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PTN Signaling: Components and Mechanistic Insights in Human Ovarian Cancer

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Abstract

Molecular vulnerabilities represent promising candidates for the development of targeted therapies that hold the promise to overcome the challenges encountered with non-targeted chemotherapy for the treatment of ovarian cancer. Through a synthetic lethality screen, we previously identified pleiotrophin (PTN) as a molecular vulnerability in ovarian cancer and showed that siRNA mediated *PTN* knockdown induced apoptotic cell death in epithelial ovarian cancer (EOC) cells. Although it is well known that PTN elicits its pro-tumorigenic effects through its receptor, protein tyrosine phosphatase receptor Z1 (PTPRZ1), little is known about the potential importance of this pathway in the pathogenesis of ovarian cancer. In this study we show that PTN is expressed, produced, and secreted in a panel of EOC cell lines. PTN levels in serous ovarian tumor tissues are on average 3.5-fold higher relative to normal tissue and PTN is detectable in serum samples of patients with EOC. PTPRZ1 is also expressed and produced by EOC cells and is found to be up-regulated in serous ovarian tumor tissue relative to normal ovarian surface epithelial tissue ($p < 0.05$). Gene silencing of *PTPRZ1* in EOC cell lines using siRNA mediated knockdown shows that PTPRZ1 is essential for viability and results in significant apoptosis with no effect on the cell cycle phase distribution. In order to determine how PTN mediates survival, we silenced the gene using siRNA mediated knockdown and performed expression profiling of 36 survival-related genes. Through computational mapping of the differentially expressed genes, members of the MAPK (mitogen-activated protein kinase) family were found to be likely effectors of PTN signaling in EOC cells. Our results provide the first experimental evidence that PTN and its signaling components may be of significance in the pathogenesis of epithelial ovarian cancer and provide a rationale for clinical evaluation of MAPK inhibitors in PTN and/or PTPRZ1 expressing ovarian tumors.

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Keywords

Epithelial ovarian cancer (EOC); pleiotrophin (PTN); survival; MAP kinase signaling; PTPRZ1 (protein tyrosine phosphatase receptor Z1)

INTRODUCTION

Epithelial ovarian cancer (EOC) is the second most common gynecological cancer, and the fifth leading cause of death among women in the U.S., with an estimated 22,240 newly diagnosed cases and 14,030 deaths in 2013 [1]. Among the different histotypes of epithelial ovarian cancer, including serous, mucinous, clear cell and endometrioid [2–4], the majority of deaths occur in patients with advanced stage, high-grade epithelial serous ovarian cancer [4]. Despite high response rates to the initial standard surgery and chemotherapy regimen, the majority of the patients exhibit recurrent disease. Furthermore, the overall survival rate for women with ovarian cancer has not dramatically improved over the last 30 years [5]. These statistics highlight a need for novel therapeutics. This can be best achieved by improving our understanding of the molecular pathways that are instrumental in driving the disease and therefore serve as the basis of the promising targeted therapeutic approaches.

We performed a synthetic lethality RNA mediated interference (RNAi) screen across ovarian tumorigenic and non-tumorigenic cells and identified pleiotrophin (PTN), a heparin binding growth factor, as one of the molecular vulnerabilities [6]. Moreover, our *in-silico* data analyses across two independent cohorts, TCGA (The Cancer Genome Atlas, [7]) and NCI (Dr. Michael J. Birrer, [8]) showed PTN to be overexpressed in 37–40% of high-grade serous EOCs. Although, PTN mediated pro-tumorigenic effects have been reported previously [9], its potential role in the pathogenesis of human ovarian carcinoma is unexplored.

PTN also known as HARP (Heparin Affinity Regulatory Protein), is a developmentally controlled 136 amino acid secreted heparin binding cytokine [10]. Pleiotrophin is expressed in embryonic cells during different developmental periods [11]. PTN is up regulated in keratinocytes during the process of wound repair and healing [12]. Although it is highly expressed during embryogenesis, there is very limited expression in adult tissues, where it is largely restricted to the brain [12]. The *PTN* gene is considered a proto-oncogene [13] and is overexpressed in a number of cancers including pancreatic cancer [14] and myelomas [9]. PTN is involved in cell transformation, growth, survival, migration and angiogenesis [12]. *PTN* is also up-regulated in cells following stress and injury and is important for new tissue formation during recovery from injury [15]. PTN exerts its biological activity through interaction with cell surface proteoglycans, such as syndecans [16] or through binding to its selective cell surface receptor, protein tyrosine phosphatase receptor Z1 (PTPRZ1) [17]. PTN ligand binding to PTPRZ1 diminishes its phosphatase activity, putatively through ligand-induced dimerization of the active D1 domain of PTPRZ1 [17]. This ligand-receptor interaction lead to accumulation of tyrosine phosphorylation of multiple downstream proteins including SRC kinase [18], calmodulin [19], and β -catenin [17] which in turn have been related to activation of multiple pro-tumorigenic signaling cascades. Furthermore,

PTPRZ1 has been shown to have oncogenic functions [19, 20] and is overexpressed in small cell lung cancer [19], cervical carcinoma [21], breast cancer [22], astrocytoma [23] and cutaneous melanomas [24].

In this study, we assessed the putative role of PTN and its signaling components in ovarian cancer cell growth and survival. We report that the PTN and its pathway members are aberrantly expressed in ovarian cancer and are important for EOC cell survival and therefore potential therapeutic targets.

