Osteopontin-c isoform inhibition modulates ovarian cancer cell cisplatin resistance, viability and plasticity

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Abstract. Osteopontin (OPN) is upregulated in several types of tumor and has been associated with chemoresistance. However, the contribution of OPN splicing isoforms (OPN-SIs) to chemoresistance requires further investigation. The present study aimed to evaluate the expression patterns of each tested OPN-SI in cisplatin (CDDP)-resistant ovarian carcinoma cell lines, focusing on the role of the OPN-c isoform (OPNc) in drug resistance. ACRP ovarian cancer cells resistant to CDDP, as well as their parental cell line A2780, were used. Analyses of the transcriptional expression of OPN-SIs, epithelial-mesenchymal transition (EMT) markers and EMT-related cytokines were performed using reverse transcription-quantitative PCR. OPNc was silenced in ACRP cells using anti-OPNc DNA oligomers and stably overexpressed by transfecting A2780 cells with a mammalian expression vector containing the full length OPNc cDNA. Functional assays were performed to determine cell proliferation, viability and colony formation. The results demonstrated that among the three tested OPN-SIs, OPNc was the most upregulated transcript in the ACRP cells compared with the parental A2780 cells. In addition, the expression levels of P-glycoprotein multidrug transporter were upregulated in CDDP-resistant ACRP cells compared with those in A2780 cells. OPNc knockdown sensitized ACRP cells to CDDP treatment and downregulated P-gp expression levels compared with those in the negative control group. Additionally, silencing of OPNc impaired cell proliferative and colony formation abilities, as well as reversed the expression levels of EMT markers and EMT-related cytokines compared with those in the negative control cells. Notably, although stable OPNc overexpression resulted in increased A2780 cell proliferation, it notably increased CDDP sensitivity compared with that in the cells transfected with a control vector. These results suggested that OPNc silencing may represent a putative approach to sensitize resistant ovarian cancer cells to chemotherapeutic agents.

Introduction

Ovarian carcinoma, which is one of the most common gynecological cancers, is the seventh most lethal malignancy worldwide (1). The efficacy of platinum-based therapy such as cisplatin (CDDP) in ovarian carcinoma is hindered by the occurrence of drug resistance, a phenomenon often associated with an increased metastatic potential (2,3). Shedding light onto the mechanisms of chemoresistance may render insights into novel therapeutic targets and improved treatment strategies (2-5).

A number of aberrantly expressed gene products mediate tumor chemoresistance, including the upregulation of ATP-binding cassette transporters, such as P-glycoprotein (P-gp) (6). Additional mechanisms, including apoptosis evasion and tumor cell survival, may also contribute to CDDP resistance, among other cancer hallmarks, including tumor cell heterogeneity, redundancy of growth-promoting pathways, increased mutation rate and/or epigenetic alterations (4).

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Similarly, a process termed the epithelial-mesenchymal transition (EMT) has been demonstrated to contribute to drug resistance (7-9). A recent study has also reported that interleukins produced by the tumor microenvironment promote the EMT through altered expression of several inducers of this process, including Snail and STAT transcription factors (5).

Osteopontin (OPN) emerges in the interface between chemoresistance and EMT; it is a matrix glycophosphoprotein that serves important roles in tumor cell proliferation and chemotherapy resistance (10-15). The OPN primary transcript undergoes alternative splicing, generating at least three main splicing isoforms (OPN-SIs), termed OPNa, OPNb and OPNc (16). In addition, OPN is subjected to post-translational modifications, generating several additional isoforms (17,18). The sum of all these isoforms comprises total OPN (tOPN). Although tOPN has been implicated in cancer cell resistance to a wide range of anticancer agents (10), the underlying molecular mechanisms through which the full-length OPN and each OPN-SI specifically perform their roles in chemoresistance remain unclear.

Nakamura *et al* (19) have demonstrated that prostate cancer cells overexpressing OPNb and OPNc are more resistant to docetaxel compared with cells transfected with an empty vector and exhibit a typical mesenchymal phenotype. Our recent study demonstrated that OPNc was upregulated in distinct B-acute lymphoblastic leukemia (B-ALL) cell lines (20). Our other previous study revealed that OPNc expression levels in B-ALL cells were significantly increased in response to treatment with chemotherapeutic agents that have been used in several backbone treatment strategies for B-ALL, namely vincristine or etoposide (21). Based on these findings, the present study aimed to investigate whether different OPN-SIs may differentially modulate chemoresistance in an ovarian carcinoma cell line model as well as their potential functional roles in the chemoresistant phenotype.

Materials and methods

Study design. The present study used ACRP, an ovarian cancer cell line resistant to CDDP, as well as its corresponding parental control cell line A2780 as models. Some data obtained using the ACRP cell line have been validated by also testing OVCar-8/Dox^R, an ovarian cancer cell line resistant to doxorubicin (Dox), which originated from OVCar-8 cells. Both ovarian cancer cell lines were used to assess the roles of OPNc in chemoresistance. The expression of OPN-SIs and P-gp was assessed using reverse transcription-quantitative PCR (RT-qPCR). After evaluating OPNc expression in the CDDP and Dox resistance models, the OPNc isoform was silenced in order to evaluate its roles in the resistant phenotype by transfecting ACRP and OVCar-8/Dox^R cells with a specific anti-OPNc DNA oligomer modified with phosphorothiotates. In these cell lines, functional assays were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue and clonogenic assays. The biological effects were validated by analyzing the mRNA expression levels of EMT markers and cytokines. To validate the cytotoxicity results observed using the knockdown approach, experimental assays were performed in the A2780 parental cell line ectopically overexpressing OPNc (OPNc$^+$). OPNc and P-gp expression levels were determined in the A2780 OPNc$^+$ cell line, and additional functional assays were performed, including MTT, trypan blue exclusion and clonogenic assays in the absence or presence of CDDP.

Cell lines and culture conditions. The epithelial ovarian cancer cell line A2780 and the corresponding CDDP-resistant cell line ACRP were generously provided by Dr Pat J. Morin (National Institutes of Health, Bethesda, MD, USA). ACRP cells were selected for progressive resistance to CDDP as previously described (22). The cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2. The human ovarian cell line OVCar-8 was acquired from the American Type Culture Collection. The OVCar-8 cell line resistant to Dox, termed OVCar-8/Dox^R resistant cell line, was originated by progressively culturing OVCar-8 cells with increasing concentrations of Dox for 6 months. The doses were incrementally increased upon selection of Dox-resistant clones up to 17 μM Dox, which was used to maintain the OVCar-8/Dox^R cells.

Isolation of total RNA and RT-qPCR. Total cellular RNA was isolated from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA was reverse-transcribed using SuperScript™ II Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. mRNA expression analysis was performed by qPCR using the Eco Real-Time PCR System (Illumina, Inc.) and Power SYBR® Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial incubation at 50˚C for 2 min and 95˚C for 10 min, followed by additional incubation at 94˚C for 5 min; 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 45 sec; and final melting curve analysis at 72˚C for 15 sec, 90˚C for 5 sec, 55˚C for 5 sec and 90˚C for 5 sec. Fold-changes in the expression levels of each mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (23). GAPDH or β-actin were used as the normalization controls. The PCR primers sequences are listed in Table I.

