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Cancer Genes and Genomics

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Osteopontin-c Splicing Isoform Contributes to Ovarian Cancer Progression

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Abstract

Ovarian carcinoma is one of the most aggressive gynecological diseases and generally diagnosed at advanced stages. Osteopontin (OPN) is one of the proteins overexpressed in ovarian cancer and is involved in tumorigenesis and metastasis. Alternative splicing of OPN leads to 3 isoforms, OPNa, OPNb, and OPNc. However, the expression pattern and the roles of each of these isoforms have not been previously characterized in ovarian cancer. Herein, we have evaluated the expression profiling of OPN isoforms in ovarian tumor and nontumor samples and their putative roles in ovarian cancer biology using *in vitro* and *in vivo* functional assays. OPNa and OPNb were expressed both in tumor and nontumor ovarian samples, whereas OPNc was specifically expressed in ovarian tumor samples. The isoform OPNc significantly activated OvCar-3 cell proliferation, migration, invasion, anchorage-independent growth and tumor formation *in vivo*. Additionally, we have also shown that some of the OPNc-dependent protumorigenic roles are mediated by PI3K/Akt signaling pathway. OPNc stimulated immortalized ovarian epithelial IOSE cell proliferation, indicating a role for this isoform in ovarian cancer tumorigenesis. Functional assays using OPNc conditioned medium and an anti-OPNc antibody have shown that most cellular effects observed herein were promoted by the secreted OPNc. According to our data, OPNc-specific expression in ovarian tumor samples and its role on favoring different aspects of ovarian cancer progression suggest that secreted OPNc contributes to the physiopathology of ovarian cancer progression and tumorigenesis. Altogether, the data open possibilities of new therapeutic approaches for ovarian cancer that selectively down regulate OPNc, altering its properties favoring ovarian tumor progression. *Mol Cancer Res; 9(3); 280–93. ©2011 AACR.*

Introduction

Ovarian carcinoma is composed of a heterogeneous group of tumors derived from surface epithelia or inclusions (1). A vast majority of ovarian tumors arise due to accumulation of genetic damage, but the specific genetic pathways involved on the development of epithelial ovarian tumors are largely unknown. An improved understanding of the molecular basis of ovarian carcinogenesis and tumor progression could allow the establishment of new ovarian cancer markers and also the identification of new targets for better treatment options.

Many gene products are involved in ovarian cancer progression, as recently reviewed (2, 3). Among those genes, osteopontin (OPN, 2ar, Spp1) is recognized as a key prognostic marker during ovarian cancer progression (4, 5), which is overexpressed in ovarian cancer in relation to normal ovarian tissues (6). An increase in OPN levels has been correlated to tumor staging, invasiveness, and grade (7–9), as well as to the presence of ovarian cancer peritoneal metastasis (10). Furthermore, plasma OPN has been shown to have potential clinical utility in detecting recurrent ovarian cancer (11).

OPN mRNA is subject to alternative splicing, resulting in isoforms that are smaller than full-length OPN (12, 13). Alternative splicing leads to deletions in the N-terminal portion of OPN, located upstream from the central integrin and C-terminal CD44 binding domains (14). OPNa is the full-length isoform, whereas OPNb lacks exon 5 and OPNc lacks exon 4 (15). OPN splicing isoforms present tissue and tumor-specific roles (16–19). All current available data about OPN in ovarian cancer relates to OPN in general,

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without distinguishing among the various isoforms, and OPN splicing in this tumor has not been characterized previously. Here we examine the putative roles of each splice variant in ovarian cancer biology by using *in vitro* and *in vivo* tumor models. Our data show that the overexpression of OPNc increases OvCar-3 cell growth, migration, invasion, anchorage-independence and tumor formation *in vivo*, suggesting a possible functional role for OPNc in ovarian cancer progression. Our observations indicate that OPNc promotes ovarian cancer progression through activating the Phosphatidylinositol-3 Kinase (PI3K)/Akt signaling pathway.

Material and Methods

Tumor specimens

We evaluated nontumoral ovarian tissues (11 samples), benign tumors (8 samples), borderline tumors (6 samples), and ovarian carcinoma (15 samples). Tumors were classified according to the standard criteria from FIGO (20). Ovarian tissues samples were collected at Instituto Nacional de Cancer, Brazil from May, 2006 to January, 2008. This study was approved by the local Ethics Committee and informed consent was obtained from all patients. Ovarian tumor samples were obtained from patients who underwent resection of a tumor lesion of the ovary. Ovarian nontumoral tissue samples were histopathologically evaluated and were negative for tumor cells. These were obtained from patients who underwent hystectomy or anexectomy due to other gynecologic malignancies, such as endometrial carcinoma tumors, cervical uterus tumors, or an ovarian follicular cyst. Histology of patient tissue specimens and some features of those samples are presented on Supplementary Table S1. Ovarian tissues samples were stored in RNA Later (Ambion) at −20°C until processing. Patients presenting ovarian borderline tumors were followed up from 10 to 37 months and presented no evidence of ovarian disease.

Cell culture and the generation of OPN isoform overexpressing ovarian cancer cells

As a model to examine the putative roles of OPN isoforms in ovarian carcinoma, we used herein ovarian cancer and normal ovarian cell lines. We used 3 ovarian cancer cell lines, OvCar-3 (ovarian papillary adenocarcinoma), ES2 (clear cell carcinoma), and MDAH 2774 (endometrioid ovarian cancer), and an immortalized ovarian surface epithelium (IOSE) normal cell line. Cell lines were cultured in standard conditions. OvCar-3, ES2, and MDAH 2774 cell lines were supplied from Clinical Research Service, Instituto Nacional de Cancer, Brazil. IOSE cell line was a kind gift from Dr. Nelly Auersperg from University of British Columbia, Canada. Cell lines were cultured in a humidified environment containing 5% CO_2 at 37°C. All cell lines were cultured in RPMI medium supplemented with either 20% (OvCar-3) or 10% (ES2, MDAH 2774, and IOSE) fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin. For all functional assays, OvCar-3 was cultured in RPMI with 2% FBS for 48 hours.

The open reading frame of OPN splice variants, OPNa, OPNb, and OPNc were cloned into pCR3.1 mammalian expression vector as previously described (17) and these DNA constructs were used for transfection into OvCar-3 cells. Transfections were carried out using Lipofectamine 2000 (Invitrogen). The OPN isoforms/pCR3.1 plasmids or the vector alone were transfected into OvCar-3 cells and the stably expressing cell clones were selected with 600 μg/mL of G418 in the culture medium. Six OPN isoform overexpressing clones (OPNa1, OPNa4, OPNb1, OPNb3, OPNc1, and OPNc5) and one empty vector control clone (empty vector) were selected. Each clone was tested individually on functional assays, as indicated.

Quantitative real-time RT-PCR

RNA was extracted from the cell lines and tumor tissues using the RNeasy kit (Qiagen). cDNA synthesis was carried out using SuperScript II (Invitrogen). OPN splice variants were amplified with specific primer pairs (Supplementary Table S2). All PCR reactions were conducted using the SYBR Green detection reagent (Applied Biosystems). Conditions for OPN isoforms PCR amplification were 50°C for 2 minutes, 94°C for 5 minutes followed by 10 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; at the end of each cycle the temperature decreased 0.5°C, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 sec, 72°C for 15 minutes, and finally a melting curve analysis (60–90°C with a heating rate of 0.2°C/sec and continuous fluorescence measurement). Product purity, size, and absence of primer dimers were confirmed by the DNA melting curve analysis and agarose gel electrophoresis. Relative gene expression of the target gene was calculated by using the ΔCT method. GAPDH and actin amplification were used as normalization controls for OPN isoforms, MMPs, or VEGF transcription level evaluation. Conditions for amplification of these genes are detailed in Supplementary Information.

Cell proliferation, sub-G_0, cell migration, invasion, and soft agar growth assays

Details about all these assays are presented in the Supplementary Information. Cell proliferation was analyzed by crystal violet, exclusion of trypan blue, and incorporation of (^{3}H) thymidine assays. Sub-G_0-G_1 analyses were conducted by using propidium iodide staining and fragmented DNA content using flow cytometry. Cell proliferation assays for OPN depletion using anti-OPNc antibody were evaluated in the absence or with the addition every other day of anti-OPNc antibody (Gallus Immunotech) at 4 μg/mL. Proliferation assays were also carried out using anti-rabbit IgG goat antibody (Pierce) as immunoglobulin control (4 μg/mL). Cell migration assays were evaluated by *in vitro* wound closure assays, as described by others (21). Transwell Invasion Assays were carried out as reported (22) and anchorage independent growth was analyzed in soft agar medium.

