Abstract. Insulin-like growth factor binding protein 6 (IGFBP6) is a secreted protein with a controversial role in human malignancies, being downregulated in most types of human cancer, but upregulated in selected tumors. Ovarian cancer (OC) is a human malignancy characterized by IGFBP6 downregulation; however, the significance of its low expression during ovarian carcinogenesis is still poorly understood. In the present study, IGFBP6 expression and activation of its associated signaling pathway were evaluated in two matched OC cell lines derived from a high-grade serous OC before and after platinum resistance (PEA1 and PEA2 cells, respectively). A whole genome gene expression analysis was comparatively performed in both cell lines upon IGFBP6 stimulation using Illumina technology. IGFBP6 gene expression data from human OC cases were obtained from public datasets. Gene expression data from public datasets confirmed the downregulation of IGFBP6 in primary and metastatic OC tissues compared with normal ovarian tissues. The comparative analysis of platinum-sensitive (PEA1) and platinum-resistant (PEA2) cell lines showed quantitative and qualitative differences in the activation of IGFBP6 signaling. Notably, IGFBP6 enhanced ERK1/2 phosphorylation only in PEA1 cells, and induced more evident and significant gene expression reprogramming in PEA1 cells compared with in PEA2 cells. Furthermore, the analysis of selected genes modulated by IGFBP6 (i.e., FOS, JUN, TNF, IL6, IL8 and EGR1) exhibited an inverse regulation in PEA1 versus PEA2 cells. In addition, selected hallmarks of TNFα signaling via NFκB, TGFβ signaling, P53 pathway and IL-6 signaling were positively regulated in PEA1 cells, whereas they were inhibited in PEA2 cells in response to IGFBP6. These data suggested that dysregulation of IGFBP6 signaling may serve a role in the progression of OC, and is likely associated with the development of platinum resistance.

Introduction

Ovarian cancer (OC) is the most common and fatal gynecological malignancy worldwide with most of patients diagnosed in locally advanced stage due to lack of symptoms (1). Surgery along with platinum-based chemotherapy is the standard treatment recommended by International Guidelines for patients with advanced stage OC. However, 5-year survival is only about 30-40% in most countries (2). Indeed, while OC is considered a platinum-sensitive tumor with significant and prolonged responses to platinum-based chemotherapy, the occurrence of platinum resistance represents a critical step in OC progression, being the prognosis of platinum-resistant OC extremely unfavorable. Thus, the identification of molecular mechanisms of platinum resistance onset and novel reliable therapeutic targets are needed to improve the OC clinical outcome.

Insulin-like growth factor binding proteins (IGFBPs) are a family of secreted proteins originally characterized as passive high-affinity carriers of two Insulin-like Growth Factors (IGF-I and II) in the circulation, composed of 7 members (IGFBP1, 2, 3, 4, 5, 6, 7) (3). Apart from functions within the IGF system, they play various roles in the extracellular and intracellular compartment to modulate cell proliferation and apoptosis and survival (4). The relationships between IGFBPs and cancer is sometimes contradictory and divergent, according to different
tumor types (5). Their role has been investigated in many cancers, which may be especially relevant since IGF-II is frequently an autocrine cancer growth factor (6). In most studies, IGFBP6 expression was lower in malignant than in normal cells, consistent with the idea that it may act as an inhibitor of tumorigenic pathways, including those driven by an excess of IGF-II activity. However, few studies showed IGFBP6 upregulation in cancer cells, which may represent a compensatory response to increased IGF-II activity or may reflect IGF-independent actions. IGFBP6 was detected in 38 of 41 OCs (7), and a microarray study showed that IGFBP6 mRNA levels were lower in OC than in non-cancerous tissue (8). Consistently, IGFBP6 plasma levels have been found downregulated in patients with OC compared to those without cancer. In a biological perspective, recent findings suggest that IGFBP6 may have opposing effects on the migration of two OC cell lines, this further supporting heterogeneous responsiveness of OC cells to IGFBP6 (9). Furthermore, several findings suggest that the IGFs/IGFBPs axis is involved in modulating drug sensitivity in cancer cells (10) and, specifically, IGFBP6 is responsible for the proliferation of chemoresistant tumor cells is human glioblastoma (11).