OPNc knockdown using anti-OPNc oligomers. After reaching 90-95% confluency, ACRP or OVCar-8 Dox^R cells were transfected with 100 nM anti-OPNc phosphorothiotate-modified antisense DNA oligomer (ASO anti-OPNc; 5'-A*C*A*AC*GC ATTCTGCTTT*T*C*C-3') or with a non-specific/scrambled control sequence (ASO SCR; 5'-C*C*T*T*TTCGTCTTACGA C*A*C*A-3'), using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The phosphorothiotate-modified bases are marked by an asterisk in the ASO sequences. Transfections were performed with 24 μg oligomers and 24 μl Lipofectamine® 2000 diluted in RPMI-1640 medium and Opti-MEM in 10-cm plates in a 5% CO_2 incubator at 37˚C for 24 h. The transfected cells were collected at 24 h post-transfection for subsequent experiments. The downregulation of the OPNc mRNA expression levels was confirmed by RT-qPCR.

Table I. Forward and reverse oligonucleotide sequences.

Gene	Sequence (5'→3')
P-gp	F: CCCATCATTGCAATAGCAGG
	R: GTTCAAACTTCTGCTCCTGA
OPNa	F: ATCTCCTAGCCCCACAGAAT
	R: CATCAGACTGGTGAGAATCATC
OPNb	F: CTCCTAGCCCCACAGACCCT
	R: TATCACCTCGGCCATCATATG
OPNc	F: CTGAGGAAAAGCAGAATG
	R: AATGGAGTCCTGGCTGT
GAPDH	F: TCCCATCACCATCTTTCAGGAGCA
	R: TTCTACATGGTGGTGAAGACGCCA
β-actin	F: GGCGGCACCACCATGTACCCT
	R: AGGGGCCGGACTCGTCATACT
E-cadherin	F: GAATGACAACAAGCCCGAAT
	R: GAC CTCCATCACAGAGGTTCC
Cytokeratin-18	F: GCGAGAAGGAGACCATGCA
	R: GGTGTTCCCGGATTTTGATCT
Vimentin	F: GACAATGCGTCTCTGGCACGTCTT
	R: TCC TCCGCCTCCTGCAGGTTCTT
N-cadherin	F: GGTGGAGGAGAAGAAGACCAG
	R: GCA TCAGGCTCCACAGT
Claudin-3	F: CTGCTCTGCTGCTCGTGTCC
	R:TTAGACGTAGTCCTTGCGGTCGTAG
Slug	F: TTCGGACCCACACATTACCT
	R: GCAGTGAGGGCAAGAAAAAG
Snail	F: TTCCAGCAGCCCTACGACCAG
	R: CTTTCCCACTGTCCTCATC
Twist	F: CCCAACTCCCAGACACCTC
	R: CAAAAAGAAAGCGCCCACC
IL-6	F: CATTTGTGGTTGGGTCAGG
	R: AGTGAGGAACAAGCCAGAGC
IL-8	F: CTTGGCAGCCTTCCTGATTT
	R: GGGTGGAAAGGTTTGGAGTATG
IL1-α	F: CATCCTCCACAATAGCAGACAG
	R: GAGTTTCCTGGCTATGGGATAAG
IL1-β	F: CAAAGGCGGCCAGGATATAA
	R: CTAGGGATTGAGTCCACATTCAG
GP130	F: TGCCTCCAGAAAAACCTAAAAA
	R: TTTGTCTCCAAGTGTGTTTCC

OPN, osteopontin; P-gp, P-glycoprotein; F, forward; R, reverse.

OPNc overexpression. To generate a cell line stably overexpressing OPNc (A2780 OPNc$^+$), 4x10^5 A2780 cells were plated in 6-well plates and maintained for 24 h in a 5% CO_2 incubator at 37˚C. Following adhesion, the cells were transfected with 2 μg pCR3.1 expression plasmid containing the complete cDNA encoding OPNc or with the empty vector plus 4 μl Lipofectamine® 2000, which were diluted in RPMI-1640 medium and Opti-MEM. The cells were then maintained in a 5% CO_2 incubator at 37˚C for 24 h. Subsequently, 300 μg/ml geneticin was added to the culture medium to select the transfected clones. The culture medium containing 300 μg/ml geneticin was changed every 2 days until clones stably expressing the OPNc isoform were selected, which lasted 15 days. Subsequent experiments were performed following the selection or A2780 OPNc$^+$ cell line in selection culture media. Following selection, A2780 OPNc$^+$ and A2780 pCR3.1 cells were routinely maintained with 300 μg/ml geneticin to ensure OPNc overexpression.

MTT assay. The MTT assay (Uniscience Corp.) was used to measure the cytotoxic effects of CDDP (Libbs Farmaceutica, Ltda.) on ovarian cancer cell lines. Briefly, ACRP, OVCar-8 Dox^R, A2780 OPNc$^+$ cells or A2780 cells transfected with the pCR3.1 empty vector were seeded in 96-well plates (1x10^4 cells/well). After adhesion, the cells were exposed to 10, 20, 40, 60 and 100 μM CDDP or drug-free complete medium for 24 or 96 h. Drug-free medium was added to the control wells. A total of 20 μl MTT (5 mg/ml) was added to each well 4 h prior to the end of the time intervals. MTT solution was then removed at 24 or 96 h, and the formazan crystals were dissolved in 150 μl DMSO (Sigma-Aldrich; Merck KGaA). The optical density at 570 nm was determined using a SpectraMax 190 microplate reader. All experiments were performed in triplicate.

Clonogenic assay. To determine clonogenicity, a total of 2x10^3 ACRP, OVCar-8 Dox^R, A2780 OPNc$^+$ cells or A2780 cells transfected with the pCR3.1 empty vector were seeded in 6-well plates and maintained in culture for ~14 days, until colonies could be observed. Colonies were fixed in absolute ethanol for 10 min and stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The crystals were dissolved in 33% glacial acetic acid solution, and the optical density was measured at 595 nm using a SpectraMax 190 microplate reader.

Statistical analysis. Data are presented as the mean ± SD of three independent experiments. Differences in various parameters between two groups were analyzed by two-tailed Student's t-test. GraphPad Prism version 5.02 (GraphPad Software, Inc.) was used to plot the graphs. Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

Results

OPNc is upregulated in ACRP chemoresistant cells. As a first approach to determine whether the three tested OPN-SIs differently modulated CDDP resistance in ovarian cancer cell lines, the transcriptional levels of each OPN-SI were analyzed in ACRP cells and compared with those in the corresponding parental cells. Among the three tested OPN-SIs, only OPNc expression levels were upregulated in ACRP cells compared with those in the parental A2780 cell line, although the results were not statistically significant (Fig. 1A).