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Preparation of cell lysates

Cells were harvested and rinsed twice with PBS. Total cell extracts were prepared with Cell Lysis Buffer (Cell Signaling Technology), sonicated and cleared by centrifugation at 15.000 g, 4°C. Total protein concentration was measured using the BCA assay kit (BioRad) with bovine serum albumin as a standard, according to the manufacturer's instructions.

Treatment of cells with LY294002 inhibitor and immunoblotting

LY294002, a PI3K inhibitor, was obtained from Cell Signaling Technology. Nontransfected OvCar3 cells and those overexpressing each OPN splice variant or empty vector control were cultured as described in Supplementary Information and above for cell proliferation, migration, and soft agar colony assays and then treated with 50 μmol/L of LY294002, according to the following protocols. For proliferation assays, 4 hours after cells were seeded, the medium was removed and replaced with culture medium in the presence of either LY294002 dissolved in DMSO and diluted in media was maintained for all proliferation kinectics. On cell migration assays, culture media containing or not LY294002 inhibitor was added just after conducting streaks on cell culture. Finally, on soft agar colony assays, culture media containing or not LY294002 was changed every 3 days.

Total cell lysates containing 50 μg total protein was subjected to 10% SDS/PAGE and the resolved proteins transferred electrophoretically to nitrocellulose membranes (Millipore). To examine PI 3-Kinase activation, we analyzed the levels of Ser^{473} phosphorylation of Akt, a downstream effector of PI 3-Kinase. After blocking with PBST (phosphate buffered saline containing 0.05% Tween 20) containing 5% bovine serum albumin for 1 h at room temperature, membranes were incubated with Akt and Phospho-Akt antibodies (Cell Signaling Technology) according to the manufacturer's instructions. The horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Pierce, Rockford) were diluted 1:1000 in PBST containing 5% bovine serum albumin and incubated for 1 h at room temperature. Chemoluminescensce detection (Amersham Biosciences) was conducted in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 (Media Cybernetics, Silver Spring, MD) and normalized to that of loading control as appropriate. Blots were conducted in triplicate in at least 3 independent experiments. Mean densitometric values were calculated.

In vivo studies

For the analysis of tumor progression *in vivo* we used athymic BALB/$c^{nude/nude}$ mice. Transfected cell clones were trypsinized, washed, and resuspended in PBS. Mice were injected subcutaneously (s.c.) into the left flank with 5×10^6 cells. Tumor volumes (V) were analyzed every 5 days using the following formula: $V\ (mm^3) = \text{width} \times \text{length}^2 \times 0.52$. A total of 25 mice were randomly assigned to five groups (5 mice/group) corresponding to five transfectant clones (wild type OvCar-3, OvCar-3 cells transfected with either OPNa, OPNb, OPNc, or empty vector). Pictures were taken 15 days after injection.

Data and statistical analysis

All results are presented as mean ± standard error of at least 3 independent experiments. For *in vitro* and *in vivo* data assays, statistical comparisons among the groups were conducted by the Student's t-test or ANOVA. $P < 0.05$ was considered significant. P values are indicated in the Figure legends.

Results

OPNc is specifically expressed in ovarian tumor samples

As a first step to investigate the expression profiling of distinct OPN splice variants in ovarian carcinoma, we analyzed OPNa, OPNb and OPNc RNA levels in ovarian cell lines and human ovarian tumor and nontumor tissues. OPNa and OPNb were expressed in tumor and nontumor cell lines and tissues, whereas OPNc was only expressed in tumor samples (Fig. 1A and B). In ovarian tumor cell lines, OPNa and OPNb present higher expression level as compared with an immortalized ovarian surface epithelium (IOSE) cell line. In ovarian malignant and borderline tumor samples, these isoforms presented variability in expression levels. Notably, OPNc expression was not detected in benign as well as in normal ovarian tissues (Fig. 1B). These findings suggest that OPN isoforms present differential expression patterns in tumor and nontumor ovarian samples, with OPNc only expressed in tumor samples. These data, together with previous reports showing that OPN isoforms have tumor-specific expression and functional properties (16–19), prompted us to investigate their functional roles in ovarian cancer biology.

Ectopic expression of OPNc is associated with increased cell proliferation

To examine the contributions of each OPN isoform in ovarian cancer progression, we used *in vitro* and *in vivo* gain-of-function experiments. We chose the approach to ectopically overexpress each of these splicing isoforms in OvCar-3 cells, once these cells are a well-established model for ovarian cancer functional studies (23, 24).

A number of cellular events are associated with tumor progression and an increase of cell proliferation is one of them. We first asked whether the proliferation rates of OvCar-3 cells overexpressing OPNa, OPNb and OPNc were altered as compared with an empty vector control clone. As shown in Fig. 2A, OPNc overexpressing cells present higher proliferation rates as compared with OPNa, OPNb, empty vector, and OvCar-3 nontransfected cell clones in the range of 4 to 96 hours of cell culture. The same proliferation behavior was observed when testing stably expressing clones presenting different OPN isoform

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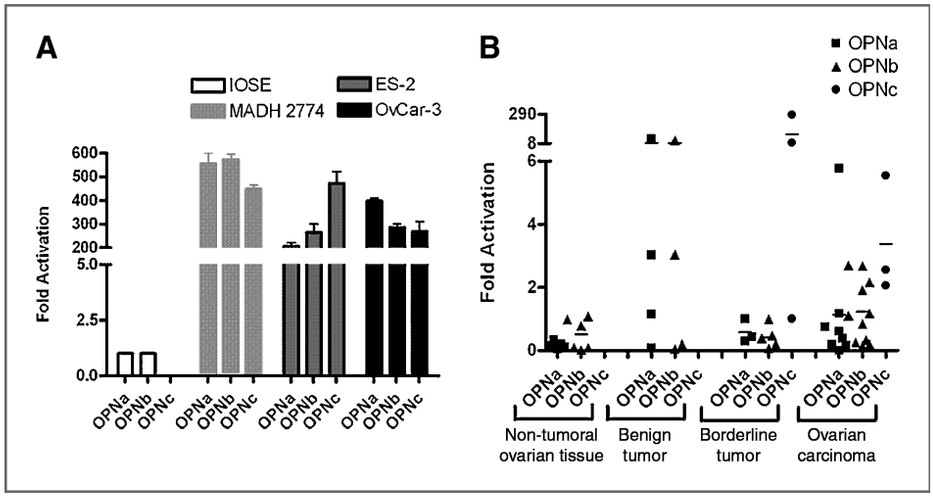

Figure 1. OPNc is specifically expressed in ovarian tumor samples. OPN isoform expression levels were analyzed by real-time PCR using isoform specific primers and were represented by fold activation and GAPDH amplification as a control for template amount. Fold activation represents the increase of expression level of each OPN isoform in relation to IOSE cell line and an ovarian nontumor tissue. A, OPN splice variants present variable expression levels in different ovarian cell lines. Ovarian tumor cell lines (MADH 2774, ES-2, and OvCar-3) and an immortalized ovarian surface epithelium (IOSE) normal cell line (IOSE) were tested. OPNc was only expressed in the ovarian tumor cell lines, but not in the IOSE cell line, whereas OPNa and OPNb were expressed in all cell lines analyzed. Results are representative of 3 independent experiments and horizontal bars represents standard deviations (SD) B, OPNa, OPNb, and OPNc expression levels in different ovarian tissue samples, as represented by the legends on the right and on abscissa axis. Each OPN isoform is represented by a different symbol as indicated. Patient data are shown on Supplementary Information. Horizontal bars represent the median splice variant expression level. OPNc was only expressed in ovarian carcinoma and ovarian borderline tumor samples, and not in ovarian benign tumors or normal tissues, while OPNa and OPNb were expressed in all ovarian tissues analyzed.

expression levels, as shown on Figure 2A and Supplementary Figure 1. Similar results were obtained when analyzing proliferation rates by tritiated thymidine incorporation (Fig. 2B) and trypan blue exclusion (data not shown) assays. We further tested whether OPNc could affect cell proliferation even under serum-starving conditions (Fig. 2C), which are known to induce cell cycle arrest and apoptosis in ovarian cancer (25, 26). In this experimental condition, OvCar-3 cells overexpressing OPNa, OPNb, empty vector, and OvCar-3 nontransfected cells exhibited reduced proliferation rates as compared with OPNc overexpressing clones. These results suggest that OPNc increases the ability of OvCar-3 cells to grow independently of growth factors, a typical feature of a protein involved in tumor progression (27). Consistently, these results support the hypothesis that expression of OPNc modulates the growth of ovarian cancer cells, even in serum-starved conditions. We then hypothesized that the higher proliferation rates observed for OPNc overexpressing clones could be explained by induction of cell death promoted by either OPNa or OPNb overexpression. Hence, we assessed the susceptibility to cell death of OvCar-3 cells transfected with either OPNa, OPNb, OPNc, or with empty vector. All these cell clones presented a low proportion of cells presenting fragmented DNA content, as shown by flow cytometry, which is indicative of a low proportion of cells undergoing apoptosis (Supplementary Fig. S2A and B). These results further confirm the potential role of OPNc on favoring ovarian cancer cell proliferation, which is not associated with stimulated cell death promoted by OPNa or OPNb.