Based on this evidence, our study evaluated the role of IGFBP6 in two matched cell lines of high-grade serous OC (HGSOC) obtained from the same patient before and after the development of clinical platinum resistance (PEA1 and PEA2 cells, respectively) (12). A comparative gene expression profiling of these cell lines was performed to evaluate, at molecular level, the differential response of OC cell lines to IGFBP6 in two different contexts of OC progression, before and after the onset of platinum resistance.

Materials and methods

Cell lines, chemicals. The human ovarian adenocarcinoma cell lines PEA1 and PEA2 were purchased from European Collection of Authenticated Cell Cultures (ECACC). PEA1 and PEA2 cells were derived from a malignant effusion from a patient with an HGSOC. PEA1 cell line was collected prior to treatment with cisplatin and prednimustine; PEA2 were collected on relapse after the development of clinical platinum resistance. IGFBP6 in two different contexts of OC progression, before and after the onset of platinum resistance.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA extraction was performed by Pure Link RNA Mini Kit (Invitrogen, Cat. No. 12183018A). RNA was first quantified through optical density measurements at 260 and 280 nm, and subsequently converted in complementary DNA (cDNA) using a SuperScript Vilo IV Reverse Transcriptase (Bio-Rad, Cat. No. 1725271) and Real-Time PCR CFX96 Touch (Bio-Rad). The reaction was conducted according to the following amplification protocol: 95°C for 3 min, 39 cycles at 95°C for 10 sec, 60°C for 30 sec and 65-95°C for 5 sec. β-Actin and α-Tubulin were chosen as internal controls. The results were calculated using the ΔΔCT (where CT is threshold cycle) method (13). Gene expression was quantified through densitometric analysis, using the ImageJ software and normalized according to the expression of housekeeping genes.

Microarray experiments and data analysis. Total RNA from PEA1 and PEA2 cells treated or not with IGFBP6 (1 µg/ml) for 2 h was extracted using TRIzol reagent (Invitrogen) and evaluated for quality and integrity number by the 2100 Bioanalyzer (Agilent Technologies). For each sample, 300 ng...
of total RNA were reverse transcribed and used for the synthesis of cDNA and biotinylated cRNA according to the protocol of the Illumina TotalPrep RNA amplification kit (Ambion; Life Technologies Cat. No AMIL1791).

A total of 750 ng of each cRNA were hybridized on the Illumina HumanHT12 V4_0_R2 Expression BeadChip array (Illumina inc.). Staining was performed according to standard protocols (14). The BeadChip was dried and then scanned with the Illumina HiScanSQ System (Illumina Inc.). All analyses were performed in triplicate.

Raw data from Illumina HumanHT-12 V4_0_R2 microarray were normalized using negc function in limma package and low-quality annotation probes were excluded. Differentially expressed genes (DEGs) were obtained on the linear model fit of the microarray data. The statistical significance was established on P<0.05.

The differences of gene expression levels were shown in terms of LogFC and the cut-off of +/-0.58 was based on its correspondence to +1.5/-1.5 fold-change (2^|0.58|=|1.5|FC), showing the number of upregulated or downregulated genes, respectively (15).

The enrichment analysis was carried out by the Gene Set Enrichment Analysis (GSEA) method in clusterProfiler package (16) and on the base of the gene sets collections (Hallmark, Pathway) in MSigDB (Molecular Signatures Database) (17,18). The statistical significance was established on adjusted P<0.05 for Hallmark collections and on P<0.05 for Pathway collections. All the steps were performed according to the ‘microarray analysis’ best practice using R well known packages (19,20).

Microarray data are available at the Gene Expression Omnibus (GEO) repository under accession number GSE189717.