Since tOPN can modulate P-gp expression (6), P-gp expression was subsequently tested in ACRP cells. The results demonstrated that the mRNA expression levels of P-gp were also upregulated in ACRP cells compared with those in the

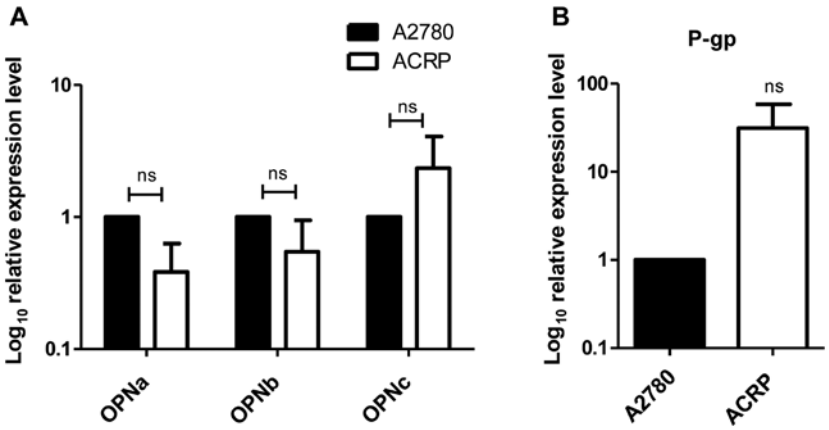

Figure 1. OPNc and P-gp are non-significantly upregulated in cisplatin-resistant ACRP ovarian cancer cells. (A) RT-qPCR was used to analyze the mRNA expression levels of OPNa, OPNb and OPNc isoforms. (B) P-gp mRNA expression levels were analyzed in ACRP cells by RT-qPCR. Log_{10} relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to those of β-actin. ns, non-significant. OPN, osteopontin; RT-qPCR, reverse transcription-quantitative PCR; P-gp, P-glycoprotein.

parental control cells, although the results did not reach statistical significance (Fig. 1B).

OPNc knockdown sensitizes ACRP cells to CDDP treatment. To further characterize the involvement of OPNc in CDDP-resistant ACRP cells, the OPNc isoform was knocked down using the ASO anti-OPNc. OPNc mRNA expression levels were reduced compared with those in cells transfected with the ASO-SCR (Fig. 2A). By contrast, OPNc knockdown in ACRP cells resulted in the upregulation of OPNa and OPNb mRNA expression levels compared with those in the ASO-SCR group, although this result did not achieve statistical significance (Fig. S1). Notably, following OPNc silencing, P-gp expression levels were also downregulated compared with those in cells transfected with the ASO-SCR (Fig. 2B), suggesting that OPNc may modulate P-gp expression.

The present study then tested whether OPNc knockdown may modulate ACRP cell viability in response to CDDP exposure. Following OPNc knockdown, ACRP cells were more sensitive to 10 and 40 mM CDDP treatment compared with the ASO SCR-transfected cells 24 h after drug treatment (Fig. 2C and D).

To further determine whether the roles of OPNc in drug resistance were dependent on the cell line model and the class of chemotherapeutic drug, the same experiments were performed in the OVCar-8 Dox^R cell line. The results demonstrated a strong but non-significant downregulation of the P-gp expression levels following OPNc knockdown in these cells compared with those in cells transfected with the ASO SCR (Fig. S2A and B), which was similarly associated with Dox sensitization following 24-h (0.05 and 0.1 μM) and 96-h (0.1, 0.2, 0.5 and 1 μM) drug exposure (Fig. S2C and D). These results suggested that OPNc may modulate drug resistance and cell viability in distinct models of drug-resistant ovarian cancer cells.

OPNc modulates cell viability and colony formation in ACRP cells. The effects of OPNc knockdown on the chemoresistant ovarian cancer cell line viability were next assessed. Notably, the number of ACRP cells was decreased in response to OPNc silencing compared with that of the ASO SCR-transfected cells (Fig. 2E). The colony formation capacity of ACRP cells transfected with the ASO anti-OPNc was analyzed, and the results demonstrated that OPNc knockdown reduced the colony formation capacity in ACRP cells compared with that of the control group, but the difference was not statistically significant (Fig. 2F). Similarly, the cell number and clonogenicity were impaired in OVCar-8 Dox^R cells transfected with the ASO anti-OPNc compared with those in the control cells (Fig. S3A and B). These results suggested that OPNc may not only modulates drug sensitivity, but also promote cell viability.

OPNc affects the expression of EMT markers in ACRP cells. The present study further investigated whether OPNc may modulate the EMT, and the RT-qPCR results demonstrated that ACRP cells exhibited an intermediate or partial EMT phenotype compared with that of the parental A2780 cell line. In ACRP cells, the expression levels of the epithelial markers E-cadherin, claudin-3 and cytokeratin-18 were upregulated compared with those in A2780 cells (Fig. 3A). In addition, vimentin and N-cadherin levels were also upregulated, whereas Slug, Snail and Twist exhibited similar or lower expression levels compared with those in A2780 cells (Fig. 3B). However, none of these differences were statistically significant. In response to OPNc silencing, changes in the expression patterns of the epithelial and mesenchymal markers were observed. E-cadherin expression levels were non-significantly upregulated in ACRP cells following OPNc silencing compared with those in the control cells. By contrast, the levels of claudin-3 and cytokeratin-18 were non-significantly downregulated in the same experimental conditions (Fig. 3C). Among the mesenchymal markers, the vimentin and N-cadherin transcriptional levels remained largely unchanged following OPNc silencing in ACRP cells, whereas the levels of Slug, Snail and Twist transcripts were downregulated compared with those in the ASO SCR-transfected cells (Fig. 3D). These results demonstrated alterations in the EMT marker transcriptional patterns in response to OPNc downregulation in ACRP resistant cell line, suggesting that OPNc may modulate CDDP resistance-associated EMT in these cells.