OvCar-3 cells overexpressing OPNc present faster migration, invasion, and increased MMP2, MMP9, and VEGF expression

Next we examined whether OPN splicing isoforms are able to induce additional altered phenotypes associated with tumor progression in OvCar-3 cells. Cell migration, a component of cellular invasion, contributes to several important steps in tumor progression. We asked whether full-length OPNa or the splice variants, OPNb and OPNc, are able to activate OvCar-3 cell migration. OvCar-3 cells overexpressing OPN splicing isoforms or empty vector were subjected to an *in vitro* wound closure assay (Fig. 3A). The assays were conducted over 72 hours and in the presence of mitomycin C, which suppresses the proliferation of rapidly growing cells. Migration of OPNc expressing cells was more extensive as compared with OPNa, OPNb, empty vector-expressing cells and OvCar-3 nontransfected cells. Eighteen hours after scratching, OPNc activated OvCar-3 cell motility into the wound. After 24 hours, OPNc expressing cells completely closed the wound edges. In contrast, OPNa, OPNb, vector controls and OvCar-3 cells did not completely close the wound until 72 hour time point (data not shown).

Higher cancer cell mobility combined with increased expression of proteases that degrade the extracellular matrix is generally predictive of invasive capability (2). We next tested the effects of OPN isoforms overexpression on OvCar-3 invasive capacity by using transwells coated with Matrigel. OPNc overexpression stimulated OvCar-3 invasion capacity by at least 5.0-fold ($P < 0.001$), as compared with cells overexpressing OPNa, OPNb and empty vector control (Fig. 3B and C).

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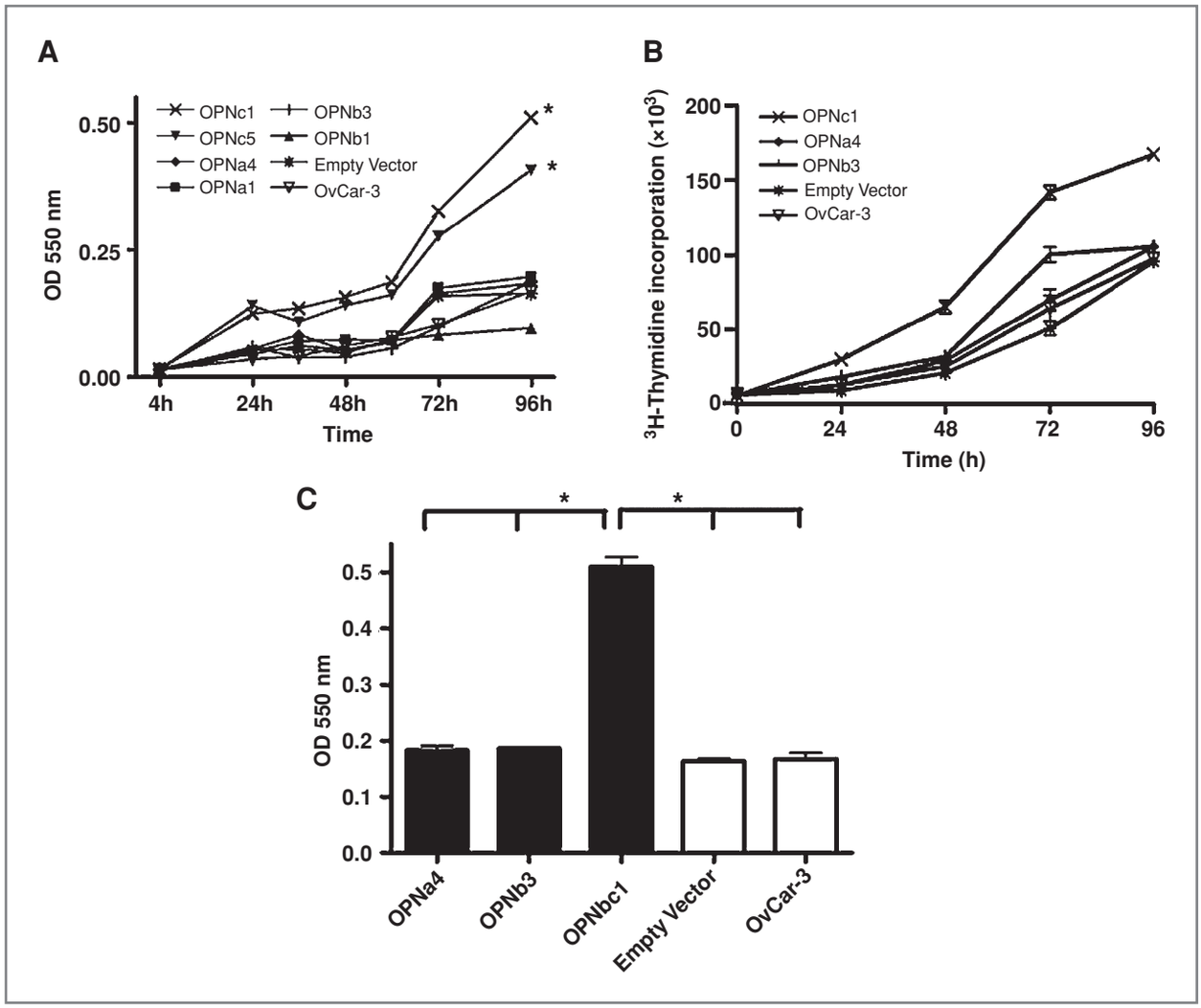

Figure 2. OPNc alters the proliferative profile of OvCar-3 cells. (A) Stably transfected cells with either empty vector, OPNa, OPNb, OPNc, or OvCar-3 nontransfected cells were plated as indicated in Material and Methods and Supplementary Information. On every consecutive day, 3 wells per cell line were harvested and the total number of cells was measured. Proliferation kinetics analysis was evaluated by crystal violet staining. OPNc had a higher proliferation rate as compared with OPNa, OPNb, and empty vector. Clones OPNa4, OPNb3 and OPNc1 had similar expression levels of each OPN splicing isoform, whereas clones OPNa1, OPNb1, and OPNc5 present differential expression levels (Supplementary Figure 1). *, $P < 0.05$ vs. empty vector control clone B, cell proliferation analyzed by (^{3}H) thymidine incorporation. ^{3}H-thymidine was added and the cells were washed, fixed and counted. C, OPNc accelerates cell proliferation under reduced-serum conditions. Cells were grown in 0.2% FBS and cell numbers were counted by crystal violet staining at 96 hours after plating. O.D., optical density measured at 550 nm. *, $P < 0.0001$ versus empty vector control clone.

Matrix metalloproteinase (MMP)-2 (gelatinase A) and MMP-9 (gelatinase B) are key players in the process of degrading extracellular matrix during tumor cell invasion. Ovarian cancer cells express MMP2, MMP9 and vascular endothelial growth factor (VEGF) and their increased expression is associated with their invasive and metastatic potential. Besides, a functional interplay between VEGF and MMP2/MMP9 expression has been described in ovarian carcinomas (28). There was a significant increase in MMP-2, MMP-9 and VEGF mRNA levels ($p < 0{,}002$) in OvCar-3 cells overexpressing OPNc as compared with OPNa, OPNb and empty overexpressing clones (Fig. 3D–F).

Secreted OPNc is involved in tumor progression features

Because OPN is predominantly a secreted protein, we sought to investigate whether the effects observed on ovarian cell proliferation, migration and invasion were mainly associated with the secreted OPN splicing isoforms.