MATERIALS AND METHODS

Cell Culture

EOC cell lines (A1847, A2780, OVCAR3, OVCAR5 and SKOV3) [25–28] were grown in RPMI 1640 (Invitrogen), [supplemented with 10% FBS (Hyclone, Gibco), 2 mM L-glutamine (Invitrogen), 100 IU/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen)]. The cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO₂ [29].

Reagents and antibodies

Goat anti-PTN was obtained from Abcam. Mouse anti-phospho ERK1/2 and anti-ERK1/2 were obtained from Cell Signaling Technology. Mouse anti-PTPRZ1 was obtained from BD. HRP-conjugated anti-goat antibodies were obtained from Santa-Cruz Biotechnology. HRP-conjugated anti-mouse antibodies were obtained from Bio-Rad. Recombinant human PTN was obtained from R&D Systems. Mouse anti-β-actin antibody was obtained from Sigma.

Analysis of secreted PTN levels by direct ELISA

A1847, A2780, OVCAR3, OVCAR5 and SKOV3 cells were seeded at a cell density of 30,000 cells/100 µL per well in a 96-well plate in complete medium. After 24 h, the media was changed to serum free and the cells were starved for additional 48 h. The conditioned media was collected and spun down at 3,000×g for 5 min. The cell free supernatant was analyzed by direct ELISA as described previously.[9] Briefly conditioned media were incubated in Immulon flat-bottomed 96-well plates overnight at 4 °C. Plates were then washed 3 times with phosphate buffered saline (PBS) and blocked with PBST (PBS with 0.1% Tween-20, supplemented with 1% bovine serum albumin) at room temperature for 2 h. Next, the plates were incubated with 100 µL/well of primary antibody (0.5 µg/mL biotinylated anti-PTN, R&D Systems) at 4 °C overnight, washed 3 times with PBST incubated with 100 µL secondary antibody (alkaline-phosphatase conjugated streptavidin, 200 mg/mL, 1:200, KPL) for 1 h at room temperature and washed 4 times with PBST. Bound proteins were detected using Blue-Phos microwell phosphatase substrate (KPL) following manufacturer's directions and plates were read using an Infinite M200Pro plate reader (Tecan) at 620nm. Experiments were performed in triplicates. A standard curve was generated using 0 to 2 ng/mL rhPTN (recombinant human PTN, R&D Systems).

qRT-PCR and PCR array

To measure *PTN* and *PTPRZ1* mRNA levels across cell lines, total RNA was isolated using TRIzol reagent as described previously [6]. Reverse transcription was performed to prepare cDNA [30] followed by specific target amplification (14 cycle PCR reaction) for each sample using 100 ng cDNA combined with the pooled TaqMan primers in Pre-Amp Mastermix (Life Technologies) using manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed using the Fluidigm BioMark microfluidic real-time PCR platform. To quantify the mRNA levels of a specific gene, the cycle threshold (C_t) values were determined from the amplification curves for each sample and then normalized to the level of *PPIA* (internal control reference gene) in that sample. Gene expression profiles in cells treated with siRNA targeting *GL2* or *PTN* mRNA were characterized using a RT² ProfilerTM PCR Array (Qiagen PAHS027Z, commercially targeted array). Since we observed an apoptotic phenotype on PTN knockdown, therefore a panel of 36 survival-related genes (shortlisted by enrichment in biological functions of cell death and-survival using IPA as described previously [31, 32]) were analyzed (Supplementary Table S5) for differential expression. qRT-PCR was performed on a Bio-Rad CFX96 C1000 Touch real-time detection apparatus (Bio-Rad Laboratories) using a RT SYBR Green/Fluorescein qPCR Master Mix (SABiosciences). The conditions used for PCR amplification were: 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C for 45 cycles, and fluorescence was measured after each amplification cycle. To quantify the mRNA levels of a specific gene, the C_t values were determined from the amplification curves for each sample and then normalized to the mean C_t values of the five housekeeping genes (*ACTB*, *GAPDH*, *HPRT1*, *B2M*, and *RPL13A*) in each sample. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method using the web-based software (SuperArray Biosciences Corp) in *PTN* siRNA treated as compared to *GL2* siRNA (negative control) treated A2780 cells.

Western blotting

Cell lysates were prepared in RIPA buffer (10 mM NaPi, pH 7.8, 60 mM NaCl, 1% Triton X100, 0.5% deoxycholic acid, 0.1% SDS, 10% glycerol, 25 mM β -glycerol phosphate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate) and analyzed by immunoblotting using primary antibodies specific for PTN (1:500), PTPRZ1 (B D, 1:250), ERK (1:250), pERK1/2 (1:2000), and β -actin (1:10,000). The blots were quantified by densitometry using Alpha View software, version 3.3 (Cell Biosciences).

siRNA reagents

PTN siRNA (Qiagen), PTPRZ1 siRNA (Qiagen), reagents were purchased from Qiagen. Each siRNA was targeted at a final concentration of 50nm that comprised of equimolar concentration of two independent siRNA sequences (PTN: product ID SI03093083 & SI00043750, PTPRZ1: product ID SI00044457 & SI00044471).