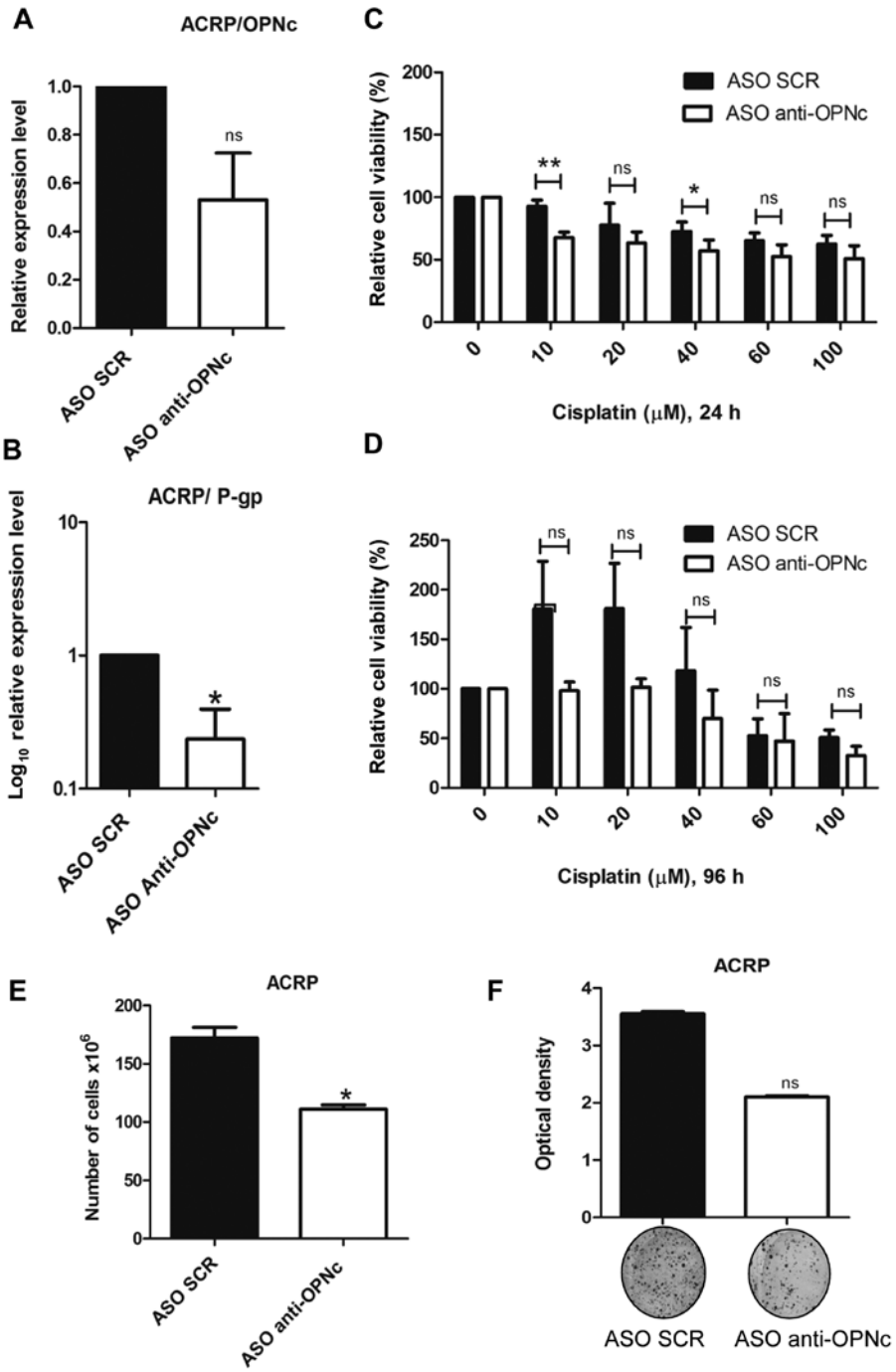

Figure 2. Knockdown of OPNc downregulates P-gp mRNA expression levels and sensitizes ACRP cells to cisplatin, impairing cell viability and colony formation. (A and B) ACRP cisplatin-resistant ovarian cancer cells were transfected with ASO SCR or ASO anti-OPNc, and reverse transcription-quantitative PCR was used to determine (A) OPNc and (B) P-gp mRNA expression levels. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to those of β-actin. (C and D) At 24 h post-transfection, ACRP cells were treated with increasing concentrations of cisplatin for (C) 24 and (D) 96 h, and cell viability was measured by the MTT assay. (E) Cell viability was also evaluated by trypan blue exclusion analysis at 24 h post-transfection. (F) ACRP cells were assessed for clonogenicity following 14-day culture by crystal violet staining. Optical density was measured at 595 nm. $^{*}P\leq0.05$ and $^{**}P\leq0.01$; ns, non-significant. OPNc, osteopontin-c isoform; ASO, antisense oligonucleotide; SCR, scramble; P-gp, P-glycoprotein.

OPNc affects the expression of EMT-related cytokines. The present study next tested the expression levels of the EMT-related cytokines IL-6, IL-8, IL-1α, IL-1β and the GP130 receptor. Notably, a general non-significantly upregulated expression pattern was observed for the EMT-related cytokines in ACRP cells compared with that in the parental cell line A2870 (Fig. 3E). Consistently, the expression levels of IL-6, IL-8 and IL-1α were significantly downregulated, and the levels of IL-1β and the GP130 receptor were non-significantly downregulated in the OPNc-silenced ACRP cells compared with those in the cells transfected with the ASO SCR (Fig. 3F). In addition, decreased levels of EMT-associated cytokines were observed in OVCar-8 Dox^R cells following OPNc knockdown compared with those in ASO SCR-transfected control cells (Fig. S4), further suggesting that OPNc may be associated with drug resistance as well as with the EMT phenotype.