We evaluated proliferation-kinetics of nontransfected OvCar-3 and IOSE cells in the presence of conditioned culture medium produced by OPNa, OPNb, OPNc, and empty vector overexpressing cell lines (Figs. 4A and B). IOSE cells were also tested in order to investigate the putative roles of secreted OPN isoforms on activating proliferation in nontumoral ovarian cells, as indicative of

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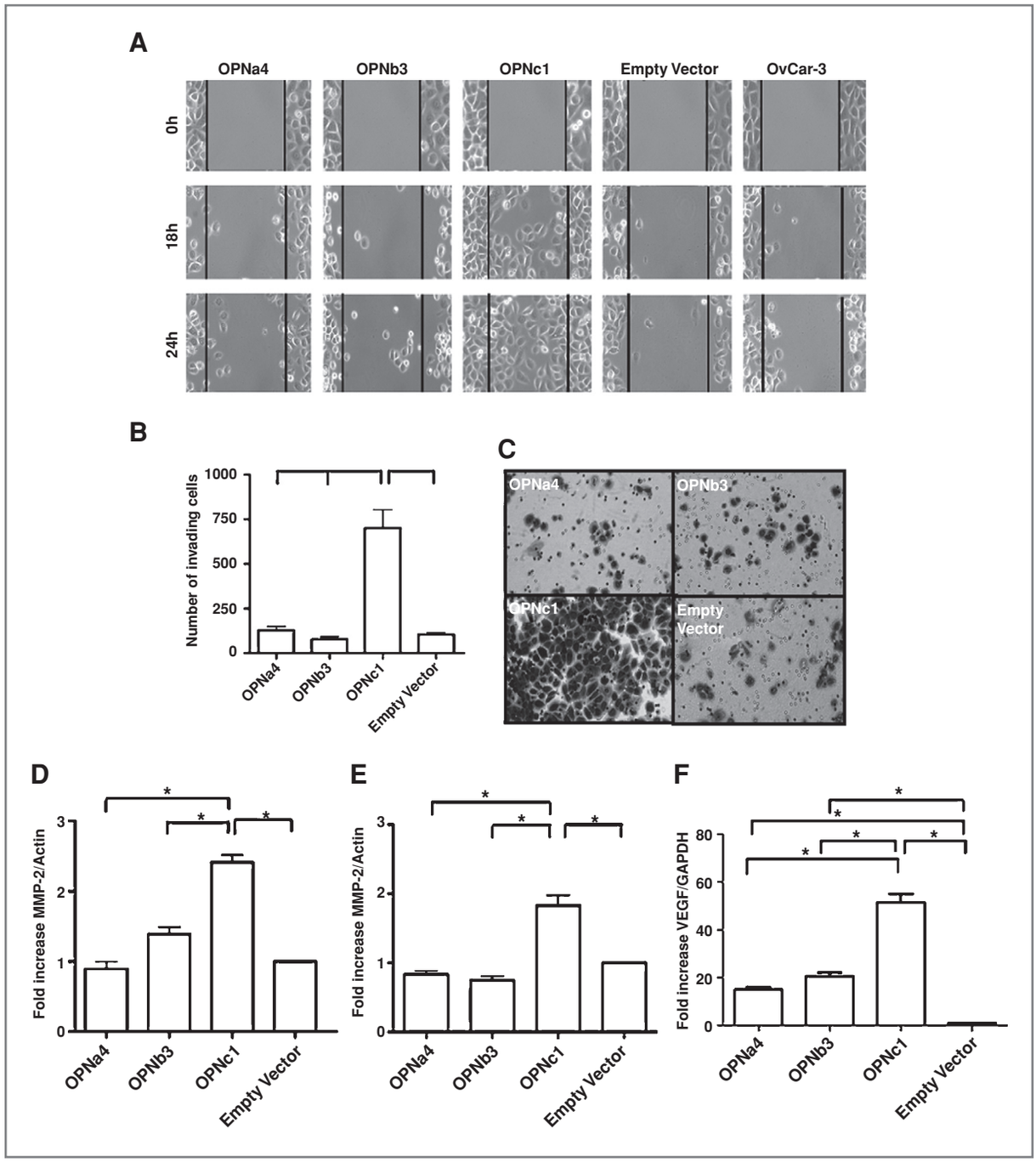

Figure 3. Osteopontin-c supports cell migration and invasion. Cell clones of OPNa1, OPNb3, OPNc1, empty vector, and OvCar-3 s nontransfected cells were plated as indicated in Supplementary Information and analyzed for cell migration and invasion by wound closure and transwell invasion assays, respectively. A, OPNc activates OvCar-3 cell migration. Phase-contrast micrographs photographs were taken at 0, 18, and at 24 hour after subjected to migration are shown. For each cell line, 3 plates were used per experiment. B, OPNc activates OvCar-3 cell invasion. The effects of OPN splice variants overexpression in OvCar-3 cell invasion capacity was determined by transwell invasion assays. Invading cells were stained with crystal violet and the number of cells manually counted. The data are reported as average of number of invading cells $\pm$ S.D. of 3 independent experiments and six microscopic fields were counted per insert. *, $P < 0{,}001$ C, representative image of invading cells stained with crystal violet described in B; D–E, induction of MMP2 (D), MMP9 (E), and VEGF (F) mRNA expression in cells overexpressing OPNc. Total RNA from cells overexpressing OPNa, OPNb, OPNc, and empty vector control was prepared to conducted quantitative real-time PCR (qRT-PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin as internal controls. The amount of targets was analyzed using the comparative CT method, where the threshold cycle (CT) values of each target sequence are given by the $2^{-\Delta\Delta CT}$ formula. We present the data as log *n*-fold change in gene expression normalized to the endogenous reference genes (GAPDH or actin) relative to the expression of cells overexpressing empty vector control.*, $P < 0.002$.

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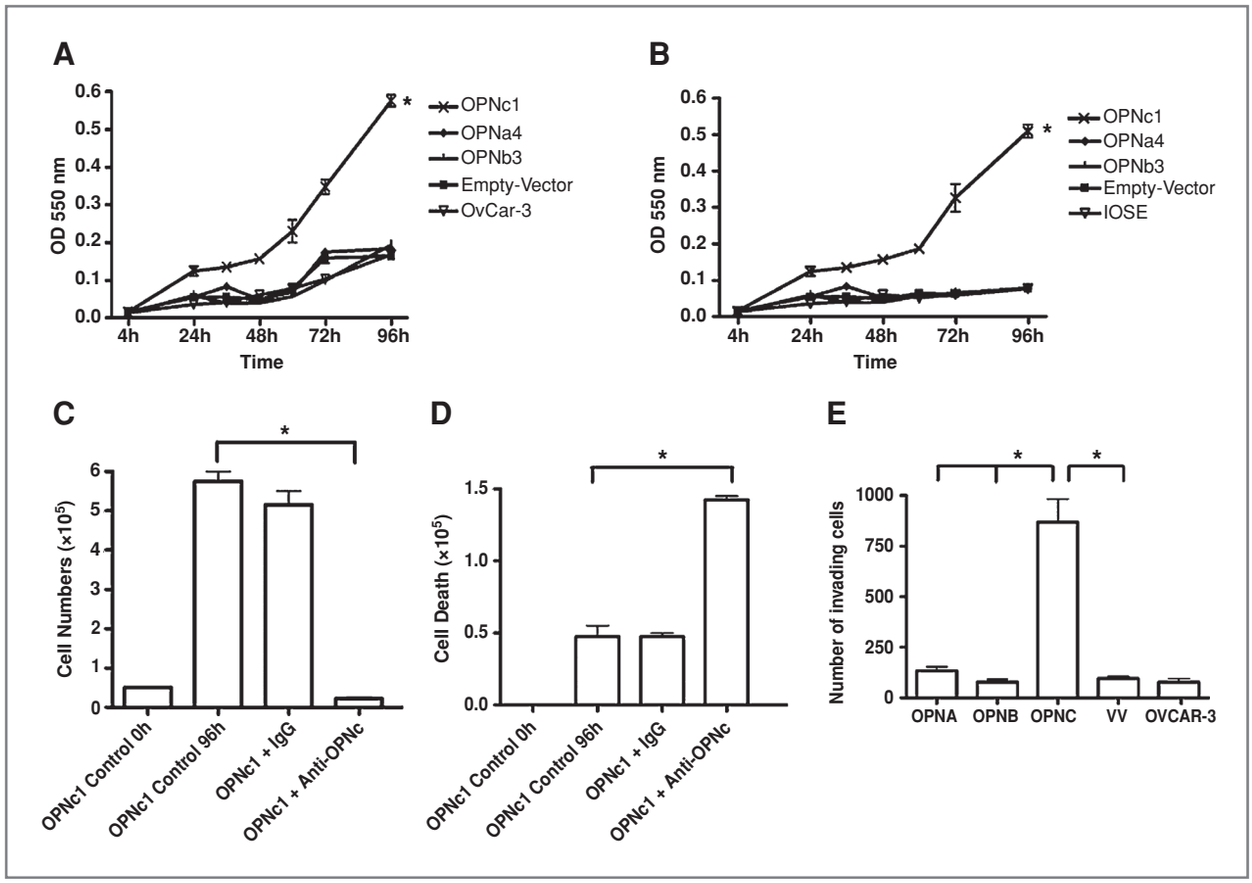

