

Original Research

Prostaglandin $F_{2\alpha}$ -induced Prostate Transmembrane Protein, Androgen Induced 1 mediates ovarian cancer progression increasing epithelial plasticity



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Abstract

The role of prostaglandin (PG) $F_{2\alpha}$ has been scarcely studied in cancer. We have identified a new function for $PGF_{2\alpha}$ in ovarian cancer, stimulating the production of Prostate Transmembrane Protein, Androgen Induced 1 (PMEPA1). We show that this induction increases cell plasticity and proliferation, enhancing tumor growth through PMEPA1. Thus, PMEPA1 overexpression in ovarian carcinoma cells, significantly increased cell proliferation rates, whereas PMEPA1 silencing decreased proliferation. In addition, PMEPA1 overexpression buffered TGF signaling, via reduction of SMAD-dependent signaling. PMEPA1 overexpressing cells acquired an epithelial morphology, associated with higher Ecadherin expression levels while catenin nuclear translocation was inhibited. Notwithstanding, high PMEPA1 levels also correlated with epithelial to mesenchymal transition markers, such as vimentin and ZEB1, allowing the cells to take advantage of both epithelial and mesenchymal characteristics, gaining in cell plasticity and adaptability. Interestingly, in mouse xenografts, PMEPA1 overexpressing ovarian cells had a clear survival and proliferative advantage, resulting in higher metastatic capacity, while PMEPA1 silencing had the opposite effect. Furthermore, high PMEPA1 expression in a cohort of advanced ovarian cancer patients was observed, correlating with Ecadherin expression. Most importantly, high PMEPA1 mRNA levels were associated with lower patient survival.

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Introduction

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is an arachidonate biosynthetic pathway endproduct, which ratelimiting step is catalyzed by cyclooxygenases (COX), enzymes implicated in various disease states including cancer [1]. $PGF_{2\alpha}$ has been scarcely studied on cancer although it has been detected in several tumor types and cancer patient body fluids [24], recently has been mechanistically associated with colon cancer progression [5].

Previous studies have shown increases of COX, prostaglandin synthases, prostaglandins and receptors in epithelial ovarian cancer (EOC) [6,7]. EOC, which comprises 90% of all ovarian malignancies, is the leading cause of death from gynecological cancer, due to late diagnosis, in developed countries [8,9].

PMEPA1 gene expression has been found in several primary and metastatic tumor types [1012]. Depending on the tumor tissue origin, PMEPA1 has been shown to have a protumor or antimetastatic role. Thus, in prostate cancer, it is well established as a part of a negative feedback loop of the Androgen Receptor (AR), which induces PMEPA1, that participates in the degradation of the receptor through an E3 ubiquitin ligase complex [13]. Depending on whether the prostate cancer cells are positive or negative to AR, PMEPA1 has a growth inhibitory or a growthpromoting role [1317], while some studies have shown that it inhibits prostate cancer metastases to bone [14]. On the other hand, PMEPA1 has been already shown to have protumorigenic effects y breast and lung cancer [1821] and high expression levels in other types, such as kidney and colorectal cancer [10,22,23]. PMEPA1 can also be induced by transforming growth factor (TGF) [10]. PMEPA1 downregulates TGF signaling by sequestering RSMAD and promoting lysosomal degradation of TGF receptor [24]. PMEPA1, through a negative feedback loop, is described to switch TGF from tumor suppressor to tumor promoter in breast cancer [12]. In addition, TGFdependent growth of aggressive breast cancer has been suggested to depend on increased expression of PMEPA1 gene [11]. TGF has been implicated in physiological and pathological processes in the ovary [25,26]. In ovarian cancer, TGF has been shown to control cell proliferation [27].

Here, we identify, PMEPA1 as a COX2/ $PGF_{2\alpha}$ upregulated gene through the induction of TGF and we have deciphered its role in ovarian cancer progression. We have found that $PGF_{2\alpha}$ induced TGF β 1 and PMEPA1 and we provide new evidence of its important role in ovarian cancer progression. Moreover, our results indicate that PMEPA1 is a critical regulator of epithelial plasticity, conferring a growth advantage in ovarian cancer cells.

Materials and methods

Ovarian samples

A series of 19 normal, 51 primary tumors and 37 metastatic/relapse ovarian samples were collected at the MD Anderson Cancer Center Biobank (Madrid; record number B.0000745, ISCIII National Biobank Record), the centers ethical committee approved the study, and a complete written informed consent was obtained from all patients. The sample characterization was performed by a pathologist (ARS), who determined the histological cancer subtype according to the World Health Organization (WHO) criteria [28], and the stage and grade (Supplemental Table 1).