Analysis of public datasets. IGFBP6 expression analysis in OC samples, compared with normal tissue, was performed using the TNMplot database (https://www.tmnplot.com/). The platform directly compares tumor and/or metastasis and normal samples and performs a Mann-Whitney U or Kruskal-Wallis tests or a paired Wilcoxon test (in case of availability of paired normal and adjacent tumor) for statistical significance (21). The expression correlation between IGFBP6 and selected FOS and EGR1 genes was revealed by R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) using the Tumor Ovarian Bowtell-285-MAS5.0-u133p2 dataset. The R2 software evaluated the statistical significance with the Pearson’s correlation analysis. The expression of FOS and EGR1 genes was analyzed in ovarian serous adenocarcinomas compared to normal tissues using TNMplot (RNA seq data) and GEPIA (TCGA normal vs GTEx data) databases. GEPIA (http://gepia.cancer-pku.cn) is a network-based tool for processing the RNA expression information, collected from carcinomas and healthy specimens from GTEx project and TCGA (22).

Statistical analysis. The unpaired Student's t-test was used to establish the statistical significance in IGFBP6-stimulated versus unstimulated cells. The densitometric analysis data were analyzed by two-way ANOVA and Bonferroni post hoc test. Statistically significant values (P<0.05) are reported.

Table I. Forward and reverse primers used for all reverse transcription-quantitative PCR analyses.

| Gene   | Primer          | Brand       |
|--------|-----------------|-------------|
| c-FOS  | F 5'-CAGTTATCTCCAGAAGAAGAAG-3' R 5'-CTTCTAGTTGGTCTGTCCTC-3' | KiCqStart® |
| c-JUN  | F 5'-AAAGGATAGTGCAGTTGTTTTC-3' R 5'-TAAATCTGCCACCAAATCACC-3' | Sigma-Aldrich |
| IL-8   | F 5'-CCAACACAGAATAATTGTGAAACG-3' R 5'-TGAATCTCAGGCCCTCTCCAA-3' | Invitrogen |
| TNF-α  | F 5'-CTCCAGGCGTGTGTTGCTTTC-3' R 5'-CAGGCAGAAGAGGCTGTTG-3' | Invitrogen |
| IL-6   | F 5'-CAGTTGCCTCTCCCTGGG-3' R 5'-TGAGTGGTGGTGTGTGTTGGG-3' | Eurofin MWG |
| IGFBP-6| F 5'-GGAAGCTAGGCTGCTGCTC-3' R 5'-GTCTCTGGGTGTTCACTTCT-3' | Operon |
| EGR1   | F 5'-GCAGAGTCTCTTTCTCTGAC-3' R 5'-TTGCTATGCTCTCACTAGG-3' | Sigma-Aldrich |
| β-ACT  | F 5'-GACGACATGAGAAATACTG-3' R 5'-ATGATCGTGGGCTGTCTTCT-3' | Sigma-Aldrich |
| TUBB   | F 5'-CTTTGTATTTGGTCAGTCTG-3' R 5'-ATCTCGTCTGATAAGGAGAGT-3' | Sigma-Aldrich |

ICOS, Icos, IL-12, interferon-γ; TNF-α, tumor necrosis factor-α; IFN-β, interferon-β; IL-6, interleukin-6; IGFBP6, insulin-like growth factor binding protein 6; EGR1, early growth response 1; β-ACT, β-actin; TUBB, tubulin β class I.

Microarray data are available at the Gene Expression Omnibus (GEO) repository under accession number GSE189717.

Analysis of public datasets. IGFBP6 expression analysis in OC samples, compared with normal tissue, was performed using the TNMplot database (https://www.tmnplot.com/). The platform directly compares tumor and/or metastasis and normal samples and performs a Mann-Whitney U or Kruskal-Wallis tests or a paired Wilcoxon test (in case of availability of paired normal and adjacent tumor) for statistical significance (21). The expression correlation between IGFBP6 and selected FOS and EGR1 genes was revealed by R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) using the Tumor Ovarian Bowtell-285-MAS5.0-u133p2 dataset. The R2 software evaluated the statistical significance with the Pearson’s correlation analysis. The expression of FOS and EGR1 genes was analyzed in ovarian serous adenocarcinomas compared to normal tissues using TNMplot (RNA seq data) and GEPIA (TCGA normal vs GTEx data) databases. GEPIA (http://gepia.cancer-pku.cn) is a network-based tool for processing the RNA expression information, collected from carcinomas and healthy specimens from GTEx project and TCGA (22).