siRNA transfections in 96 well plates and cell viability

All siRNA transfections were done using the reverse transfection method as described previously [6]. Briefly, DharmaFECT-1 was diluted in reduced-serum media (OptiMEM, Invitrogen) and added to the siRNAs arrayed in v-bottom 96-well dilution plates using a

bulk reagent microplate dispenser. The concentration of siRNA pools was 50 nM. The siRNA-lipid complexes were allowed to form for 30 min at room temperature followed by addition of cells in an antibiotic-free medium using a bulk reagent microplate dispenser (100 μ L final volume per well). Following 72 h of incubation at 37 °C, cell viability was determined by using Cell Titer-Blue (CTB, Promega). The CTB reagent was diluted 3-fold in PBS prior to its addition to the assay plates (20 μ L per well added using a bulk reagent microplate dispenser). Fluorescence intensity was measured by using an Infinite M200Pro plate reader (Tecan) 3 h following addition of the CTB reagent. Data were analyzed using the viability score method as described previously [6] where viability in test well was normalized to the viability in the negative control wells. Parameters which affect efficiency of transfection such as the dilution factor of the DharmaFECT-1 lipid reagent, the concentration of the siRNA, and the cell seeding density were optimized for each of the five cell lines as described previously [6].

siRNA treatment in 10 cm plates

Cells were seeded ($4 \times 10^5 - 6 \times 10^5$) in 10 cm plates overnight. Next day, media was changed to OptiMEM (Invitrogen) for 4 h. Transfection complexes were then added to the plate with final siRNAs (50 nM). After 48–72 h, cells were harvested and lysates were prepared for immunoblotting.

Apoptosis and Cell Cycle analysis

A2780 and OVCAR5 cells were transfected with *GL2* (negative control) and *PTPRZ1* siRNA in 96-well plates using the transfection method as described above for the 96-well format. After 72 h, cells were trypsinized and processed for apoptosis and cell cycle analysis using the Guava Nexin and Guava Cell Cycle assays, respectively, following the manufacturer's instructions (Guava Technologies, Millipore).

Analysis of genomic data sets

The log₂ ratios for copy number and β -values for DNA methylation were downloaded from TCGA portal (<http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>) for a total of 494 cases for the *PTPRZ1* gene. A mean β -value was calculated since the results from 2 probes were provided

Serum and Tissue samples from ovarian cancer patients

All tumor (high-grade serous ovarian cancer) and non-tumor serum and tissue samples (that were homogenized in lysis buffer and used for immunoblotting experiments) were obtained from the KU Cancer Center's Biospecimen Repository Core Facility (BRCF). Tumor grades and histological subtypes were obtained from the pathology reports without reference to the patient's personal information. Written informed consent was obtained by the members of the BRCF from each patient and extensive precautions were taken to preserve the privacy of the participants. The University of Kansas Medical Center's Human Subjects Committee approved the use of these tissues. All safety and ethical guidelines were followed.

Tumor samples for Immunostaining

Ovarian tissue microarrays (TMAs) were constructed by the KUCC BRCF and contained duplicate cores of both normal ovarian tissue samples (n = 26) and serous ovarian tumor cases (n = 56).

Immunohistochemistry

The method for IHC has been described previously.[33] Briefly, rehydrated TMA sections were treated with target retrieval solution (pH 6.0, Dako). Sections were then incubated with primary antibody (PTPRZ1 at 1:100 dilution) for overnight at 4 °C and successively conjugated with peroxidase-conjugated polymer directed against mouse (Dako) and diaminobenzidine (DAB) substrates. Stained sections were imaged using Nikon 80 microscope (Nikon Inc.). Both the epithelial ovarian cancer (EOC, serous subtype) tissue cores and normal ovarian surface epithelia (OSE) were assessed for intensity of staining by two independent pathologists (visualization experiment 1 (VE1) and visualization experiment 2 (VE2)) and scored as 0 (for undetectable staining), 1 (for weak intensity), 2 (for moderate intensity) or 3 (for strong intensity) as well as for the percentage of positively staining cells. In addition, staining was separately assessed for subcellular location. Spearman's correlation test was performed to analyze correlations between VE1 and VE2.

Bioinformatics and enrichment analysis

Functional analysis and network generation was done using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com)

Statistical analysis

Descriptive statistics including mean and standard deviation, student's two-tailed t-tests and Fisher's exact test were computed using Graph Pad Prism 5.0 software. P values < 0.05 were considered significant.

RESULTS

PTN expression in EOC cell lines and serous ovarian carcinoma tissue

In order to dissect the potential role of PTN in ovarian cancer, we determined the PTN expression levels across a panel of five EOC cell lines (A1847, A2780, OVCAR3, OVCAR5 and SKOV3). We evaluated expression of PTN mRNA using qRT-PCR which showed differential levels of expression in the EOC cell lines (normalized C_t values, Figure 1A). PTN was detected as a band with a molecular weight (MW) of 18 kDa, corresponding to the migration of recombinant human PTN (rhPTN), across these EOC cell lines by immunoblotting (Figure 1B). Primary human fibroblasts derived from normal ovaries (HFNO-402) or from ovarian tumors (HFOT-208) [34] have undetectable levels of PTN (Supplementary Figure S1A). We next measured secreted levels of PTN across the EOC cells and a positive control glioblastoma cell line, LN229, by ELISA. PTN was detected in all the EOC cell lines evaluated, showing an average level of 0.49 ng/mL (range ~0.30 – 0.65 ng/mL, n = 4, Figure 1C) and was 2- to 3-fold higher than levels detected in the positive control LN229 cell line (0.19 ng/mL). Based on these results, it was apparent that

PTN is expressed, produced and secreted by the ovarian cancer cells evaluated. We next evaluated PTN levels in tumor tissue and serum samples from patients with ovarian carcinoma. Using a small sample set that included normal ovarian tissue (n = 2) and high-grade serous ovarian tumor tissue (n = 5), protein extracts were prepared and analyzed for PTN expression using immunoblotting (Figure 1D). PTN was detected in 4 out of the 5 tumor lysates with 3 of the samples exhibiting 2-fold or higher expression levels than the normal tissues (Figure 1D). The mean level of PTN across the five tumor tissues was ~3.5-fold higher than the mean level in normal ovarian tissue (Supplementary Figure S1B). However, when we measured circulating levels of PTN in serum samples from patients with high grade serous ovarian cancer (grade III, n = 10) and normal human age- and gender-matched controls (n = 10), no statistically significant difference was observed (p = 0.63) (Supplementary Figure S1C), suggesting that the circulating levels of PTN as measured in this study are not a strong discriminator of disease state.