Figure 4. Secreted OPNc is mostly involved in OPNc pro-proliferative features. OvCar-3 A, and IOSE non-transfected cells B, were assayed for cell proliferation rates by crystal violet staining after incubation with conditioned medium from OPNa, OPNb, OPNc, and empty vector overexpressing cells. Legend on the right indicate cell clones tested. All results are representative of at least 3 independent experiments. O.D., optical density measured at 550 nm. The standard deviations (error bars) indicate the variability within each experiment. *, $P < 0.05$ vs. IOSE nontransfected cells C, D, 5×10^4 of OvCar-3 cells overexpressing OPNc (clone OPNc1) were plated and the total number of cells as indicative of cell proliferation (C) and cell death (D) was measured by trypan blue exclusion staining. Cell number was measured at 0 hour time point (bar OPNc1 control 0 h) and after 96 of cell culture (bar OPNc1 control 96 hours). Cells were treated with anti-OPNc antibody (bar OPNc1 + anti-OPNc) or with rabbit anti-human IgG (bar OPNc1 + IgG) at the concentration of 4 μg/mL for 96 hours. Significant differences in cell number are represented by asterisk. *, $P < 0.0001$ vs. OPNc1 control 96 hour. E, OPNc conditioned media is a chemoattractant for OvCar-3 cells invasion capacity. Matrigel invasion assays were conducted using conditioned media form cells overexpressing OPNa, OPNb, OPNc, and empty vector control as chemoattractant for OvCar-3 cell invasion thorugh transwell invasion assays. Invading cells were stained with crystal violet and cell counting was carry out manually. The data are reported as average of number of invading cells ± S.D. of 3 independent experiments and six microscopic fields were counted per insert. *, $P < 0.05$.

early steps of tumorigenesis. These assays were conducted in serum-free conditions, in order to eliminate skewing of the results by cell activation promoted by growth factors. OvCar-3 and IOSE cells cultured in OPNc conditioned medium displayed higher proliferation rates as compared with cells cultured with OPNa, OPNb, or empty vector conditioned medium (Figs. 4A and B). A serial dilution analysis of OPNc conditioned medium promoted a proportional decrease on OvCar-3 and IOSE cell proliferation rates (Supplementary Figure 3).

We then show that these changes in cell proliferation were directly dependent on the secreted OPNc protein, as the addition of a neutralizing polyclonal anti-OPNc antibody, but not control immunoglobulin, to the cultures every other day with the exchange of medium, significantly suppressed proliferation rates promoted by OPNc OvCar-3 overexpressing cells (Fig. 4C). Accordingly, OPNc OvCar-3 overexpressing cells treated with the anti-OPNc antibody, also presented an increase in cell death, further evidencing a survival role for OPNc in this ovarian tumor cell line (Fig. 4D). In contrast, the antibody incubation had no significant effects on cell proliferation and cell death of control clones formed by nontransfected or OPNa OvCar-3 overexpressing cells (Supplementary Figure 4A–D), indicating that cell death induced by the depletion of OPNc using the anti-OPNc neutralizing antibody was specific for cells overexpressing this isoform.

Altogether, these data indicate that secreted OPNc not only activates the proliferation of an ovarian tumoral cell, such as OvCar-3, but also stimulates proliferation of ovarian normal cells, indicating a role of secreted OPNc not only on

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ovarian tumor progression, but also in ovarian tumorigenesis and cell survival.

To obtain further functional insights related to migration and invasion profiles modulated by OPN isoforms, we next evaluated whether the effect on cell migration and invasion was mediated by the secreted OPN isoforms. Wound closure assays were carried out with nontransfected OvCar-3 cells cultured in the presence of conditioned culture medium from clones transfected with each OPN splicing isoform. Similarly to OPNc overexpressing clones, as observed on Figure 3A, OvCar-3 cells cultured in the presence of OPNc conditioned medium completely closed the wound edge 18 h after scratching (Supplementary Figure 5). On the other hand, cells cultured with conditioned medium from with either OPNa, OPNb or empty vector-overexpressing cells showed limited migration (Supplementary Figure 5) and did not reach this stage until 72 hours after scratching (data not shown). Similarly, when testing conditioned media from cells overexpressing each isoform as chemoattractant for nontransfected OvCar-3 cells on transwell invasion assays, conditioned media from cells overexpressing OPNc significantly stimulated OvCar-3 cell invasion (Fig. 4E).

Taken together, these results indicate that secreted OPNc acts as an activating factor for ovarian cell proliferation, migration and invasion, typical features of tumor progression.

OPNc enhances soft agar colony formation

Soft agar colony formation reflects a combination of growth rate (cell cycle progression) and anchorage independence (antianoikis). The ability to form colonies in semisolid medium correlates well with tumor malignancy and metastasis formation. We further analyzed the effect of OPN splicing isoform overexpression on the potential of OvCar-3 to form colonies in soft agar. OPNc significantly increased the number of colonies formed, whereas OPNb inhibited this process. OPNa isoform promoted no significant effect on the number of colonies formed (Fig. 5A). OPNa, OPNb, and OPNc promoted an increase in the size of colonies formed (Fig. 5B) as compared with control cells, but for OPNc overexpressing cells these effects were even stronger. These data indicate that OPNc can act as a strong activating factor on soft agar colony formation, whereas OPNb can inhibit the number of colonies formed.

OPNc enhances tumor growth *in vivo*

To assess the roles of OPN splicing isoforms on ovarian progression *in vivo*, equal numbers of OvCar-3 cells stably transfected with OPNa, OPNb, OPNc, empty vector, or nontransfected OvCar-3 cells were implanted subcutaneously into athymic nude mice and the growth of the implanted tumors was measured. The animals were monitored for tumor growth and xenograft tumors volumes were measured. Five days after the injections, small tumors were observed in all the groups of mice. Notably, cells overexpressing OPNc resulted in extremely rapid tumor growth and formation of larger ($\geq$200 mm^3) tumors between 10 and 15 days after s.c. injection (Fig. 5C and 5D). In these tumors, 3 known markers of tumor progression, MMP2, MMP9, and VEGF were consistently upregulated ($P < 0.001$; Fig. 5E). In contrast, tumors resulting from OPNa, OPNb or empty vector expressing clones were of comparable size to those generated with OvCar-3 nontransfected cells. Taken together, these results indicate that overexpression of OPNc is able to enhance tumor growth rates and progression of xenograft tumors formed by OvCar-3 cells.

OPNc mediates ovarian carcinoma progression features through the PI3K/Akt pathway

OPN bind to a variety of cell surface integrins, CD44 and epidermal growth factor receptor (EGFR) and function as signal transducers to promote cell proliferation, adhesion, motility and survival through activating kinase cascades, and transcription factors (2). Among signaling pathways typically activated on ovarian tumor progression, phosphatidylinositol 3-kinase (PI3K) present an important prosurvival role (29–33). Previous reports indicate that the gene encoding the p110α catalytic subunit of (PI3K) is increased in copy number in ovarian cancer cells (32, 33). PI3K binds to and is activated by several receptor and non receptor tyrosin kinases and its oncogenic form can induce cellular transformation. The best known downstream target of PI3K is the serine-threonine Akt, which transmits the angiogenic and oncogenic signals from growth factors. In ovarian tumorigenesis, this pathway plays critical roles in invasion and metastasis (32). Given these observations, we sought to determine if the overexpression of each OPN isoform would modulate PI3K activity and Akt phosphorylation (Ser^{473}) and whether this signaling pathway is important to mediate specific OPN splicing variant effects on ovarian cancer progression.

OPNc overexpression, but not the remaining isoforms, resulted in a 2-fold increase in Akt (Ser^{473}) phosphorylation (Fig. 6A). ERK 1/2 phosphorylation was not observed on cell extracts overexpressing OPNa, OPNb and OPNc splice variants (data not shown). Having shown that OPNc overexpression induces activation of PI3K/Akt pathway, we next examined the effects of inhibiting this pathway on cells overexpressing each OPN isoform. We used a specific small molecule inhibitor of PI3K, LY294002, to assess the effects of inhibiting PI3K on OvCar-3 cells proliferation, migration, and soft agar colony formation (Fig. 6 B–E).