Cell lines

SKOV3lucD6 cells, stably expressing Firefly Luciferase, were obtained from Caliper Life Sciences. SKOV3 and TOV112D cells were from ATCC and A2780 cell line was provided by SigmaAldrich. OVCAR8 cell

line was a gift from Dr. JM Cuezva (CBMSO). All cell lines were grown in the recommended conditions.

Reagents

All generic reagents were from Invitrogen or SigmaAldrich. Oligonucleotide and antibody details can be found in Supplemental Tables 2 and 3. Plasmids and Lentiviral vector transduction methods are listed in Supplemental Materials and Methods.

Cell assays

Cell proliferation assays were performed plating 20,000 cells in various 35 mm wells and each day of the experiment cells from a different well were counted. Growth was also quantified by Crystal Violet staining: Glutaraldehyde fixed cells were stained with 0.5% Crystal Violet/50% methanol for 20. Stain was dissolved in 10% Acetic Acid after washing and O.D. measured at 570 nm.

Substrate independent cell growth assays were performed by seeding 50,000 cells/well in ultralow attachment surface 24well plates (Costar) in normal growth medium. At the end of the experiment relative cell numbers were estimated by Alamar Blue staining (Invitrogen).

RNA isolation and RTqPCR analysis were performed as described [5].

Immunofluorescence and Western blotting were performed as described before [5]. Images were taken using a Zeiss Axioscop2 plus with a color CCD camera or the Zeiss LSM710 confocal microscope. For nuclear/cytoplasmic/membrane fractionation please see Supplemental Methods. For WB, densitometry quantification of the bands obtained is included in Supplemental Figures S6 and S8.

Luciferase assays were performed as described before [5].

Tumor growth in nude mice

In all cases, the minimum cell number indicated for each xenograft model was used. In the case of A2780 and TOV122D cell lines, 10^6 and 5×10^4 cells were injected subcutaneously in 6 week old female Rag2/Il2rg Double Knockout mice (R2G2, ENVIGO, France). For the SKOV3Luc subcutaneous and intraperitoneal xenograft models, 6weekold female Swiss Nude mice (CrI:NU (Ico)Foxn1^{nu}, Charles River Laboratories) were injected with 1×10^6 cells, following the instructions of the provider of the cells, Caliper LS. Mice were weighted once a week and the tumor volume was estimated with a digital caliper by measuring: length width height. Tumor growth estimation was made as described before [5,29] using a Xenogen IVIS Lumina CCD camera (Caliper Life Sciences). In the orthotopic mouse model of ovarian cancer, animals were injected with 5×10^5 in the left ovary.

Histological analysis and immunohistochemistry

Tumors from mice and patients were fixed in 4% phosphate buffered formalin (pH 7.4) and 2 m paraffinembedded sections were immunostained, performed as previously described [5,29]. Antibodies used for immunohistochemistry: antiPMEPA1 (1:50) as primary antibody (SigmaAldrich) and secondary antibody conjugated with HRP (Envision + Dual link System HRP, Dako). Finally, sections were developed using DAB solution (Liquid DAB + substrate chromogen system, DAKO K3468) and images were taken with a LEICA DMD108 Digital Microimaging Device (Leica Microsystems).

Databases and genomics analysis was performed through the UCSC Xena Browser as indicated in Supplemental Methods.

Statistical analysis

Results are expressed as mean SEM. The Students t test, the ANOVA test or Welch's t test were used for comparisons, where necessary. * $p < 0.05$ and ** $p < 0.01$ denote statistical significance. For gene expression correlation/covariance, Pearson's correlation coefficient was calculated. The statistical analysis was performed using the GraphPad Prism 4.0 statistical software.