Expression of PTPRZ1 in EOC cell lines and serous ovarian carcinoma tissue

We next evaluated the expression levels of the PTN receptor, PTPRZ1, in EOC cells. As with *PTN* transcript levels, *PTPRZ1* mRNA could be detected across all of the EOC cell lines (Figure 2A). Immunoblotting revealed the presence of a transmembrane protein with an approximate MW of 300 kDa in all EOC cells, similar to one that was observed in the positive control cell line LN229 [19] (Figure 2B). In comparison, the primary human ovarian fibroblast lines, HFNO-402 and HFOT-208, appear to have very low levels of PTPRZ1 (Supplementary Figure S2). Furthermore, TCGA ovarian dataset [7] was analyzed for *PTPRZ1* DNA methylation and copy number across 494 high grade serous adenocarcinoma specimens (Figure 2C and 2D). β -values for DNA methylation status of *PTPRZ1* indicate that the gene was hypomethylated (β -values < 0.25 [35]) across 96% of the specimens. These results imply that the gene is actively transcribed across the majority of the tumor specimens. Copy number data showed that *PTPRZ1* exhibits low level copy number gains ($\log_2$ tumor/normal ratio > 0.3) across 30% of the samples.

We further evaluated PTPRZ1 expression in clinical samples by immunostaining. The ovarian TMAs examined contained epithelial components from a total of 26 normal ovarian tissue samples and 54 ovarian tumor (serous histotype) cases (two cores per case) (Table 1, patient characteristics). Each core was independently scored by two pathologists (VE1 and VE2) as described in the Methods section. PTPRZ1 could be detected in 100% of the tumor samples (Supplementary Table S2) with PTPRZ1 staining noted in 60%-100% of the epithelial cells (Supplementary Table S2). In contrast, PTPRZ1 staining was undetectable in the majority of normal ovarian surface epithelial (OSE) cells with measurable staining being typically weak (Supplementary Table S1). The sub-cellular localization of PTPRZ1 was not uniform across the serous ovarian cancers or the normal ovarian tissues studied. Fifteen percent of the tumor samples showed strong membranous staining at the tumor-stromal junction (Table 2 Fisher's exact test p-value < 0.05; Supplementary Tables S1 and S2 and Figure 3A (left) for a representative image). No membranous staining was observed in normal OSE cells. PTPRZ1 expression was elevated in the cytoplasm of all the transformed tissues with weak, moderate and strong staining noted in 15%, 59% and 26% of the samples respectively. The tumor epithelial components scored higher when compared to normal OSE

(Table 3 Fisher's exact test p-value < 0.0001; Supplementary Tables S3; Figure 3A for a representative image; and Figure 3B)). Nuclear PTPRZ1 expression was elevated in the tumor epithelium as compared to normal OSE (Table 4 Fisher's exact test p-value = 0.0018; Supplementary Table S4; Figure 3A for a representative image; and Figure 3C). Nuclear staining for PTPRZ1 was undetected in 42% of normal OSE samples and was weakly positive for 54% of normal OSE samples with only one sample staining moderately. Nuclei of 89% of tumor samples stained positive with 39% staining weakly, 45% staining moderately and about only 5% staining strongly. A statistically significant positive correlation between VE1 and VE2 scores for the cytoplasmic ($r = 1$) and nuclear ($r = 0.93$, $p < 0.001$) PTPRZ1 intensity across 26 OSE cores was observed (Supplementary Table S1). Similarly, a statistically significant positive correlation between VE1 and VE2 scores for the cytoplasmic ($r = 0.86$, $p < 0.001$) and nuclear ($r = 0.61$, $p < 0.001$) PTPRZ1 intensity across 54 EOC cores was observed (Supplementary Table S2). Although a weakly positive correlation ($r = 0.3$) between VE1 and VE2 scores for the membranous PTPRZ1 intensity across the fifteen percent of the tumor samples was observed (Supplementary Table S2), but the VE1 and VE2 scores did not significantly overlapped due to non-uniform staining patterns. Furthermore, Kaplan-Meier survival analysis of the IHC staining intensities (undetected/weak vs. moderate/strong) with the corresponding clinical survival data from each patient was performed to determine the prognostic value of PTPRZ1 expression at all three cellular sites (cytoplasm, membrane and nucleus). We did not observe statistical significance ($p > 0.05$) in the survival curves for any of the sub-cellular stains, with nuclear staining, although not statistically significant, showing the greatest difference in survival between the two staining intensities (Supplementary Figure S3).

