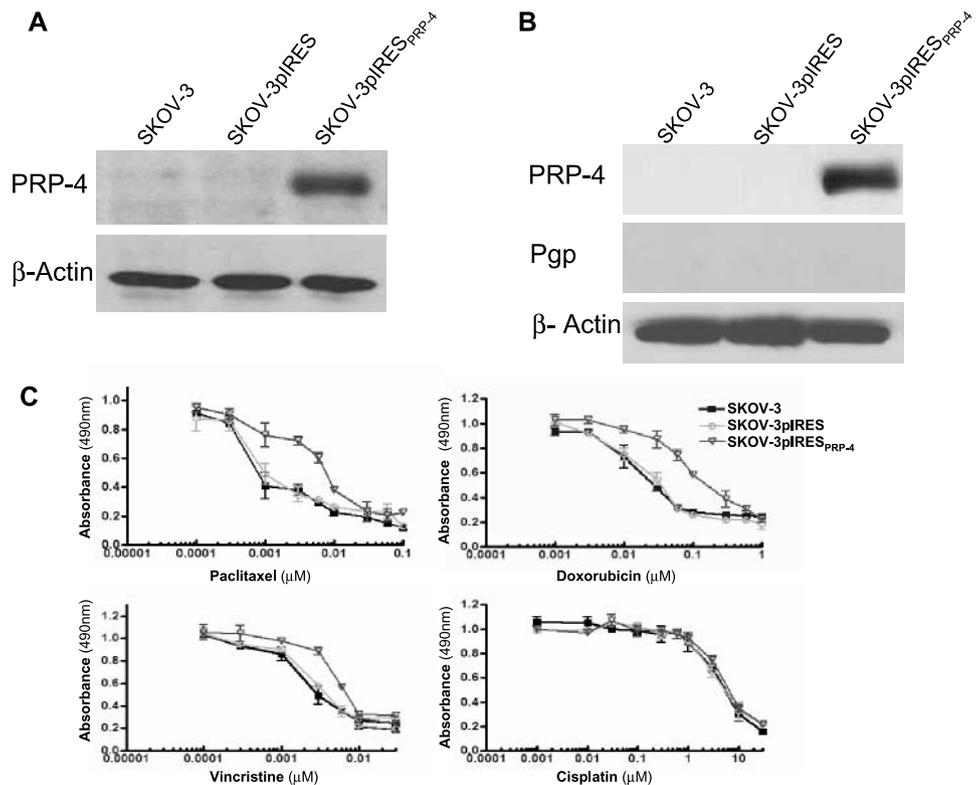
In this study, our goal was to use a lentiviral shRNA-mediated genetic screen to identify genes that are important in paclitaxel resistance in ovarian cancer cell lines. We extend previous studies of cDNA microarray analysis using paclitaxel resistance associated genes. We observed that knockdown of PRP-4 and five additional genes (MDR1, survivin, THOC1, MDM2, and Krit1) are associated with significant reversal of paclitaxel resistance. We further validated the functional role of PRP-4 in paclitaxel resistance. Previous studies showed that up-regulated expression of PRP-4 is broadly associated with multidrug resistance, and increased PRP-4 transcript levels are detected in paclitaxel resistant cell lines. We confirm these observations in the ovarian cancer cell line SKOV-3$_{TR}$; importantly, we show that overexpression of the PRP-4 protein in a paclitaxel-sensitive ovarian cancer cell line led to a modest increase in paclitaxel, doxorubicin, and vincristine resistance. These data imply an important role for PRP-4 in the development of the chemotherapy drug resistance.