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| c-JUN  | F 5'-AAAGGATAGTGCAGTTGTTTTC-3' R 5'-TAAATCTGCCACCAAATCACC-3' | Sigma-Aldrich |
| IL-8   | F 5'-CCAACACAGAATAATTGTGAAACG-3' R 5'-TGAATCTCAGGCCCTCTCCAA-3' | Invitrogen |
| TNF-α  | F 5'-CTCCAGGCGTGTGTTGCTTTC-3' R 5'-CAGGCAGAAGAGGCTGTTG-3' | Invitrogen |
| IL-6   | F 5'-CAGTTGCCTCTCCCTGGG-3' R 5'-TGAGTGGTGGTGTGTGTTGGG-3' | Eurofin MWG |
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| TUBB   | F 5'-CTTTGTATTTGGTCAGTCTG-3' R 5'-ATCTCGTCTGATAAGGAGAGT-3' | Sigma-Aldrich |

ICOS, Icos, IL-12, interferon-γ; TNF-α, tumor necrosis factor-α; IFN-β, interferon-β; IL-6, interleukin-6; IGFBP6, insulin-like growth factor binding protein 6; EGR1, early growth response 1; β-ACT, β-actin; TUBB, tubulin β class I.
in Figure Legends. All experiments were independently performed at least three times.

Results

IGFBP6 is downregulated along ovarian carcinogenesis. Since the role of IGFBP6 in human OC carcinogenesis is controversial, IGFBP6 expression levels were preliminary explored using gene expression data from public datasets. Thus, TNMplot gene chip data were used to analyze the differential expression of IGFBP6 in normal human ovarian samples (n=46), non-metastatic OCs (n=744) and metastatic OCs (n=44). Interestingly, while normal ovarian tissues were characterized by high IGFBP6 expression, a significant downregulation of IGFBP6 mRNA was observed from normal ovarian tissue to non-metastatic OC tissues (Fig. 1) (P=1.77e-07, Kruskal-Wallis test and post hoc Dunn's test). No further down-regulation was observed between primary tumor tissue and metastatic OCs. These data suggest that IGFBP6 expression is downregulated at early stages during ovarian carcinogenesis.

Platinum-sensitive PEA1 HGSOC cells are more sensitive to IGFBP6 stimulation compared to platinum-resistant PEA2 HGSOC cells. To study the activity of IGFBP6 during OC progression, IGFBP6 mRNA and protein expression levels were examined in both platinum-sensitive (PEA1) and platinum-resistant (PEA2) OC cell lines by quantitative RT-PCR and immunoblot blot analysis, respectively. IGFBP6 mRNA levels were significantly higher in OC PEA2 cells compared to PEA1 cells (Fig. 2A), while IGFBP6 protein expression was similar in both cell lines (Fig. 2B).

In the effort to explore the effects of IGFBP6 on ERK pathway, which is known to play a major role in OC pathogenesis (23), ERK1/2 phosphorylation was evaluated in PEA1 and PEA2 cell lines in response to exogenous IGFBP6. Interestingly, a 10 min stimulation with IGFBP6 recombinant protein increased ERK1/2 phosphorylation in PEA1 cells and induced no effects on ERK pathway activation in PEA2 cell line (Fig. 2C). These preliminary data suggest that IGFBP6 signaling is more active in PEA1 OC cells.

IGFBP6 elicits a wider gene expression reprogramming in platinum-sensitive PEA1 compared to platinum-resistant PEA2 cell lines. To further characterize IGFBP6 activity and identify differentially expressed genes in PEA1 and PEA2 OC cell lines following IGFBP6 perturbation, a full genome gene expression profiling was performed by Illumina technology after cell stimulation with IGFBP6 recombinant protein for 2 h. Data allowed the identification of significantly modulated genes and were also analyzed to understand the role of IGFBP6 in the regulation of pathways of interest.