To elucidate whether PI3K affects the proliferation of OvCar-3 cells overexpressing each OPN isoform we evaluated proliferation kinectics by crystal violet assay in the absence and presence of LY294002. Optical density at 550 nm, reflecting total cell number, was counted 24, 48, 72, and 96 hours after the incubation. As shown on Figure 6B, proliferation rates of OvCar-3 cells overexpressing OPNc was greatly increased over the period of 96 h in culture in the absence of LY294002. The proliferation of OPNc overexpressing cells was slightly decreased by the addition of 50 μmol/L LY294002 24 and 48 hours after the treatment. However, after 72 and 96 hours of the treatment,

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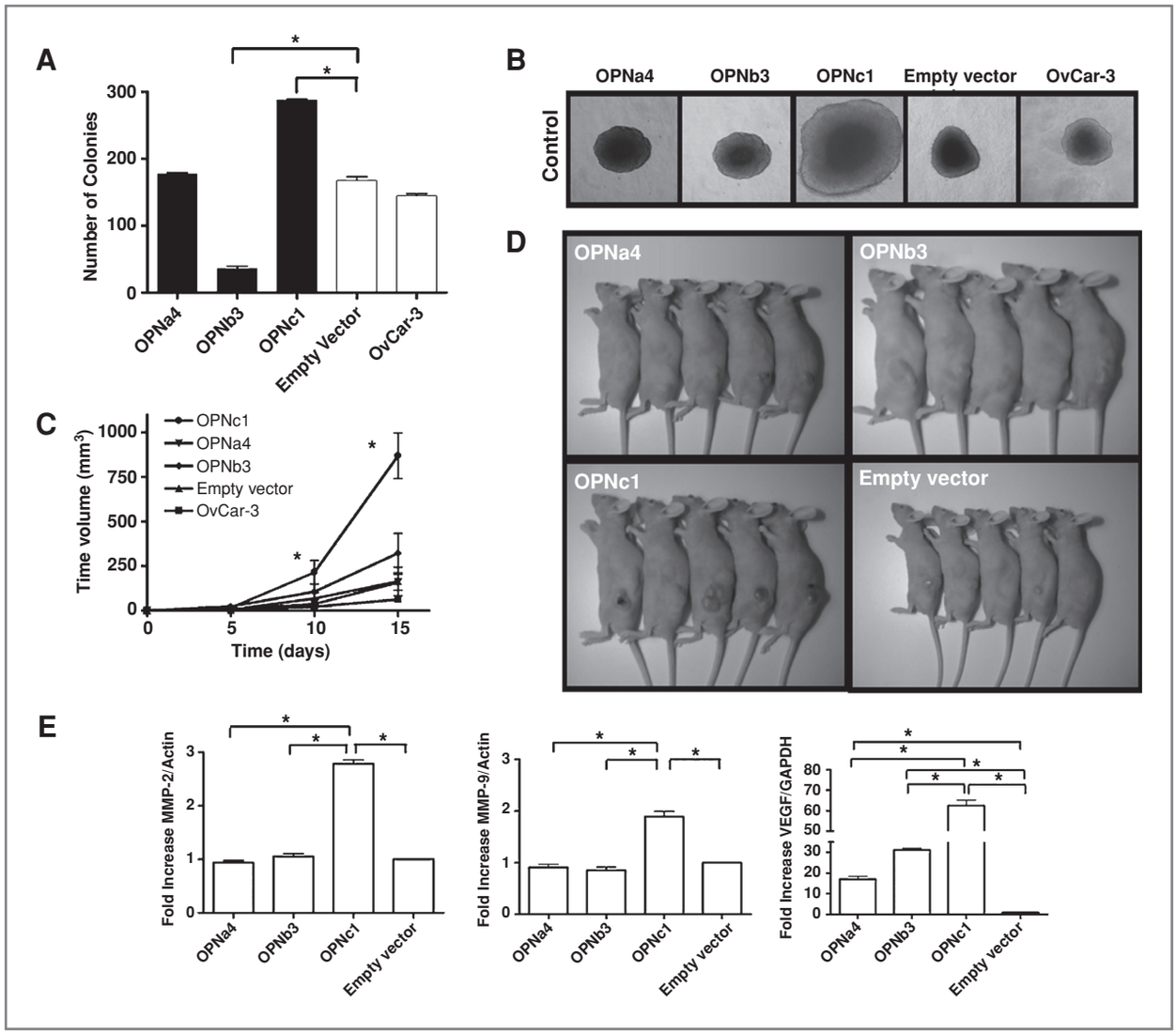

Figure 5. Expression of the OPNc enhances the colony formation in soft agar and potentiates tumor *in vivo* formation. Soft agar assay was carried out to assess the ability of stably transfected cell lines to grow in an anchorage independent environment as described in Supplementary Information. Plates were examined microscopically for growth after 30 days. A, quantification of the number of OvCar-3 cell colonies grown in semisolid agarose medium transfected either with empty vector, OPNa, OPNb, or OPNc expression vectors. *, $P < 0.01$ vs. empty vector cells. B, phase-contrast microscopy of 2 representative OvCar-3 colonies. Cell overexpressing OPNc form colonies with larger size than those overexpressing OPNa, OPNb, empty vector, or OvCar-3 nontransfected cells. Pictures were obtained in 5× magnification. All results are representative of at least 3 independent experiments. C, OvCar-3 transfected and nontransfected cells were implanted s.c. in the right flanks of BALB/c nude mice and the animals were monitored for tumor formation. Tumor growth rates were measured every 5 days. Cells overexpressing OPNc present higher tumor volumes. *, $P < 0.01$ vs. empty vector cells. D, enhanced tumor growth in mice injected with OvCar-3 cells overexpressing OPNc. Representative pictures of tumors grown in nude mice. Tumor development was monitored over 15 days. E, induction of MMP2 (left panel), MMP9 (middle panel) and VEGF (right panel) mRNA expression in xenograft tumors overexpressing OPNc. Total RNA from xenograft tumors grown from cells overexpressing OPNa, OPNb, OPNc, and empty vector control was prepared to conduct quantitative real-time PCR (qRT-PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin as internal controls. The amount of targets was analyzed using the comparative CT method, where the threshold cycle (CT) values of each target sequence are given by the $2^{-\Delta\Delta CT}$ formula. We present the data as log *n*-fold change in gene expression normalized to the endogenous reference genes (GAPDH or actin) relative to the expression of cells overexpressing empty vector control. *, $P < 0{,}001$.

the proliferation of the OPNc overexpressing cells was significantly inihibited by LY294002. These results indicate that PI3K may play a role in the induction of proliferation promoted by OPNc splicing isoform. Cells overexpressing OPNa, OPNb, and empty vector control as well as nontransfected OvCar-3 cells present no modification on proliferation rates after LY294002 treatment (Fig. 6B).

To determine whether the inhibition of PI3K activity by LY294002 also affects OvCar-3 cell migration, cells overexpressing each OPN isoform were treated with LY294002