Knockdown of PTPRZ1 effects viability of EOC cells

In our previous study [6] we showed that gene silencing of *PTN* by RNAi approaches affected EOC cell viability and decreased survival. In this study we evaluated whether knockdown of its receptor, *PTPRZ1* affected EOC cell viability as well. We transfected A1847, A2780, OVCAR3, OVCAR5 and SKOV3 with *PTPRZ1* siRNA and a negative control siRNA (*GL2* siRNA targeting firefly luciferase) and measured the effect on cell viability after 72 h. All EOC cell lines exhibited variable sensitivity to knockdown of *PTPRZ1* with normalized viability scores ranging from 0.40–0.89 (decrease in viability ranged from 11–60%) across the five EOCs as compared to respective controls (Figure 4A). More than 40% reduction in cell viability was observed in 3 out of the 5 cell lines. Among these cell lines, A2780 and OVCAR5 were the most sensitive to PTPRZ1 knockdown. Phase contrast images of *GL2* siRNA treated and *PTPRZ1* siRNA treated A2780 cells (Figure 4B) and OVCAR5 cells (Supplementary Figure S4) are shown. As an additional check on the ability of *PTPRZ1* siRNA to correctly target *PTPRZ1*, we performed immunoblotting to determine the level of PTPRZ1 protein 72 h after siRNA treatments in A2780 cells (Figure 4C). An approximate 80% decrease in PTPRZ1 level was observed in *PTPRZ1* siRNA treated as compared to *GL2* siRNA treated A2780 cells.

Since cell viability measurements provide limited information about the mechanism of decreased cell viability following gene silencing, we investigated the functional aspects of targeting *PTPRZ1* on survival (apoptosis and necrosis) and cell cycle progression in EOC

cells. A2780 and OVCAR5 cells were transfected with *PTPRZ1* siRNA and *GL2* siRNA (50 nM) in 96- well plates. Cells were harvested 72 h post transfection. Thereafter, effect on survival (using the Guava Nexin assay which combines annexin V staining to measure apoptosis and 7-Amino-actinomycin D (7-AAD) staining to measure necrosis) and on cell cycle progression (using the Guava Cell Cycle assay) were analyzed (Figures 4D and 4E, respectively). A significant 1.4-fold increase in annexin V positive and a significant 1.3-fold increase in 7-AAD positive cells was observed in *PTPRZ1* siRNA treated as compared to *GL2* siRNA treated A2780 cells (Figure 4D, **left panel**). In OVCAR5 cells, there was a significant 1.2-fold increase in annexin V positive and a significant 1.4-fold increase ($n = 3$, $p < 0.05$) in 7ADD positive cells in *PTPRZ1* siRNA treated as compared to *GL2* siRNA treated cells (Figure 4D, **right panel**). However, no significant effect on the cell cycle phase distribution was observed following knockdown of *PTPRZ1* gene in either of the two cell lines (Figure 4E).

PTN mediates its pro-survival effect through ERK1/2 (extracellular signal-regulated kinase) in EOC cells

We have previously shown that knockdown of *PTN* induces significant apoptosis in A2780 cells [6]. To investigate the potential signaling pathways induced by PTN in EOC cells, we adopted an integrated experimental and computational approach. First, we evaluated the differences in expression across a panel of 36 survival related genes in *PTN* siRNA treated A2780 cells as compared to negative control siRNA treated A2780 cells. Fold-change in gene expression was calculated (Supplementary Table S5) and it was found that 24 genes exhibited a 1.5-fold or greater change in expression level following *PTN* silencing relative to *GL2* siRNA treated cells (Figure 5A). Of these, 15 genes were down-regulated by 1.5-fold or more following *PTN* depletion (highlighted in green in Supplementary Table S5), and were subsequently analyzed for signaling effectors responsible for the observed changes in their gene expression using a computational method (IPA network analysis, Figure 5B). Functional mapping and network characterization of these 15 genes was performed (shaded nodes, Figure 5B). The results showed that the network with the highest significance [36, 37] to which the genes mapped exhibited downstream effects through multiple signaling effectors (non-shaded nodes, Figure 5B) namely ERK1/2, RB, and cyclin A. Of these, PTN has been previously shown to exert its pro-tumorigenic effects through ERK1/2.[38, 39] Moreover, ERK1/2 is a well-known effector protein that has been implicated as an important mediator of growth factor induced oncogenic pathways in ovarian cancer [40–42].

Based on these observations, we evaluated whether ERK1/2 is involved in PTN mediated signaling in EOCs. First, we investigated whether PTN knockdown affects ERK1/2 signaling in A2780 cells. *PTN* siRNA treatment down-regulated the cellular PTN levels by more than 50% at 48 h in A2780 cells (Figure 5C) and secreted PTN levels as measured by ELISA were decreased by more than 75% (Supplementary Figure S5). Furthermore, *PTN* siRNA treatment in A2780 cells reduced phosphophorylated ERK1/2 (pERK1/2) levels by 86% and 100% at 24 h and 48 h after siRNA transfection, respectively (Figure 5C). Similar to *PTN* siRNA treatment, *PTPRZ1* siRNA treatment in A2780 cells also reduced phosphophorylated ERK1/2 (pERK1/2) by 70% at 48 h after siRNA transfection (Supplementary Figure 6A and 6B).

Next, we examined the effect of PTN stimulation on ERK1/2 phosphorylation. The three cell lines A2780, OVCAR3 and OVCAR5 that showed maximum sensitivity to *PTN* siRNA treatment (Supplementary Figure S7A) were tested. Cells were seeded overnight and serum starved for 48 h followed by treatment with PTN (100 ng/mL). In A2780, peak induction of pERK1/2 levels was observed at 5 min (2.4-fold increase in pERK1/2 levels as compared to unstimulated cells) following PTN treatment (Supplementary Figure S7B). OVCAR3 and OVCAR5 also showed pERK1/2 induction following PTN treatment (Supplementary Figures S7C and S7D). Finally, this induction of pERK1/2 by PTN treatment was attenuated by treatment with a MEK inhibitor U0126 (30 μ M [30], 2 h) (Figure 5D) suggesting that induction was directly related to PTN stimulation.