The analysis of PEA1 cell line treated with IGFBP6 compared to its unstimulated control identified 2,440 significantly modulated genes, 1,348 up- and 1,092 down-regulated (P<0.05). Therefore, performing a filter based on absolute logFC>0.58 (15), this list was restricted to 37 up- and 36 down-regulated (P<0.05). Moreover, performing a filter based on absolute logFC>0.58, this list was restricted to 37 up- and 36 down-regulated genes (Table SII).

The enrichment analysis was focuses on the Hallmarks and Pathways categories (Table SIII). We identified 16 hallmarks statistically significant (adjusted P<0.05) and with ES>0.3 upon IGFBP6 stimulation in PEA1 cells and, among them, TNF-α signaling via NF-κB was the most significant (Table II). Conversely, only two hallmarks were statistically significant (adjusted P<0.05) and with ES>0.3 in PEA2 cells stimulated with IGFBP6 (Table III).

In order to further study the differential activation of IGFBP6 pathway in OC cell lines, six genes (i.e., FOS, JUN, EGR1, TNF, IL-6, and IL-8) were selected among the top 20 significantly upregulated genes in PEA1 data set (Tables IV and SI). Considerably, all the selected genes enriched the 16 hallmarks reported in Table II, while three of them (i.e., FOS, JUN, and EGR1) were also in the list of IGFBP6-modulated genes in PEA2 cells (Tables V and SII). Noteworthy, while all these genes were significantly upregulated in PEA1 cells upon IGFBP6 stimulation (Fig. 3A; Table IV), FOS, JUN and EGR1 were downregulated in PEA2 cells after exogenous IGFBP6 stimulation (Fig. 3B; Table V). The expression of these genes was further validated by quantitative RT-PCR in PEA1 and PEA2 cells stimulated with IGFBP6, confirming the significantly upregulation of all genes in PEA1 cells (Fig. 3A) and the downregulation of FOS and EGR1 in PEA2 cells (Fig. 3B).

Since these data suggest that IGFBP6 induces a wider gene expression reprogramming in platinum-sensitive PEA1 compared to platinum-resistant PEA2 cells, we comparatively evaluated gene expression data at basal level between PEA2 and PEA1 cell lines. This comparative analysis of PEA2 versus PEA1 cell lines in basal conditions identified 9,372 significantly modulated genes, 4,477 up- and 4,895 down-regulated (P<0.05). Moreover, performing a filter based on absolute logFC>0.58, 2,871 genes up-regulated and 3,238 down-regulated were identified. Hallmarks enrichment
analysis in PEA2 versus PEA1 cells identified 16 positive and 9 negative statistically significant hallmarks (adjusted P<0.05, ES>0.3, ES<0.3) and, among other, the TNF-α signaling via NF-κB pathway, which is known to favor platinum resistance (24) (Tables SIV and SV). Consistently, the comparative expression analysis of FOS, JUN, EGR1, TNF, IL-6 and IL-8 showed that these genes are upregulated in platinum-resistant PEA2 compared to platinum-sensitive PEA1 cells (Tables SVI and SVII). Altogether, these data suggest that IGFBP6 stimulation of platinum-sensitive PEA1 cells results in the activation of gene/pathways that are already constitutive active in platinum-resistant PEA2 cells and that this is a potential explanation of the poorer response of PEA2 cells to IGFBP6 stimulation.

To further validate the relationship between IGFBP6 and the expression of selected genes observed in PEA1 and PEA2 cells, gene expression correlation analysis of IGFBP6 with FOS and EGR1 genes, was performed using R2 software. In particular, the correlation analysis was carried out interrogating the microarray dataset Tumor Ovarian Bowtell, which provides the gene expression profiling of 285 OC samples (25). Interestingly, the analysis revealed positive association between mRNA levels of IGFBP6 and expression of both FOS (R=0.222, P=1.61e-04) and EGR1 (R=0.232, P=7.69e-05) in OC specimens (Fig. 3C). In addition, since IGFBP6 is down-regulated from normal ovarian tissue to OC, we evaluated the hypothesis that FOS and EGR1 may have a similar behavior being IGFBP6-modulated. The expression of FOS and EGR1 genes was analyzed in ovarian serous cystadenocarcinomas in TMMplot database (RNA seq data), comparing normal tissue (133 samples) to tumor tissue (374 samples) (FOS P=1.52e-11, EGR1 P=3.07e-07). The down-regulation of FOS and EGR1 expression was observed in tumors compared to normal samples (Fig. S1A). These results were further confirmed