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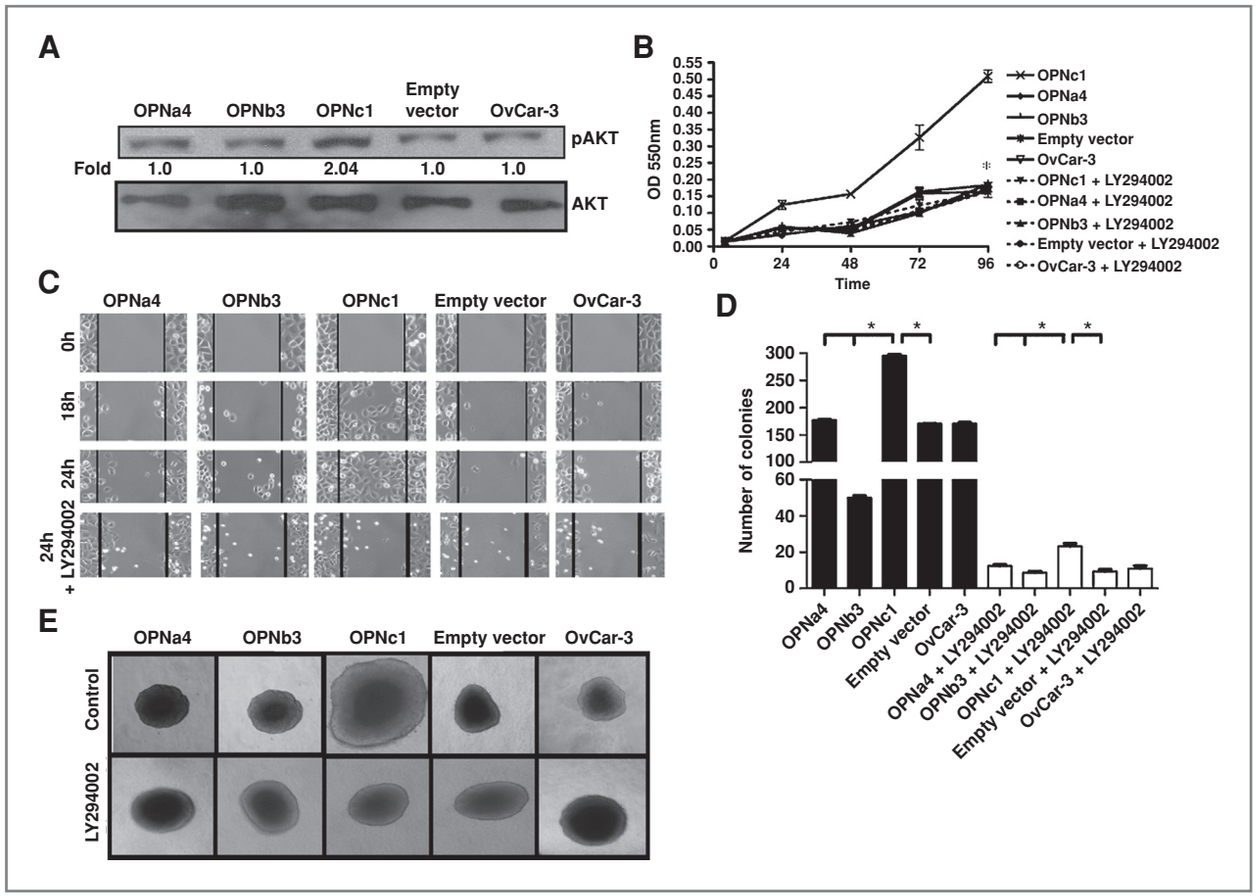

Figure 6. PI3K/Akt signaling pathway mediate ONPc roles on activating tumor progression features. A, PI3K/Akt signaling is activated in OvCar-3 cells overexpressing OPNc. Total Akt and p-Akt levels in OvCar-3 cells overexpressing OPNa, OPNb, OPNc, and empty vector control were measured using Western blotting using their specific antibodies. Non phospho Akt were used as a loading control. Fold changes were calculated based on densitometric quantification; B, OPNc-induced OvCar-3 cell proliferation is specifically blocked by the PI3K inhibitor. Growth curves of OvCar-3 overexressing clones and nontransfected OvCar-3 cells cultured with or without PI3K LY294002 inhibitor are plotted. LY294002 was added 4 hours after cell plating and culture media maintained for all proliferation kinectics as measured by crystal violet assay.). *, $P < 0.0001$ vs. empty vector control clone; C, OPNc-induced OvCar-3 cell migration is blocked by the PI3K inhibitor. OvCar-3 cells were seeded, and migration capacity was evaluated after carrying out streaks in wound closure assay. LY294002 inhibitor was added just after carrying out the streaks and cell migration was analyzed at the 24 hour time point; D, OPNc overcomes LY294002 effects on inhibiting the number of OvCar-3 cells colonies formed in soft agar. Colonies were cultured in soft agar in the presence or absence of LY294002 inhibitor added to culture medium, which was changed every 3 days until reaching 30 days of culture. *, $P < 0,002$; E, the increase of size of colonies of OvCar-3 formed in soft agar promoted by OPNc overexpression was reversed by the PI3K inhibitor. OvCar-3 cells overexpressing OPNa, OPNb, OPNc, and empty vector control were cultured in the presence and absence of LY294002 and the the size of colonies was evaluated until reaching 30 days of cell culture in soft agar. Original magnification 5×.

as described above and cell migration was evaluated by wound closure assay. The treatment of OvCar-3 cells with 50 μmol/L of LY294002 for 24 hours specifically inhibited the migration of cells overexpressing OPNc (Fig. 6C).

Similarly, the inhibition of PI3K activity significantly reduced the size of colonies of anchorage independent growth formed by OvCar-3 cells overexpressing OPNc. The number of colonies formed decreased 5- to 12-fold after LY294002 treatment on cells OvCar-3 overexpresing either OPNa, OPNb, OPNc, empty vector control, and wild-type OvCar-3 cells. However, cells overexpressing OPNc treated with LY294002 could better overcome the effects of this inhibitor, once the number of colonies formed was ∼50% higher as compared with clones overexpressing OPNa, OPNb, emtpty vector, and OvCar-3 cells nontransfected cells. These results indicate that OPNc probably exert an antagonist effect over LY294002 treatment on soft agar colony formation, probably through OPNc roles on activating ovarian cancer cell survival. Taken together, these results indicate that the PI3K signaling pathway is required for some OPNc oncogenic features related to ovarian cancer progression.

Discussion

We have shown here the first description of OPN alternative splicing expression in ovarian carcinoma cell lines and human tissue samples. We further show that the OPNc

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isoform, when overexpressed in OvCar-3 cells, is able to promote several aspects of ovarian cancer tumor progression, both *in vitro* and *in vivo*. Additionally, this paper is the first description of the involvement of PI3K signaling pathway mediating OPN isoform-specific roles on promoting tumor progression features. Ovarian malignant transformation is caused by genetic modifications that disrupt the regulation of proliferation, programmed cell death and senescence, but the specific genetic pathways involved in these processes are poorly understood (34, 35). Alternative pre-mRNA splicing is an important molecular mechanism associated with tumorigenesis and cancer progression (36) and has been shown to be one of the mechanisms by which cancer cells alter the structure and function of OPN (16–19, 37). Based on these assumptions, we believe that describing the functional significance of OPN splicing isoforms in ovarian cancer would improve the understanding of signaling pathways involved in the progression of this complex neoplasic disease. Alternative splicing of OPN has been described in mesothelioma, breast cancer, hepatocellular carcinoma, glioma, lung cancer and head and neck squamous cell carcinoma (HNSCC) and the roles of different splicing isoforms on cancer growth and progression has been reported (16–19, 37). However, OPN splicing and its resulting products has not been examined previously in ovarian cancer.

To address these issues, we firstly explored the occurrence of OPN isoforms in ovarian tumor and nontumor cell lines and tissues. Our results have shown that OPNa and OPNb are expressed in all ovarian cell lines analyzed, both normal and tumor, but OPNc is only expressed in tumor samples, including tumor cell lines and malignant and borderline tumor tissues. Therefore, our data indicate that OPNc isoform is specifically expressed in ovarian tumor samples, as previously described for breast cancer cells (16, 17). In contrast, OPNc is barely detected in mesothelioma tumors and in lung cancer samples, whereas OPNa and OPNb showed upregulation in these samples (18, 19). Our data further support previous findings showing that OPN isoforms present tumor-specific expression patterns.

The differential expression profiling of OPNa, OPNb and OPNc expression in ovarian samples and the important cellular effects due to OPNc overexpression observed herein, could be the basis for future studies to better investigate their potential use of OPNc as an ovarian cancer biomarker. Examining the expression of these isoforms in an appropriate representative sample of ovarian tissues containing different tumor subtypes and clinical behavior could provide additional information about the involvement of these isoforms in ovarian cancer tumorigenesis and progression.

We then examined the functional roles of each OPN splicing isoform in ovarian cancer progression by using *in vitro* and *in vivo* tumor models. We show here that OvCar-3 cells that overexpress OPNc have increased proliferation, migration and invasion, whereas OPNa and OPNb did not have these effects. Additionally, OPNc significantly increased the number and size of colonies formed in soft agar assay. Interestingly, OPNb inhibited the number of colonies formed, suggesting a potential negative regulatory role of this isoform in the formation of tumor metastasis. Opposing roles for OPN isoforms have also been described previously in breast cancer cells when comparing OPNc versus OPNa (17). In general, tumor cells express splice variants involved in cancer progression with different potential to support metastasis. Among these examples, only certain splice variants of HIF1a (38), Syk (39), CD44 (40) and S100A4 (41) gene products favor metastasis formation, whereas others act as inhibitors. Although we do not provide here direct evidence about the roles of individual OPN isoforms on modulating tumor metastasis, the roles of OPNc on activating MMPs, VEGF, tumor migration, and invasion give strong support to the putative roles of OPNc on stimulating ovarian cancer metastasis, once all this events act cooperatively to facilitate metastasis of cancer. Future studies with appropriate animal tumor models should be conducted in order to elucidate the roles of OPN isoforms on ovarian carcinoma metastasis formation. Altogether our results clearly show that OPNc contributes to ovarian cancer progression and malignancy.