DISCUSSION

The cancer cell secretome that includes proteins like growth factors, extracellular matrix-degrading proteinases, cell motility factors and cytokines or other bioactive molecules has been demonstrated to have an essential role in the processes of cancer cell survival differentiation, invasion, metastasis and angiogenesis by signaling through their respective receptors [43]. Growth factor-receptor pairs are well known to be instrumental in pro-tumorigenic signaling, and therefore, represent important targets for therapeutics and potential biomarker candidates. Using an RNA interference screening approach we recently demonstrated that knockdown of *PTN* is synthetically lethal across a panel of EOC cell lines leading to apoptotic cell death [6]. In this study, we explored the role of PTN mediated signaling pathways in EOC cell survival. *PTN* and its pathway members have been previously been implemented in cancer [9, 22, 24, 44]; however, PTN's potential role in the pathogenesis of human ovarian cancer has not previously been reported.

PTN has been suggested to play a role in the tumorigenesis of several cancers including glioblastoma, melanoma, and pancreatic cancer [45–47]. We detected elevated levels of PTN, both mRNA and protein in EOC cells as well as in conditioned media. We also detected abundant expression of PTN in tumor tissue samples from patients with high grade serous EOC. However, secreted PTN levels in serum samples from patients did not discriminate the disease state, i.e., a small but statistically insignificant increase in levels of PTN as compared to serum samples from healthy controls was observed. A previous study in pancreas, colon and stomach tumors has shown that in ~47% of the patients with PTN positive tumors, the amount of pleiotrophin protein produced by the tumor was not sufficient to result in elevated pleiotrophin serum levels [48]. As shown by our results, tissue-associated levels of PTN were elevated across the majority of serous ovarian carcinoma tissues, suggesting that PTN may be involved in an autocrine loop with its receptors in ovarian tumors.

PTN is known to mediate its pro-tumorigenic effects through a broad range of signaling pathways including IRS-1, AKT, ERK, STAT3, and Wnt signaling [17, 49]. Activation of these pathways is known to mediate a myriad of functions that include tumor growth, metastasis, and resistance to chemotherapy [17, 50]. In order to dissect its signaling mediators in EOC, we performed functional analysis of the survival genes that were affected following PTN gene silencing in EOC cells. Use of the IPA knowledge-base to evaluate the

functional interaction of these genes demonstrated that ERK1/2 was one of the signaling effectors in the highest scoring network. Experimental validation showed that the knockdown of *PTN* reduced levels of phosphorylated ERK1/2 in a time dependent manner. Additionally, PTN stimulation led to augmentation of phosphorylation of ERK1/2 that could be attenuated by specific drug inhibition. It appears that the relative level of activation of ERK1/2 by PTN was dependent on PTPRZ1 expression as pERK induction was modest in OVCAR5 cells as compared to A2780 and OVCAR3 cells (Supplementary Figure S7), i.e., qPCR and immunoblotting results revealed that OVCAR5 expressed lower levels of PTPRZ1 as compared to A2780 and OVCAR3 (Figure 2A and 2B).

ERK1/2 is a well-known pro-tumorigenic effector across multiple tumor types [37] and is therefore being targeted clinically. MAPK inhibitors are currently in Phase I/II clinical trials for patients with solid tumors and lymphoid malignancies [51, 52]. A promising drug, Trametinib (GlaxoSmithKline) is being considered for FDA approval for the treatment of melanoma [53]. Another MAPK inhibitor, Selumetinib (AstraZeneca), has been shown to be effective in high grade serous ovarian cancer patients [54] with a disease-control rate of more than 80% with a median progression free survival of 11.3 months. Therefore, pathways that converge on ERK1/2 signaling are of critical importance from a combinatorial therapeutics point-of-view. Modulation of sensitivity to novel targeted therapeutics by targeting MAP kinases has been proposed to be an amenable approach [55]. Our results show that PTN signaling involves ERK1/2 in EOC cells. Furthermore, A2780 cells that exhibit sensitivity to PTN and PTPRZ1 siRNA treatment followed by reduced levels of phospho-ERK, are also sensitive to MAP kinase inhibition using small-molecule inhibitors as evident through multiple studies [56–59], thereby strengthening the correlation of PTN/PTPRZ1 with phospho-ERK. Thus, interfering with PTN in combination with MAPK inhibitors is likely to be effective against ovarian tumor growth. Anti-PTN therapeutics that involve the use of PTN neutralizing antibodies to inhibit its functional effects is currently under investigation [60].

PTPRZ1, a member of tyrosine phosphatase receptor family interacts with its ligand PTN through which it mediates its proliferative and metastatic effects as shown in glioblastoma and SCLC [19, 44] tumor models. Its expression has been found to be up-regulated in multiple tumor types and has been shown to be important for growth and survival of tumor cells [20, 44]. We report that EOC cells express PTPRZ1 at both the mRNA and protein levels. A1847, OVCAR5 and SKOV3 exhibited low expression of PTPRZ1 at both mRNA and protein level. However, A2780 and OVCAR3 cells exhibited discrepancy in relative transcript and protein levels. Such divergence between gene and protein expression in tumor cells have been reported before (for e.g., lung adenocarcinomas [61, 62]) and could be attributed to multiple factors, e.g., sample processing, technological sensitivities and actual biological difference between transcript and protein abundance [63]. Moreover, it has been previously shown that in neuroblastoma cells [64] and small cell lung cancer cells [19], both core and glycosylated forms of transmembrane PTPRZ1 protein is expressed. This could be a possible explanation behind the double bands observed in our positive control glioblastoma cell line (LN229).