**Figure 2. IGFBP6 expression levels and ERK pathway analysis in HGSOC PEA1 and PEA2 cell lines.** IGFBP6 (A) quantitative PCR and (B) immunoblot analysis in PEA1 and PEA2 OC cells lines. β-actin was used as loading marker. *P<0.05. (C) p-ERK1/2 immunoblot analysis in PEA1 and PEA2 cells cultured in serum-free medium overnight and subsequently stimulated with IGFBP6 recombinant protein (1 µg/ml) for 10 min. GAPDH was used as loading marker. (B and C) Histograms represent band intensity ± SD and the ratio of phosphorylated/total proteins from the densitometric analysis of three independent experiments. *P≤0.05. IGFBP6, insulin-like growth factor binding protein 6; ERK, extracellular signal-regulated kinase; HGSOC, high-grade serous ovarian carcinoma; PEA1, pleural effusion adenocarcinoma-1; PEA2, pleural effusion adenocarcinoma-2; PCR, polymerase chain reaction; OC, ovarian cancer; p-ERK1/2, phosphorylated-extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
analyzing 426 tumor tissue samples (OC) and 88 normal tissue samples in the GEPIA (TCGA normal vs GTEX data) dataset (logFcl 0.58; P-value cutoff: 0.05) (Fig. S1B).

**IGFBP6 activity is qualitatively different in platinum-sensitive PEA1 compared to platinum-resistant PEA2 cell lines.** Since our data suggest a different response of platinum-sensitive and platinum-resistant OC cell lines to IGFBP6 stimulation, signaling pathways significantly deregulated (ES>0.4, P<0.05) by IGFBP6 in PEA1 versus PEA2 cells were subsequently analyzed. The analysis identified 1240 and 502 pathways in PEA1 and PEA2 cells, respectively (Fig. 4A) and among them, 156 in common between the two cell lines (Fig. 4B). Of note, the comparison of the top 10 common pathways in Table SIII showed a positive regulation in PEA1 cells and a negative regulation in PEA2 cells exposed to IGFBP6 (Fig. 4C). Consistently with data showing a constitutive activation of selected pathways in PEA2 versus PEA1 cells, higher levels of IL6, STAT3 and phosphoSTAT3 were observed in PEA2 compared to PEA1 cells.

Concluding, these data suggest that OC cell response to IGFBP6 stimulation is divergent in platinum-sensitive PEA1 cells compared to platinum-resistant PEA2 cells.

**Discussion**

The role of IGFBP6 in human malignancies and specifically in OC is still controversial (9). The majority of studies suggests that IGFBP6 is downregulated in human tumors compared to normal tissues, this supporting the hypothesis that IGFBP6 is characterized by oncosuppressive functions, stimulating apoptosis and inhibiting cell proliferation (6). Again, IGFBP6 expression is increased in selected tumors (26-28), along with evidence that IGFBP6 positively regulates cell proliferation and migration.

Several data suggest that IGFBP6 is downregulated along OC progression (8,29), while other authors report that IGFBP6 promotes migration in SKOV3 OC cells by activation of MAP kinase signaling, while represses migration in HEY OC cells through both IGF-dependent and IGF-independent signaling pathways.
mechanisms (9). The IGFBP6 family revealed distinct prognosis in patients with OCs (30). Indeed, it was recently reported that increased IGFBP6 mRNA levels negatively affect overall survival and progression-free survival in OC patients. Interestingly, aberrant IGFBP6 mRNA expression was correlated with significantly worse prognosis in patients receiving different chemotherapeutic regimens, suggesting IGFBP6 as a useful prognostic predictor for chemotherapeutic effect in OCs (30).