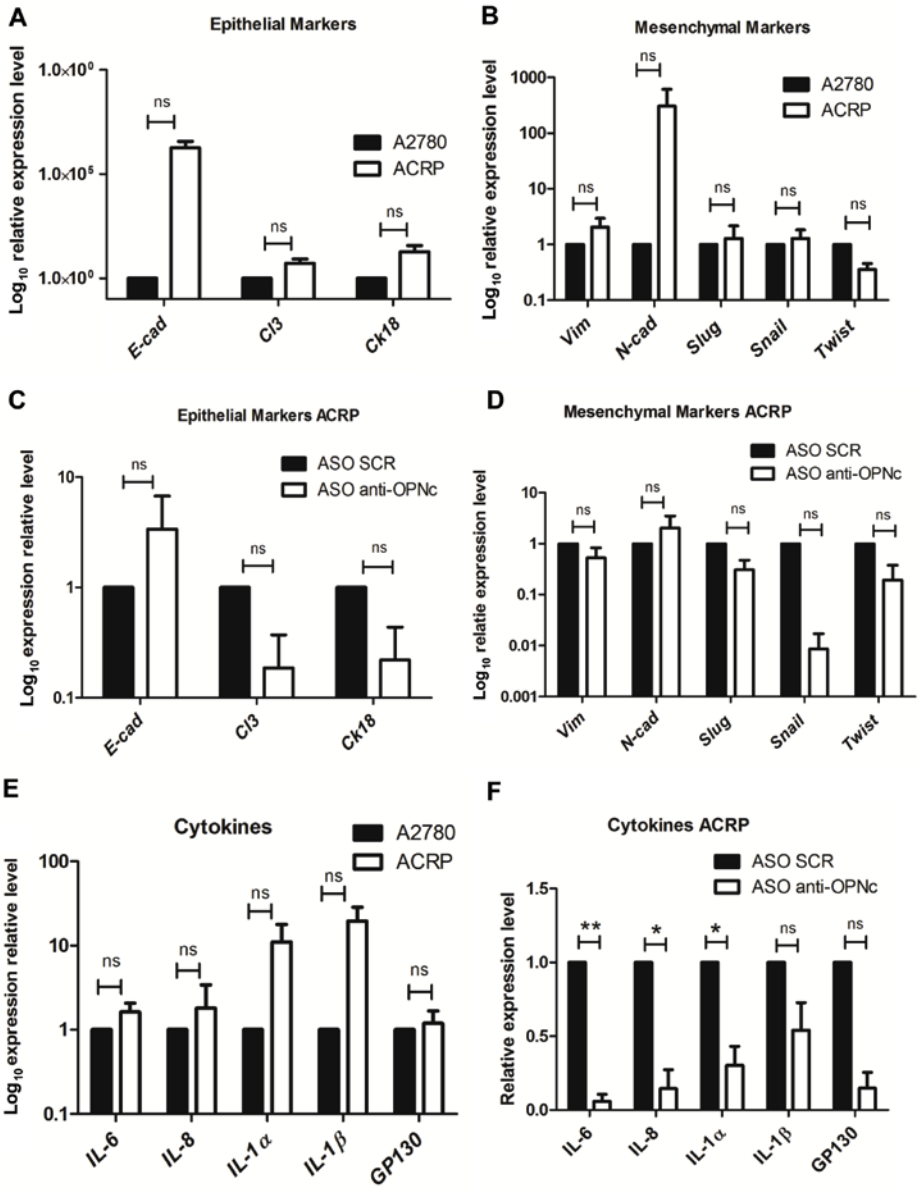

Figure 3. OPNc expression levels modulate the epithelial-mesenchymal transition marker and associated interleukin expression patterns in cisplatin-resistant ACRP ovarian cancer cells. (A, B and E) A2780 and ACRP cells were harvested, and the expression levels of (A) epithelial and (B) mesenchymal markers, as well as (E) IL-6, IL-8, IL-1α, IL-1β and the GP130 receptor were analyzed by RT-qPCR. (C, D and F) ACRP cells were transfected with ASO SCR or ASO anti-OPNc, and RT-qPCR was used to determine the mRNA expression levels of (C) epithelial and (D) mesenchymal markers, as well as (F) IL6, IL8, IL-1α, IL-1β and the GP130 receptor. The mRNA expression levels of E-cad, Cl3, ck18, Vim, N-cad, Slug, Snail and Twist were normalized to those of GAPDH, and the expression levels of IL6, IL8, IL-1α, IL-1β and the GP130 receptor were normalized to those of β-actin. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. $^{*}P\leq0.05$ and $^{**}P\leq0.01$; ns, non-significant. OPNc, osteopontin-c isoform; RT-qPCR, reverse transcription-quantitative PCR; ASO, antisense oligonucleotide; SCR, scramble; E-cad, E-cadherin; Cl3, claudin-3; ck18, cytokeratin-18; Vim, vimentin; N-cad, N-cadherin.

OPNc⁺ cells exhibit enhanced proliferative capacity and sensitivity to CDDP. To further determine the cellular effects of OPNc, this splice variant was stably overexpressed in the A2780 parental cells (Fig. 4A). The results demonstrated that the OPNc⁺ cells also displayed high P-gp expression levels (Fig. 4B). Notably, OPNc overexpression improved the cell proliferative capacity, as denoted by a higher number of cells (Fig. 4C), cell viability at 96 h (Fig. 4D) and clonogenic potential (Fig. 4E) in the OPNc⁺ group compared with those in the empty vector-transfected control group.

In order to address whether OPNc overexpression modulated the response to CDDP, OPNc⁺ cells were exposed to increasing CDDP concentrations for 24 and 96 h. Notably, OPNc+ cells became more susceptible to CDDP treatment compared with cells transfected with the empty PCR3.1 expression vector after 24-h drug treatment (Fig. 5A). These results were confirmed in a long-term assessment of cell viability, which demonstrated that OPNc+ cells formed fewer colonies in response to CDDP treatment (Fig. 5B). We then hypothesized that CDDP may negatively modulate the expression of OPNc, further sensitizing OPNc⁺ cells to this drug. The results of RT-qPCR analysis demonstrated that CDDP downregulated the OPNc mRNA levels in OPNc⁺ cells (Fig. 5C) following 24-h CDDP treatment. However, these effects were less pronounced following 96-h CDDP exposure (Fig. S5A and B). These results demonstrated that CDDP

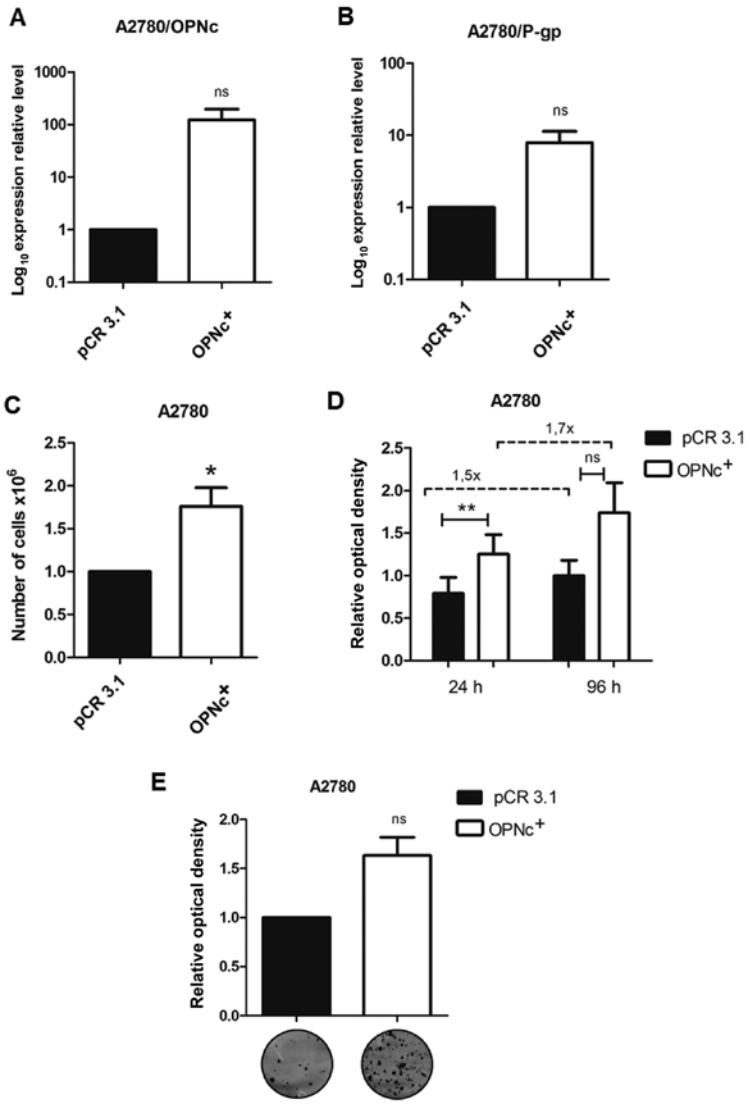

Figure 4. OPNc overexpression promotes cell viability and clonogenicity. A2780 cells were transfected with pCR3.1 or OPNc$^+$, and reverse transcription-quantitative PCR was used to analyze the mRNA expression levels of (A) OPNc and (B) P-gp. Log_{10} relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to those of β-actin. OPNc$^+$-transfected cells were analyzed for (C) cell viability by trypan blue exclusion analysis and (D) proliferation rates at 24 and 96 h. (E) OPNc$^+$- and pCR3.1-transfected cells were assessed for clonogenicity following 14-day culture by crystal violet staining. Optical density was obtained at 595 nm. $^*P\leq0.05$ and $^{**}P\leq0.01$; ns, non-significant. OPNc, osteopontin-c isoform; OPNc$^+$, OPNc overexpression vector; pCR3.1, empty plasmid; P-gp, P-glycoprotein.

inhibited OPNc expression in OPNc+ cells, increasing cell sensitivity and further decreasing cell viability.

Discussion

The present study aimed to evaluate whether OPN-SIs were differentially expressed in CDDP-resistant ovarian cancer cells compared with their parental cells. Based on the differential expression patterns, the contribution of OPNc to the modulation of chemoresistance was selected for further investigation in this cell line model. The results demonstrated that the expression levels of the OPNc splice variant were upregulated in ACRP cells compared with those in the parental cell line. Using an OPNc-specific knockdown approach in the ACRP cell line, the present study revealed that OPNc modulated various aspects of drug resistance, such as cell viability, sensitivity to CDDP, and colony formation, as well as the EMT phenotype and the expression of the associated cytokines. A number of these features were also validated by stably overexpressing OPNc in the A2780 parental cell line.