As suggested by our siRNA silencing data, PTPRZ1 is important for EOC cell survival. However, PTPRZ1 siRNA treatment did not affect cell-cycle phase distribution in ovarian cancer cells. PTPRZ1 depletion has been shown to be synthetically lethal by other studies as well [19, 44, 65]. For instance, an RNA interference study that focused on discovering novel kinases and phosphatases which may affect cancer cell survival identified PTPRZ1 as one of the important candidates [65]. However, there are no apparent efforts to develop PTPRZ1 inhibitors. In this aspect, siRNA based drugs are gaining traction clinically [66].

In support of our findings, analysis of TCGA data revealed that ~30% of the high-grade serous ovarian tumors display copy number gains at the *PTPRZ1* gene locus. *PTPRZ1* has also been reported to be amplified up to eight-fold in a renal cell carcinoma rodent model induced by chronic oxidative stress [67, 68]. Although circumstantial, we observed that the *PTPRZ1* gene was hypomethylated in the vast majority (96%) of the high grade ovarian serous adenocarcinoma specimens. We evaluated the protein levels of PTPRZ1 in an independent panel of high-grade serous adenocarcinomas by immunohistochemical staining. Most notably cytoplasmic staining was predominant in all of the tumor samples with approximately 15% of the tumor samples showing apical membranous accentuation. It has been reported that PTPRZ1 expression may correlate with tumor aggressiveness [21]. Moreover, cytoplasmic staining pattern in our results is consistent with PTPRZ1 expression pattern observed in other tumor models including cervical and breast tumors [21, 22]. This observation can be explained by the possibility that the PTPRZ1, which has been inactivated through the PTN-mediated dimerization may be processed and appear in different stages of degradation as the tumor grows and progresses [22]. Further investigation to evaluate PTPRZ1 expression pattern with disease progression might be warranted.

In HUVEC (human umbilical vein endothelial cells, a cell model unrelated to EOC) PTN-PTPRZ1 signaling has been shown to activate ERK1/2 through activation of SRC kinase [18]. Recent findings suggest that PTN mediated ERK1/2 activation is abrogated in the absence of activated SRC kinase (Sethi and Godwin, unpublished data). Moreover, SRC mediated activation of ERK1/2 is well documented [69–72]. Previously, it was reported that in ovarian cancer cells, A2780, that activation of SRC leads to activation of ERK1/2 [73]. Thus there is likelihood that PTN-PTPRZ1 signaling indirectly activates ERK1/2 through the activation of SRC kinase; however, the exact mechanism of how PTN mediates SRC activation through PTPRZ1 in EOC remains to be elucidated.

Targeted therapy is at the forefront of cancer treatments [5]. Identifying essential nodes of tumor cell survival is paramount to improve the outcome of patients with ovarian cancer. Our study reveals an important role of PTN in the pathogenesis of this disease and provides a rationale for further preclinical evaluation of MAPK inhibitors in PTN and/or PTPRZ1 expressing serous ovarian tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PTN expression across different EOC cell lines and primary tissues

A) Bar graph representing relative levels of *PTN* mRNA transcript across EOC cell lines assessed by qRT-PCR and normalized to *PPIA* mRNA levels. Mean normalized gene expression values are shown ($n = 2$, $\pm$ SD). B) Immunoblotting result of cell lysates prepared from EOC cell lines probed with polyclonal anti-PTN. Recombinant human PTN (15 ng, R&D Systems) was used as a positive control. C) Relative levels of secreted PTN measured using ELISA in the conditioned media (48 h) obtained from EOC cell lines and media ($n = 4$, $\pm$ SD), and the positive control cell line LN229. D) Immunoblot analysis of cell lysates prepared from five high grade serous ovarian tumor tissue samples (lanes T1, T2, T3, T4 and T5) and two normal ovarian tissue samples (lanes N1 and N2) probed with polyclonal anti-PTN. Recombinant human PTN (15 ng) was used as a positive control. Quantification of the immunoblot using AlphaView software ver. 3.3 (Cell Biosciences) is shown as a bar graph and represents fold-change of PTN expression in each tumor sample relative to the mean of the normal tissues. An asterisk indicates that the tumor tissue that showed a 2-fold or more increase in PTN levels as compared to normal tissues.

Figure 2. PTPRZ1 expression in EOC cell lines and evaluation of DNA methylation and copy number of *PTPRZ1* in ovarian serous adenocarcinoma

A) Bar graph represents relative levels of *PTPRZ1* mRNA transcript across EOC cell lines assessed by qRT-PCR and normalized to *PPIA* mRNA levels. Mean normalized gene expression values are shown (n=2, ±SD). B) Immunoblot analysis of cell lysates prepared from EOC cell lines probed with monoclonal anti-PTPRZ1. A cellular lysate from the LN229 cell line was used as a positive control. C) Shown is the β-values (mean of 2 probes) for DNA methylation status for *PTPRZ1* gene in 494 TCGA samples. The dotted line at y = 0.25 indicates hypomethylation of *PTPRZ1*. D) Plotted are the copy number values (log₂ tumor/normal ratio) of *PTPRZ1* across the 494 TCGA samples. The dotted line at y = 0.3 indicates copy number gain at a threshold of log₂ 0.3.