To shed light on this conflicting evidence, the role of IGFBP6 was evaluated by using gene expression data from public datasets of human OCs and by an established OC cell model of two matched cell lines derived from the same patient with HGSOC, before and after rising platinum resistance. Our data suggest that i) IGFBP6 is downregulated from normal ovarian tissues to primary OCs, and that ii) IGFBP6 signaling is quantitatively and qualitatively different in platinum-sensitive PEA1 compared to platinum-resistant PEA2 cells. Indeed, data showed a more abundant and significant gene expression reprogramming in PEA1 compared to PEA2 cells and the analysis of specific genes and signaling pathways suggest a divergent response to IGFBP6 in platinum-sensitive PEA1 versus platinum-resistant PEA2 cells. Among them, IGFBP6 activated ERK1/2 signaling only in PEA1 cells, while IL6 signaling pathway was positively regulated in PEA1 and conversely inhibited in PEA2 cells, upon IGFBP6 stimulation.

This evidence supports the hypothesis that IGFBP6 signaling is deregulated during ovarian carcinoma progression, likely playing a role in the transition from platinum-sensitive to platinum-resistant conditions. Actually, platinum-based chemotherapy is the mainstay of OC treatment in locally advanced and metastatic stages (31,32). OC is considered a chemo-responsive neoplasm, but, despite this, most patients ultimately develop recurrent disease and resistance to platinum-based chemotherapy with parallel worsening of clinical outcome (33). In such context, our data, although limited to a single model of platinum resistant/sensitive OC cell lines, suggest that the deregulation of IGFBP6 signaling may represent a mechanism associated to the development of platinum resistance and that its modulation may be ultimately a strategy to prevent/delay its onset.

Intriguingly, we observed both quantitative and qualitative differences in gene expression reprogramming between platinum-sensitive PEA1 and platinum-resistant PEA2 cell lines upon IGFBP6 stimulation. Since the differential expression of IGFBP6 between the two cell lines is minimal, it does not explain the differences observed in signal transduction and gene expression. Indeed, the mechanism used by IGFBP6 to activate its downstream signaling is complex and still largely unknown (34). The extracellular activity of IGFBP6 presents both IGF-dependent and IGF-independent mechanisms. As observed for others IGFBPs, IGFBP6 binds and inhibits IGFs preventing their interaction with the receptors, with a relatively higher propensity towards IGF-II than IGF-I. Furthermore, IGFBP6 is capable of actions independent of IGF-II, including regulation of proliferation, apoptosis, angiogenesis and cell migration upon binding with other receptors, mostly unknown (35,36). In such a context, IGFBP6 binding to prohibitin-2 (PBH2) has been proposed as a mechanism responsible for IGF-independent inhibition of cancer cell migration (36). Thus, it is reasonable to hypothesize that modifications in IGFBP6 signaling transduction machinery are responsible for the qualitative different response of PEA2 compared to PEA1 cells and are likely involved in the onset of platinum resistance.

A relevant observation is the evidence that IGFBP6 stimulation of platinum sensitive PEA1 cells induces several genes/pathways/hallmarks that are already constitutively active in platinum-resistant PEA2 cells. While this result may account for the lower response of PEA2 cells to IGFBP6 stimulation, it may also suggest that IGFBP6 is responsible for promoting platinum resistance by activating specific pathways responsible for escape from cytotoxic agents. Interestingly, ERK signaling is among signaling pathways differentially

| Symbol | Name | Entrez ID | logFC | P-value | Fold change |
|--------|------|-----------|-------|---------|-------------|
| EGR1   | Early growth response 1 | 1958  | 2.829 | 2.2E-10 | 7.106 |
| FOS    | Fos proto-oncogene, AP1 transcription factor subunit | 2353  | 3.874 | 2.9E-10 | 14.662 |
| JUN    | Jun proto-oncogene, AP1 transcription factor subunit | 3725  | 1.451 | 8.7E-08 | 2.734 |
| TNF    | Tumor Necrosis Factor | 7124  | 1.133 | 3.9E-08 | 2.193 |
| IL-6   | Interleukin 6 | 3569  | 0.679 | 4.7E-06 | 1.601 |
| CXCL8  | C-X-C motif chemokine ligand 8 | 3576  | 1.198 | 1.4E-07 | 2.294 |