The results of the present study demonstrated that ACRP cells presented with higher expression levels of OPNc compared with those in A2780 cells. Similarly to these results, previous studies have demonstrated that tOPN expression was induced in human hepatocellular carcinoma cells resistant to CDDP compared with non-resistant cells (11), in colorectal cancer cells resistant to oxaliplatin (13) and in glioma cells resistant to temozolomide (TMZ) and CDDP (15) and alkylphosphocholines (24). In a number of experimental models, tOPN expression levels were upregulated in response to drug treatment (11,13,15). In addition, tOPN knockdown sensitized cells to drug exposure (15). It has also been demonstrated that oncogenic signaling pathways, such as PI3K, NF-κB/Bcl-2 and Raf/MEK/ERK, are activated in response to tOPN overexpression, favoring drug resistance (15). Similarly to tOPN and OPNc, overexpression of other gene products have also

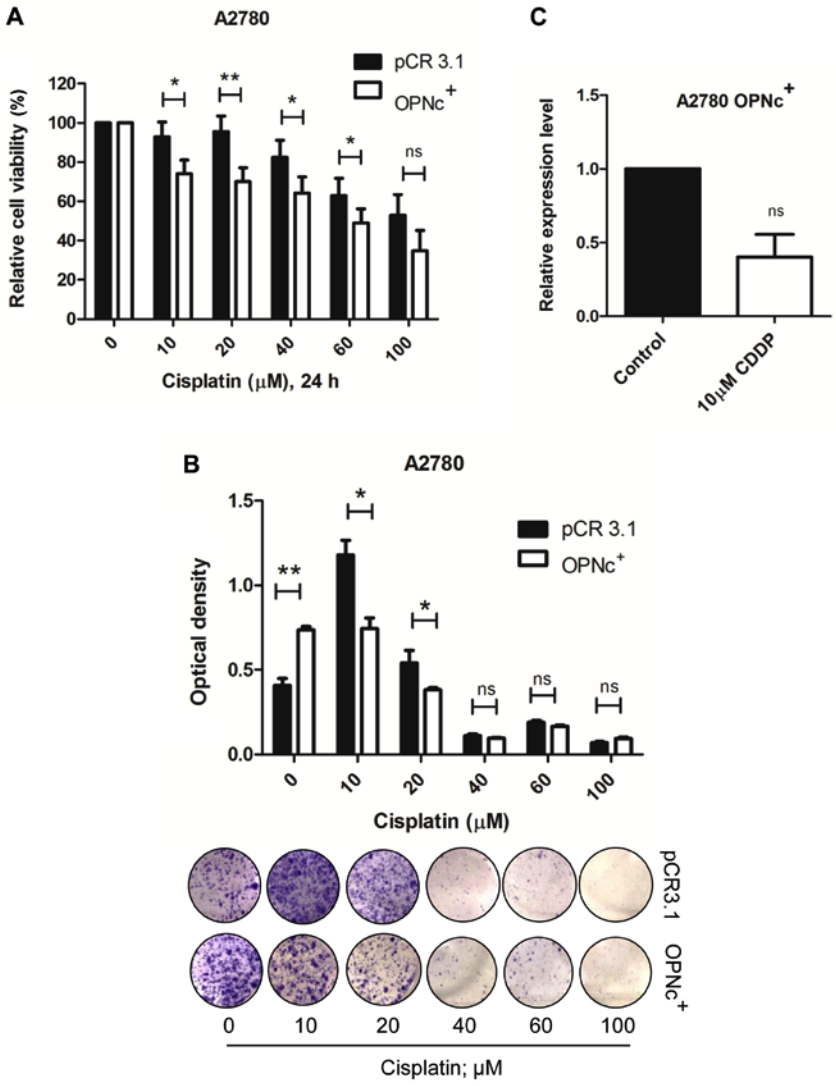

Figure 5. OPNc overexpression sensitizes ovarian cancer cells to cisplatin treatment. (A) Following 24-h transfection, the viability of OPNc$^+$- and pCR3.1-transfected cells were analyzed by MTT assay following treatment with increasing concentrations of cisplatin for 24 h. (B) Clonogenicity of OPNc$^+$- and pCR3.1-transfected cells was determined following treatment with increasing concentrations of cisplatin and 14-day culture by crystal violet staining. Optical density was obtained at 595 nm. Images are representative of three independent experiments. (C) A2780 OPNc$^+$- and pCR3.1-transfected cells were treated with 10 μM cisplatin for 24 h. Cells were then harvested for reverse transcription-quantitative PCR analysis of OPNc expression levels. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to those of β-actin. $^*P \leq 0.05$ and $^{**}P \leq 0.01$; ns, non-significant. OPNc, osteopontin-c isoform; OPNc$^+$, OPNc overexpression vector; pCR3.1, empty plasmid.

been associated with resistance to distinct therapeutic drugs, including GATA binding protein 1 (25), secretory clusterin (26), Her-2 (27) and drug efflux pumps, including P-gp (28,29). Although studies have reported an association between tOPN expression and chemoresistance (10,12-15), the functional roles of specific OPN-SIs in chemoresistant cells have not been previously determined. The results of the present study suggested a potential role for OPNc in chemoresistance in the ACRP and OVCar-8/Dox^R cell line models. In accordance with this hypothesis, our recent study demonstrated that ectopic overexpression of OPNb and OPNc in PC3 prostate cancer cells induced resistance to docetaxel (19), suggesting that overexpression of specific OPN-SIs may differentially contribute to tumor drug resistance. The expression levels of OPNa and OPNb were also upregulated in the ACRP cell line compared with those in A2780 cells in the present study, although at lower levels than OPNc. Future work should evaluate their putative contribution to the resistant phenotype. Notably, in the present study, when OPNc was knocked down, OPNa and OPNb were upregulated; the indirect effects of OPNc knockdown on the expression of the two other splice variants may be explained by cellular compensation due to low OPNc levels. However, the specific mechanisms by which OPNc may favor chemoresistance are currently unknown. Based on available data regarding the roles of tOPN in several tumor types, we hypothesize that in the experimental model used in the present study, OPNc may activate signaling pathways that contribute to the survival of ovarian tumor cells, favoring increased viability and colony-forming capacity. Similarly, our previous study demonstrated that ectopic OPNc overexpression in the ovarian cancer line OVCar-3 contributed to increased migration, proliferation, cell invasion, independent anchorage and tumor formation *in vivo* compared with those in OVCar-3 cells transfected with an empty vector (30). It was also observed