Figure 3. PTPRZ1 expression across normal ovarian surface epithelium and ovarian tumor specimens

A) Immunohistochemical analysis of PTPRZ1 expression was performed on ovarian TMAs containing normal ovarian tissue (26 samples) and tumor specimens (54 serous adenocarcinomas). Panel A, top left core is an example of a tumor tissue core exhibiting membranous staining at the tumor-stromal junction. Panel A, middle core is an example of a tumor tissue core showing cytoplasmic expression. Panel A, top right core is an example of a tumor tissue core exhibiting both cytoplasmic and nuclear expression. Normal surface epithelium is shown in the bottom left core. B) Bar graphs represent percentage of normal and tumor samples exhibiting different levels of expression of PTPRZ1 in the cytoplasm. C) Bar graphs represents percentage of normal and tumor samples exhibiting different levels of PTPRZ1 expression in the nucleus. The intensity of PTPRZ1 staining was scored using light microscopy as follows: 0 (undetected), 1 (weak), 2 (moderate) or 3 (strong). The number above each bar indicates the total number of samples evaluated in the respective category.

Figure 4. Effect of PTPRZ1 knockdown on EOC cell proliferation

A) The average normalized viability scores ($n = 2$, $\pm$ SD) following gene silencing using *PTPRZ1* siRNAs evaluated in the five EOC cell lines are shown. B) Representative phase contrast images of A2780 cells treated with *GL2* siRNA and *PTPRZ1* siRNA. C) Left, Immunoblotting was performed following *GL2* siRNA and *PTPRZ1* siRNA treatment to determine the level of knockdown of *PTPRZ1* in A2780 cells ($n = 2$, $\pm$ SD). Right, immunoblots were quantified using AlphaView software, version 3.3 (Cell Biosciences). D) A2780 and OVCAR5 cells were transfected with *PTPRZ1* or *GL2* siRNAs. Seventy-two

hours post-transfection, cells were harvested and processed for analysis of apoptosis. Cells were stained for annexin V and 7-AAD (7-aminoactinomycin) using Guava Nexin reagent (contains both the stains) followed by enumeration using a Guava flow cytometer (Millipore). The fold-change in annexin V positive cells and 7-AAD positive cells is shown (mean $\pm$ SD, n = 3, **** p-value<0.0001, *p-value<0.01). E) A2780 and OVCAR5 cells were transfected with *PTPRZ1* or *GL2* siRNAs. Seventy-two hours post-transfection, cells were harvested and processed for analysis of cell cycle progression. Cells were stained using Guava Cell Cycle reagent (propidium iodide) followed by enumeration using a Guava flow cytometer (Millipore). The fraction of cells in each phase of the cell cycle was measured by propidium iodide staining followed by enumeration using the Guava instrument. The percentage of cells in each cell cycle phase after *PTPRZ1* siRNA and *GL2* siRNA treatment (72 h) is shown (mean $\pm$ SD, n = 3).

Figure 5. ERK1/2 mediates PTN signaling in EOC cells
A) Heat map shows the fold-change in gene expression in A2780 cells treated with *PTN* siRNA (48 h) as compared to the *GL2* siRNA treated cells. Red represents increased expression (up-regulation); black represents mean expression; and green represents lower expression (down-regulation). All expression values (fold-change) fall within [−5.68 to 20.8]. B) Shown is the network with the highest score as generated using IPA. Fifteen genes (shaded nodes) for which expression levels were decreased by 1.5-fold or more in *PTN* siRNA treated cells relative to *GL2* siRNA treated A2780 cells were mapped and functional

interactions between gene products based on IPA knowledge are described. ERK1/2 (underlined) was identified as one of the signaling effectors (non-shaded nodes). C) Shown is the immunoblotting result of cell lysates from *GL2* siRNA and *PTN* siRNA treatment at 24 h and 48 h subjected to electrophoresis and probed with polyclonal anti-PTN, anti-pERK1/2, anti-total ERK1/2 and β -actin. Numbers denote the average (at least 2 independent experiments) fold-change of the ratio of PTN: β -actin and pERK1/2:ERK1/2 normalized to respective negative controls (lane1 and lane 3). D) Shown is the immunoblotting result to evaluate effect of PTN (100 ng/mL) on activation of ERK1/2 in A2780 cells in the presence or absence of ERK inhibitor, UO126 (30 μ M, 2 h). Lysates were subjected to electrophoresis and probed with anti-pERK1/2, and anti-total ERK1/2. Numbers denote the average (at least 2 independent experiments) fold-change of the ratio of PTN: β -actin and pERK1/2:ERK1/2 normalized to respective negative controls (lane1).

Table 1

Characteristics of ovarian serous cancer patients

Characteristics		Number of Patients (total patients in study, n = 54)
Age at diagnosis		
Median	61.5	
Range	26–85	
25–40		2
41–55		12
56–70		26
>70		14

Table 2

Membranous staining of PTPRZ1 in normal ovarian epithelium and ovarian serous carcinoma

	Total Cases	# of Negative	# of Positive	Fishers-exact test
Normal Epithelium	26	26	0	P = 0.048
Serous ovarian Cancer	54	46	8	

Table 3

Expression of cytoplasmic PTPRZ1 in normal ovarian epithelium and ovarian serous carcinoma

	Total Cases	# of Negative	# of Positive	Fishers-exact test
Normal Epithelium	26	14	12	P < 0.0001
Serous ovarian Cancer	54	0	54	

Table 4

Expression of nuclear PTPRZ1 in normal ovarian epithelium and ovarian serous carcinoma

	Total Cases	# of Negative	# of Positive	Fishers-exact test
Normal Epithelium	26	11	15	P = 0.0018
Serous ovarian Cancer	54	5	49	