Table IV. Expression levels of selected genes in PEA1 data set.

| Symbol | Name | Entrez ID | logFC | P-value | Fold change |
|--------|------|-----------|-------|---------|-------------|
| EGR1   | Early growth response 1 | 1958  | -0.661 | 0.003  | 0.632 |
| FOS    | Fos proto-oncogene, AP1 transcription factor subunit | 2353  | -1.299 | 0.0002 | 0.406 |
| JUN    | Jun proto-oncogene, AP1 transcription factor subunit | 3725  | -0.388 | 0.026  | 0.764 |

Table V. Expression levels of selected genes in PEA2 data set.
active between PEA1 and PEA2 and this, in our preliminary experiments, was used to establish that PEA2 cells are poorly sensitive to IGFBP6 stimulation. Since ERK signaling is one of the most relevant signaling pathway whose activation is modulated upon IGFBP6 stimulation (37), the differential activation of ERK signaling between the two cell lines may
be responsible for the difference in gene expression after IGFBP6 stimulation and likely drug resistance. In line with this hypothesis, the expression of JUN and FOS, two well established downstream targets of ERK signaling (38), is upregulated in PEA1 and downregulated/unchanged in PEA2 cells upon IGFBP6 stimulation. However, further studies are needed to address the specific role of ERK signaling in IGFBP6-dependent reprogramming of gene expression and its relationship with drug resistance.

Intriguingly, our data suggest a qualitative difference between drug-resistant PEA2 and drug-sensitive PEA1 cells lines in terms of response to IGFBP6. Indeed, IGFBP6 regulates specific genes and pathways involved in inflammation and their expression/activation was inhibited in PEA2 cells where IGFBP6 signaling is contextually downregulated. More specifically, several hallmarks/pathways involved in inflammatory processes, as IL6-JAK-STAT3 and TGF-B signaling pathways (39) are active in PEA1 and conversely inhibited in PEA2 cells, after IGFBP6 stimulation. This evidence is consistent with several studies, which suggest that inflammatory pathways along with metabolic remodeling are involved in the development of platinum resistance (40-42). In addition, specific inflammatory indexes are predictive of platinum sensitivity in OC (43,44) and are differentially expressed in platinum-sensitive versus resistant OC cell models (45). However, although the literature demonstrates
that IL6-JAK-STAT3 pathway is known to favor platinum resistance in OC (46), our data failed to demonstrate that IL6-JAK-STAT3 axis activation/inhibition is the main mechanism responsible for modulation of platinum sensitivity in response to IGFBP6 in OC cells (data not shown). Thus, based on our results, we suggest that it is unlikely that a single pathway/hallmark is responsible for modulation of platinum sensitivity in response to IGFBP6, but rather that the vast...
remodeling induced by IGFBP6 with activation/inhibition of several pathways may modulate platinum sensitivity in PEA1/PEA2 ovarian carcinoma cells. However, it is important to underline that this study does not support the use of a single model of platinum sensitive/resistant ovarian carcinoma cell lines and does not provide a final answer about the specific molecular mechanism responsible for this process. Thus, further studies are needed to further validate these results and clarify the role of IGFBP6 in platinum resistance/sensitivity.

In conclusion, this study suggests a role of IGFBP6 in OC progression, highlighting that the deregulation of its actions is likely involved in the remodeling of the gene expression response and the occurrence of platinum resistance. Further studies are needed to evaluate whether IGFBP6 may represent a target to slowdown OC progression toward drug resistance and characterize this signaling pathway as a novel source of biomarkers.

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Availability of data and materials

Gene expression data generated in this study are available at the GEO repository under accession number GSE189717 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189717).

Authors’ contributions

ML and AL designed the study. AP, VC, MP, FC and SV performed the experiments. AP, VC, FC, ARDC, GB, GC, GG and DT analyzed the data. AP and VC confirmed the authenticity of the raw data. ML and AP wrote the manuscript. AL, SV, GB, GG, VC, FC, MP and DT revised the paper. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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