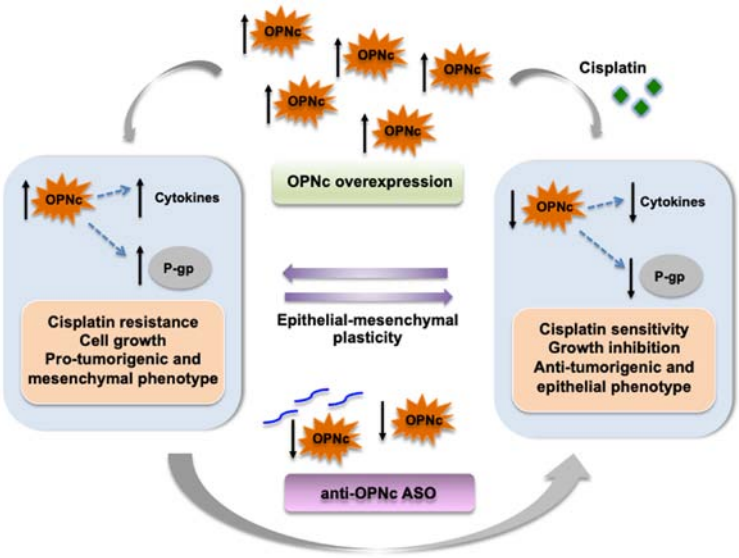

Figure 6. Schematic model for targeting OPNc for cisplatin sensitization in ovarian cancer cells. In ACRP cisplatin-resistant ovarian cancer cells, OPNc upregulation is associated with high P-pg mRNA expression levels, possibly contributing to cisplatin extrusion and drug resistance. Knockdown of OPNc levels using an anti-OPNc ASO resulted in decreased P-gp expression levels and survival rates, as well as inhibited the expression of EMT markers and EMT-related cytokines. By contrast, overexpression of OPNc in the A2780 cell line augmented P-gp expression compared with that in the control cells transfected with the empty expression vector. OPNc-overexpressing cells exhibited increased cell viability rates, which rendered them more sensitive to cisplatin exposure compared with the control cells. These results suggested that OPNc may be a preferential target for the cytotoxic effects of cisplatin in ovarian cancer cells, further reducing cell survival and viability. OPNc, osteopontin-c isoform; ASO, antisense oligonucleotide.

that certain OPNc-dependent protumorigenic functions were mediated by the PI3K/AKT signaling pathway, demonstrating the role of OPNc in ovarian cancer tumor progression through the activation of these signaling pathways (30). The protumorigenic functions of the PI3K/AKT pathway also involve the regulation of survival and apoptosis inhibition, which are associated with drug resistance in ovarian cancer cells (31,32). Therefore, we hypothesize that the inhibition of the PI3K/AKT signaling pathway may be one of the mechanisms underlying the biological effects observed in the OPNc-knockdown cells.

tOPN has been reported to be a key regulator of the EMT (33). The specific mechanism involves the modulation of the expression of transcription factors that contribute to EMT initiation, such as Twist, Snail and Slug (33). Therefore, tOPN favors EMT initiation, which in turn contributes to tumor progression and chemoresistance. Although these findings refer only to tOPN, OPNc may similarly modulate cellular plasticity in the ACRP and OVCar-8 Dox^R cell lines, further contributing to tumor progression.

tOPN modulated the expression of ILs through binding to their receptors in the tumor microenvironment, contributing to the chemoresistance and tumor progression (10). tOPN has also been demonstrated to bind to the C-C motif chemokine receptor 1 (CCR1), leading to the activation of the PI3K/AKT/hypoxia-inducible factor 1α signaling pathway in hepatocellular carcinoma cell lines (34). The same study also reported that blocking the OPN-CCR1 interaction with a CCR1 antagonist restricted the effects of tOPN on tumor progression and metastatic capacity in this tumor model (34). Therefore, OPNc upregulation may contribute to the increased expression of the cytokines analyzed in the present study through the activation of their corresponding cellular receptors. Indeed, the mRNA expression levels of the GP130 receptor were downregulated in the OPNc-knockdown cells in the present study. Thus, OPNc may act directly and indirectly by promoting the activation of signaling pathways associated with cell survival, inhibition of apoptosis, tumor progression and chemoresistance in ovarian cancer.

In addition to the upregulation of OPNc expression in ACRP cells, the results of the present study demonstrated that P-gp expression levels were upregulated in these cells compared with those in the parental A2780 cells, which has been previously demonstrated in chemoresistant cells in other tumor models (35). Hsieh *et al* (6) have reported that tOPN modulates the expression of P-gp in a concentration- and time-dependent manner through binding to αvβ3 integrin (6). However, the contribution of specific OPN-SIs to the regulation of P-gp expression has not been previously addressed. The results of the present study also demonstrated that the upregulation of P-gp in ACRP cells was reversed when OPNc was silenced. Similarly, P-gp expression was induced following OPNc overexpression. These results were in agreement with a previous study, in which knockdown of endogenous tOPN potentiated various P-gp drug substrate-induced apoptosis of prostate cancer cells (6). These results suggest that OPNc may be a modulator of P-gp expression and a potential target to modulate drug efflux by P-gp in cancer cells, facilitating tumor resistance. However, the molecular mechanisms by which tOPN, and specifically OPNc, regulates the expression of P-gp remain unknown and should be determined in future studies. High levels of P-gp have been demonstrated to induce TMZ resistance in a glioblastoma (GBM) model (36). Increasing concentrations of TMZ competed with calcein for P-gp, resulting in reduced efflux in the Adriamycin-resistant DC3F cells; however, various inhibitors of P-gp reversed TMZ resistance in two GBM cell lines by increasing the levels of active caspase-3, suggesting that P-gp may be a key regulator of TMZ resistance in GBM (36). High expression of P-gp has

been reported to confer resistance to a wide range of structurally and functionally unrelated chemotherapeutic agents (37). In the present study, OPNc was demonstrated to regulate P-gp gene expression, suggesting that targeting the OPNc/P-gp signaling axis may be a potential approach for ovarian cancer cell sensitization to Dox and CDDP.

The results of the present study also demonstrated that OPNc expression levels modulate the survival of drug-resistant ovarian carcinoma cells. In response to OPNc knockdown, the viability of drug-resistant ovarian cancer cells decreased, resulting in higher sensitivity to CDDP and Dox exposure compared with that of the control cells. These results were in accordance to a role of OPNc as a putative novel gene product able to activate the key steps towards resistance to these chemotherapeutic drugs. Similar results have been observed for other proteins that mediated resistance to chemotherapy by stimulating cell survival and inhibiting apoptosis, as well as promoting the cell cycle, including Bcl-2, inhibitor of apoptosis proteins and the heat shock protein family (5). In addition, tOPN has also been described as a modulator of cell survival in response to chemotherapeutic drugs (10,38). In light of these results, among the three OPN-SIs tested in the present study, OPNc may be a key contributor to the cellular processes mediating chemoresistance, such as cell survival and viability, at least in chemoresistant ovarian cancer cells. Future studies should further characterize the targets and pathways by which OPNc may activate cell survival in response to chemotherapeutic drugs.

Following silencing OPNc expression in OVCar-8 Dox^R cells, increased sensitivity to Dox treatment compared with that in the control cells was observed in the present study. Similarly, the results demonstrated decreased viability and colony formation capacity in these cells, along with a modulation in the expression of EMT markers and EMT-related cytokines. Considering similar experimental findings in CDDP- and Dox-resistant cell lines following OPNc silencing, we hypothesize that targeting OPNc may counteract resistance to various chemotherapeutic drugs, similarly to Dox and CDDP. However, further studies should investigate whether these findings can be extended to other drugs.