We also observed herein that OPNc stimulate OvCar-3 cell proliferation rates independently of growth factors, a typical feature of a protein involved in tumor progression (27). OPNc could stimulate growth signal autonomy by different strategies, such as alteration of extracellular growth signals, of transcellular transducers of those signals or of intracellular circuits that translates those signals into action (26). Literature has provided evidence that total OPN affect the expression of genes involved in multiple aspects of tumor progression and malignant growth, including those involved on self-sufficiency in growth signals (42, 43). In ovarian carcinoma, among all isoforms, OPNc could better stimulate these signals as compared with the remaining isoforms. As shown herein, OPNc overexpression stimulate PI3K signaling pathway, which could be one of the molecular mechanisms of activating transducer growth signals and signaling pathways activating cell proliferation and/or rescuing cells from apoptotic stimuli such as serum starvation. Our data also give support to the hypothesis that the increase in cell proliferation observed for cells overexpressing OPNc, as compared with OPNa, OPNb and vector control, is not due to differential levels of cell death promoted by these OPN isoforms, but rather to an enhancement of cell survival promoted by OPNc. This hypothesis was further reinforced by the observation that when treating OPNc overexpressing cells with an anti-OPN antibody, cells were induced to die, again supporting a role of OPNc in ovarian cancer cell survival. Previous reports have been showing that OPN is able to prevent cell death in response to diverse stress stimuli, including serum starvation (17, 37) and that OPN has been shown to inhibit apoptosis in several systems. According to our data, we propose that OPNc may enhance OvCar-3 survival due to an enhancement of proliferation. Further studies should be carried out to determine whether apoptosis evasion is also involved in OPNc survival roles in ovarian cancer. Besides promoting higher proliferating rates in OvCar-3 tumor cells, we have

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also shown that secreted OPNc stimulates IOSE nontumoral cell line proliferation. These data indicate that OPNc is not only involved in ovarian cancer progression and survival, but may also contribute to early steps of ovarian cancer tumorigenesis process.

Current available data about OPNc roles in other tumor models show that the effects of OPNc may vary among different tumor types. For example, in breast cancer cells, OPNc does not affect cell growth, but can stimulate soft agar colony formation (16). By contrast, in mesothelioma and non-small–cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC) tumors, isoforms OPNa and OPNb, but not OPNc, are able to stimulate pro-tumorigenic behaviors (18, 19, 37). In lung cancer, OPNc overexpression was associated with decreased angiogenic properties, whereas OPNa and OPNb present opposite roles (19). Therefore, OPN splicing isoforms seem to have cell, tissue and tumor specific and even opposing roles in different tumor progression models. One such example of this opposite roles has been observed when evaluating OPNa functional roles in another ovarian cancer cell line (31). In this model, it was observed that OPNa promoted ovarian cancer progression and survival, in contrast to what has been observed in our study. However, in this study they did not compared their results with the remaining OPN isoforms.

OPNc lacks exon 4, and it is thus likely that the roles of OPNc in tumor progression are related to the absence of the protein sequence contained in exon 4. However, the physiological and pathophysiological roles of the N-terminal domain of OPN, including exon 4, are still poorly understood. Some evidence about functional properties of exon 4 suggests that this region, as well as exon 5, may play a critical role in OPN solubility, making soluble OPN available for receptor ligation (17). Another layer of added complexity is that, besides alternative splicing, post translational modifications (PTMs) of OPN, especially phosphorylation, are functionally important (17, 44–46). Recent reports have also shown that the degree of phosphorylation of OPN isoforms produced by different tumor cell types can regulate its roles and receptor interactions (37). Based on these data, we propose here that the deletion of exons 4 and 5 could alter the pattern of PTMs, promoting functional modifications. According to previous reports evaluating OPN PTMs in tumor cells, OPN splicing isoforms produced by different cell types exhibited different functional properties (46). Similar functional modifications could affect OPNc and OPNb isoforms in ovarian cancer cell lines, due to alterations on their PTMs patterns as a consequence of exon deletion. Further work is required to elucidate PTMs patterns of OPN isoforms and their impact on their roles in ovarian cancer cells.

The molecular mechanisms by which OPN splice variants modulate tumorigenic roles are still unclear and very few data are currently available. Previous reports on hepatocellular carcinoma cell lines showed that OPNa and OPNb activated migration-associated signaling pathways by increasing the expression of urokinase-type plasminogen activator and the phosphorylation of p42/p44 MAP Kinase, but these pathways were not activated by OPNc (47). Another report showed that OPNa and OPNb overexpression promoted tumor growth in pancreatic cancer and fibrosarcoma cells due to less apoptosis and that these effects were signaled mainly through the activation of FAK and NF-kB (37). Most data about signaling pathways activated by human OPN relates to total OPN. Besides post translational modifications, OPN functional diversity results from differential binding to seven types of integrins and specific splice variants of CD44 (26, 47). The engagement of the integrin receptor $\alpha v\beta 3$ by OPN increases the c-Src-mediated phosphorylation of EGFR, activating PI3K-dependent Akt and mitogen-activated protein kinase 1 (MEK1)-dependent ERK1/2 pathways. In contrast, the binding of OPN to CD44 receptors activates the PLC-γ-dependent Akt pathways (47). Besides, crosstalks of these signaling pathways also exists (2). Our data showed that OvCar-3 cells overexpressing OPNc specifically activate PI3K/Akt signaling pathway and that OPNc roles on activating OvCar-3 cell proliferation, migration and soft agar colony formation are mediated by this pro-survival pathway. OPNc roles on overcoming the effects induced by the PI3K inhibitor LY294002 on the number of colonies formed in soft agar are probably related to OPNc signals on counteracting the effects of this molecule as a typical inhibitor of ovarian tumor growth by inducing apoptosis (32). In summary, these results suggest that OPN splice variants differentially couple to signaling pathways to modulate ovarian cancer progression and that previous description of PI3K/Akt pathway involvement on survival-promoting and stimulating cell cycle progression functions of OPN in ovarian carcinoma (31) are probably mainly mediated by OPNc splicing isoform. Our data also reinforce the notion that OPN splicing isoform-specific roles on tumor progression are a result of differential activation of cell surface receptors and signaling pathways.

OPN is predominantly known as a secreted protein (sOPN) and there is increasing knowledge that OPN binding to its receptors and the signaling pathways so activated, mediate different OPN functions (2). Our results suggest that the functional effects we described herein are mediated primarily by secreted OPNc. Nevertheless, based on our data, we cannot exclude the possibility that a putative intracellular OPN (iOPN), recently described (48–51), could act in addition to secreted OPNc and contribute to the effects we describe. Considering the different roles OPNc has in different tumor types, we hypothesize that besides posttranslational modification and proteolytic cleavage (46, 52), an unbalanced proportion of putative iOPN and sOPN isoforms could also modulate the tumor- and tissue-specific functional roles of this splicing isoform, as has been described by others (49, 50). Further studies will be necessary to determine the putative occurrence, regulation, and functional roles of both iOPN and sOPN in tumor cells, specially the occurrence of secreted and intracellular OPNc isoforms in ovarian cancer and their

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corresponding roles in this cancer.In conclusion, based on the observation of differential expression of OPN splicing isoforms in tumor and nontumoral ovarian samples, with OPNc identified only in tumor cell lines and tissues, whereas OPNa and OPNb expressed in tumor and non-tumor tissue and cell lines, we examined the functional roles of each isoform in ovarian cancer biology. We found that OPNc overexpression stimulating cell proliferation, migration, invasion, colony formation and tumor growth denotes its important role on ovarian cancer progression. Our data also show that at least some of these OPNc tumorigenic roles are mediated by PI3K/Akt signaling pathways. The observed involvement of OPNc in ovarian cancer progression contributes to a better knowledge about the molecular mechanisms related to this important and complex gynecological malignancy. Together with previous published results on other tumor models, our data reinforce the notion that OPN splicing isoforms may have tumor and tissue-specific roles. An in-depth knowledge of specific behavior of the OPNc isoform and its corresponding gene expression control may open the possibilities of new therapeutic approaches that selectively down regulate OPNc altering its properties which favor tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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