The PRP-4 gene encodes a 150-kDa serine/threonine protein kinase that has been implicated in the regulation of mRNA splicing, and mutations in PRP-4 lead to the accumulation of pre-mRNAs (29). PRP-4 is also involved in mitosis; the expression of a dominant truncated PRP-4 protein could induce mitotic aberrations, suggesting a dual PRP-4 function in RNA splicing and mitosis (30, 31). The catalytic domain of PRP-4 shows significant similarity to the JNK/stress-activated protein kinase type of mitogen-activated protein kinase including the TPY motif, suggest-

ing that PRP-4 may play an important role in cell differentiation (32). PRP-4 has been reported to mediate cellular signaling (33). The precise cellular function of PRP-4 in cancer cells remains unclear, although recent studies in HeLa cells have found that PRP-4 expression is important for chromosome alignment (20). Several lines of evidence support that PRP-4 belongs to the family of spindle assembly checkpoint regulatory genes (20, 33). Our results also show the important role of PRP-4 kinase in regulating cell survival and drug sensitivity. The roles of PRP-4 in mRNA splicing and mitosis do not have clear connections with its apparent role in the development of the multidrug resistance. However, the PRP-4 gene is expressed in a wide spectrum of both normal and neoplastic tissues (33), and the determination of PRP-4 function in cancer cells may clarify its association with acquired drug resistance. The precise definition of its role awaits further investigation.

Enhanced paclitaxel sensitivity and drug-induced apoptosis was not limited to PRP-4 gene knockdown; we also observed increased paclitaxel sensitivity and cell apoptosis with shRNA that was directed against the drug-resistant target MDR1, antiapoptotic protein MDM2, and survivin, which is associated with tumor cell survival and anti-apoptosis. All three genes have been associated with resistance to chemotherapy and poor prognosis (Table 1). For example, overexpression of MDR1 has been associated with resistance to paclitaxel and Adriamycin in several cancer cell lines (34, 35). Knockdown of MDR1 expression by RNA interference could partially reverse drug resistance (11). Overexpression of survivin has been associated with

drug resistance and poor prognosis in breast and ovarian cancers (36, 37). MDM2 gene encodes a protein that inhibits p53-mediated cell cycle arrest and apoptosis by binding its transcriptional activation domain (38). Overexpression of MDM2 in several types of human cancers is associated with a poor prognosis (39, 40). Because of its functions in cell cycle and apoptosis, MDM2 may also play a role in the development of drug resistance in tumor cells. It has been reported that overexpression of MDM2 resulted in expression of the MDR1 gene and its protein P-glycoprotein in human glioblastoma cells and resulting in decreased sensitivity to etoposide (VP-16) and doxorubicin (41). More recent reports showed that MDM2 bound to topoisomerase II resulted in decreased cellular enzyme content. Knockdown of MDM2 by RNA interference stabilized topoisomerase II α and decreased resistance to topoisomerase II targeting drugs (etoposide and mitoxantrone; ref. 15). Another study found that curcumin, a dietary component that has anticancer, chemosensitization, and radiosensitization effects down-regulates MDM2. Curcumin also inhibited the growth of these cells and enhanced the cytotoxic effects of gemcitabine (16, 42). The human THOC1 gene, also known as hHpr1 or p84, encodes a protein that was originally recognized as a nuclear matrix component that binds the retinoblastoma tumor suppressor protein (43). Changes in the nuclear matrix and resulting alterations in nuclear structure have been recognized to correlate with tumor progression (42). Indeed, overexpression of THOC1 has recently been documented in human breast cancer with THOC1 levels correlating with tumor size and metastases (44).

In conclusion, we identified PRP-4 and several other genes that, when knocked down, could partially reverse drug resistance in the ovarian cancer cell line SKOV-3$_{TR}$ and OVCAR8$_{TR}$. Significantly, overexpression of the PRP-4 protein in a drug-sensitive cell line was sufficient to increase paclitaxel resistance, suggesting that PRP-4 may directly participate in the development of clinical drug resistance. The apparent synergy that we observed between PRP-4 repression and paclitaxel sensitivity (Table 1), in comparison with other known drug-resistant target genes such as MDR1, MDM2, and survivin, suggest that shRNA chemosensitizer screens could find molecular components closely related to the mode of action of a given drug. All of these genes, in addition to PRP-4, may be developed as new targets in improving cancer treatment using combination chemotherapy. A combination of low-dosage paclitaxel with MDR1, survivin, MDM2, or PRP-4 inhibitors could decrease the apoptotic threshold in drug-resistant cells and may prove to be an effective anticancer strategy *in vivo*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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