The results of the present study demonstrated that OPNc regulated the EMT in the ACRP cell line. OPNc knockdown altered the pattern of the intermediate EMT phenotype observed in ACRP cells. These results were in accordance with a putative role of OPNc as a modulator of EMT, which was previously assigned to tOPN in several experimental models, such as in breast cancer, hepatocellular carcinoma, ovarian, gastric and colorectal cancer, as well as melanoma and brain tumors (33). In the lung cancer cell line A549, Huang *et al* (39) have demonstrated that OPNc overexpression induces increased cell migration and invasion, and alters the expression of EMT markers (decreases E-cadherin and increases vimentin expression) in response to TGF-β treatment, indicating that OPNc stimulates cellular plasticity of A549 tumor cells. The roles of OPNc in non-small cell lung cancer cells were mediated by Runt-related transcription factor 2 via a histone deacetylase-dependent pathway, further suggesting that OPNc may be a marker of the EMT and tumor progression in lung cancer. Our previous study in prostate cancer cells resistant to docetaxel supported these results, as cells overexpressing OPNb or OPNc presented with an EMT-related phenotype typical of chemoresistant cells, as observed in other tumor models (19). The intermediate EMT phenotype observed in CDDP-resistant ACRP ovarian carcinoma cells in the present study was in agreement with a previous study demonstrating that ovarian carcinoma cells present various degrees of EMT (40). An intermediate EMT phenotype has been demonstrated to occur in breast, colon and ovarian carcinoma (41). In addition, the results of the present study demonstrated that OPNc silencing modulate the intermediate EMT phenotype. Notably, ovarian carcinoma has unique biological characteristics, and whether ovarian cancer cells in the primary tumor undergo a complete transition to a mesenchymal state is still under discussion (40,42). In the present study, the EMT does not fully occur in ovarian carcinoma, and is even reversed in tumor cells present in malignant peritoneal and pleural effusions (40). Additionally, Carduner *et al* (43) have reported that ascites in patients with ovarian cancer undergo a shift toward an unstable intermediate state of the epithelial-mesenchymal spectrum, conferring aggressive cell behavior, depending on the initial epithelial-mesenchymal background (43). Future studies are required to fully characterize the intermediate EMT phenotype in ACRP cells and its association with OPNc expression by further evaluating the protein expression and subcellular localization of EMT markers.

In the present study, OPNc knockdown reverted the expression pattern of EMT-related cytokines in ACRP cells. It has been demonstrated that a number of interleukins or cytokines induce the EMT, including IL-6, IL-8 and TGF-β (44,45). The role of cytokines in cancer has been widely studied, particularly in ovarian cancer (46). In this context, certain cytokines have been demonstrated to induce phenotypes consistent with the EMT in transformed epithelial as well as tumor cell lines (47,48). The results of the present study demonstrated that the expression levels of OPNc appeared to modulate the expression of EMT-related cytokines in the drug-resistant cell lines, which in turn induced changes in the expression of EMT markers, as has been previously observed (44,45). A previous study has demonstrated that tOPN modulates cytokine expression, contributing to tumor progression; the interaction between tOPN-IL-6-STAT3 contributes to the invasive and migratory features of osteosarcoma tumor cells, enhancing their metastatic potential (49). Since these results refer to tOPN, the present results are the first to report the role of OPN-SIs, mainly OPNc, in the modulation of the expression of EMT-related cytokines. Further studies are needed to determine the regulatory mechanisms between OPNc and the investigated cytokines in the *in vitro* chemoresistance models. We hypothesize that upregulation of OPNc may contribute to the increased expression of the cytokines analyzed in the present study through the activation of their corresponding cellular receptors. This was observed to occur for the GP130 receptor, the mRNA levels of which were downregulated in the OPNc-knockdown cells in the present study. Thus, OPNc may act directly and indirectly by promoting the activation of signaling pathways associated with cell survival, inhibition of apoptosis, tumor progression and chemoresistance in ovarian cancer.

To validate the data from OPNc silencing experiments, the present study overexpressed OPNc in the A2780 cell line.

Cells overexpressing OPNc displayed increased cell viability and colony formation capacity compared with those in cells transfected with the empty expression vector. These data reinforced the pro-survival roles of OPNc in these ovarian cancer cells. Similar to ACRP cells in which OPNc was silenced, A2780 OPNc$^+$ cells were also more sensitive to CDDP treatment compared with the control cells. These results suggested that cells overexpressing OPNc may be preferentially targeted for CDDP sensitization. Further corroborating our hypothesis, the results of the present study demonstrated that CDDP downregulated the expression of OPNc in OPNc$^+$ cells. As samples from patients with ovarian cancer exhibit high levels of OPNc (30), and the results of the present study demonstrated that CDDP-resistant cells present upregulated levels of OPNc, we hypothesized that targeting OPNc in ovarian cancer cells with high levels of OPNc may be a promising new therapeutic approach to overcome resistance to CPPD. In addition, combining OPNc silencing with CDDP treatment may possibly enhance cytotoxicity in these resistant cells. One explanation for this effect may be that CDDP, in addition to inhibiting OPNc expression, may also somehow prevent OPNc binding to classical OPN receptors, such as CD44 and integrin heterodimers, which is worth investigating in the future.

The cytotoxicity assays performed in the present study revealed that ACRP cells became more sensitive to CDDP compared with A2780 cells when CDDP concentrations were progressively increased (data not shown). Accordingly, drug adaptation and thus, resistance, are considered to be a dose-dependent condition for certain drug classes and *in vitro* models. In addition, the present study demonstrated that OPNc silencing in ACRP cells counteracted cell viability and resistance to CDDP. Notably, OPNc$^+$ cells exhibited increased sensitivity to CDDP compared with that in the control cells, which may be associated with CDDP-induced OPNc downregulation. In the experimental settings of the present study involving the ACRP cell line which exhibited upregulation of endogenous OPNc, as well as A2780 OPNc$^+$ cells, targeting OPNc sensitized cells to CDDP. Based on these results, we hypothesized that OPNc$^+$ cells may be suitable candidates for novel approaches to CDDP treatment. Translating these findings into the clinical practice, high OPNc expression in patients with ovarian cancer at diagnosis may be a potential predictor for a favorable response to CDDP treatment. This idea follows the rationale reported for other drug targets, such as Her-2 (50-52). In addition, approaches aiming at inducing cell toxicity based on oncogene expression are already used in other tumor models, such as in Her2-positive breast, gastric and esophageal cancer, in which distinct strategies have been proposed to block Her2-induced tumor growth (53-55).

In conclusion, the results of the present study demonstrated that OPNc was upregulated in CDDP-resistant ACRP cells and may serve important roles in modulating various biological processes associated with drug resistance, including cell viability, the EMT phenotype and the expression of EMT-related cytokines. OPNc$^+$ cells were more sensitive to CDDP cytotoxicity compared with the negative control cells, suggesting that OPNc overexpression may be a potential strategy for targeted therapy to improve CDDP sensitivity in ovarian cancer cells. Therefore, we propose a model in which OPNc is a key modulator of resistance to CDDP in ovarian cancer cells (Fig. 6). These early insights on the role of OPNc in cancer drug resistance provide a rationale for using this variant as a novel potential molecular target to sensitize ovarian cancer cells to Dox and CDDP treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MCMB participated in the design of the study, performed the majority of the experiments and analyzed the data. LBF participated in the data analysis, reviewed and edited the manuscript. IDSG participated in in the design of the study and data analysis, as well as reviewed and edited the manuscript. LBAR participated in the design of the study and provided support to this work. RCM provided support to this work, reviewed and edited the manuscript. GNDM and ERPG are co-senior authors and equally participated in the design of the study, analyzed and summarized the data, provided financial support, wrote, reviewed and edited of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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