Results

TGFB1 expression correlates with the COX2/PGF_{2α}/NFAT pathway

TGF is a crucial factor for ovarian homeostasis and tumorigenesis [27]. Indeed, gene expression analysis of the TCGA TARGET GTEx patient cohort RNA sequencing data using the UCSC XENA cancer browser shows a 2fold increase of TGFB1 mRNA levels in ovarian tumor samples, compared to normal ovarian tissue (Supplementary Figure S1A). PGF_{2α} has an important role in the female reproductive system and is known to be produced in the ovary, as is produced also in ovarian tumors [7]. PGF_{2α} binds and signals through the F prostaglandin receptor (FP, PTGFR), a G protein coupled receptor, coupling mainly to Gq, thus leading to an increase in intracellular Ca²⁺ levels [30]. Consequently, PGF_{2α} binding to FP causes Ca²⁺/calcineurin activation NFAT transcription factors, implicated in a growing variety of physiological and pathological functions, including cancer [31]. Interestingly, we found that PGF_{2α} significantly increased TGFB1 mRNA levels in ovarian serous adenocarcinoma SKOV3 cells (Suppl. Figure S1B). TGFB1 mRNA levels correlated to the PTGS2 (Pearson's $r = 0.20$, $p < 0.0001$, $n = 369$) and PTGFR mRNA levels in ovarian tumors (Suppl. Figure S1C). On the other hand, the calcineurin/NFAT activator Ca²⁺ ionophore A23287 significantly increased TGFB1 mRNA levels in SKOV3 cells mimicking the PGF_{2α} effect (Suppl. Figure S1B). We found that NFATC2 is upregulated more than 2 fold in ovarian tumors, compared to healthy tissue (Suppl. Figure S1D). Moreover, NFATC2 and TGFB1 mRNA levels correlated strongly (Suppl. Figure S1E), as did also NFATC1 and TGFB1 (Pearson's $\rho = 0.41$, $p < 0.0001$, $n = 369$). As expected, TGFB1 levels also correlated with the RCAN1 levels (a *bonafide* NFAT transcriptional target) mRNA levels (Suppl. Figure S1F).

Along these lines, multiple NFATc1 binding sites were identified in the promoter region of TGFB1, using chromatin immunoprecipitation and mass sequencing (ChIPseq) data experimental from the Gene Transcription Regulation Database (GTRD), two of them in the TGFB1 gene regulatory region located in its first intron (Suppl. Figure S2), Site IDs: 345418867, indicating a possible transcriptional regulation of TGFB1 by PGF_{2α}/NFAT signaling. All these findings could indicate a close relationship between the COX2/PGF_{2α}/FP/Ca²⁺/NFAT pathway and TGFB1.

PMEPA1 levels are elevated in patients ovarian tumor samples

PMEPA1 has been proposed as a TGF induced gene [12]. Indeed, we found a strong correlation between TGFB1 and PMEPA1 mRNA levels in the TCGA ovarian cancer cohort (Suppl. Figure S1G). A similar correlation was found between PMEPA1 and RCAN1 mRNA levels (Suppl. Figure S1H), indicating the possible, direct or indirect, implication of NFAT in the PMEPA1 gene expression. Additionally, we found tumor PMEPA1 mRNA levels to be significantly higher than in normal ovary (Suppl. Figure S1I).

To confirm this, we performed immunohistochemistry on normal tissue obtained from ovaries, as well as ovarian primary tumors and relapses. Primary ovarian tumors showed strong PMEPA1 staining in tumor cells

compared to normal ovaries that showed diffuse expression in some epithelial cells (Figure 1).

The patient cohort used for determining PMEPA1 protein levels includes only highgrade tumors and most of them resulted positive for PMEPA1. Thus, no significant differences in patient survival according to PMEPA1 expression could be obtained. However, after analysis of the gene expression databases available (TCGA ovarian cancer), high PMEPA1 expression associated with lower survival probability (Suppl. Figure S3). The above observations suggest PMEPA1 could have an important role in ovarian cancer progression and it could be considered as a potential biomarker for ovarian tumor characterization and patient stratification.

Induction of PMEPA1 expression by PGF_{2α}

Treatment of SKOV3, OVCAR8, TOV112D or A2780 ovarian cancer cells with fluprostenol, a metabolically stable PGF_{2α} analog, increased PMEPA1 mRNA levels, as did TGF treatment. The combination of both treatments had a partially additive effect (Figure 2A). We transduced Skov3 cells with lentiviral shRNA vectors to knockdown PTGFR mRNA, thus decreasing PTGFR levels and signaling. When the resulting cell lines were treated with PGF_{2α} (Figure 2B) or fluprostenol (not shown) they failed to increase PMEPA1 mRNA levels as the control scrambled shRNA cells did, indicating that PMEPA1 induction depended on the PGF_{2α} signaling. Moreover, calcineurin/NFAT inhibition by cyclosporine A (CSA) could revert PGF_{2α} induced TGFB1 and PMEPA1 increase (Figure 2C). Indeed, CSA not only reverted significantly both genes induction, but also reduced their levels in the absence of PGF_{2α}, indicating a possible basal Ca²⁺/calcineurin signaling. Interestingly, the PTGFR induction of PMEPA1, but not RCAN1, a direct NFAT target, was reverted by cotreatment with an inhibitor of TGF type I receptor, LY2109761 (Figure 2D), indicating that basal TGFR signaling may be necessary for basal and Ca²⁺ stimulated PMEPA1 expression.

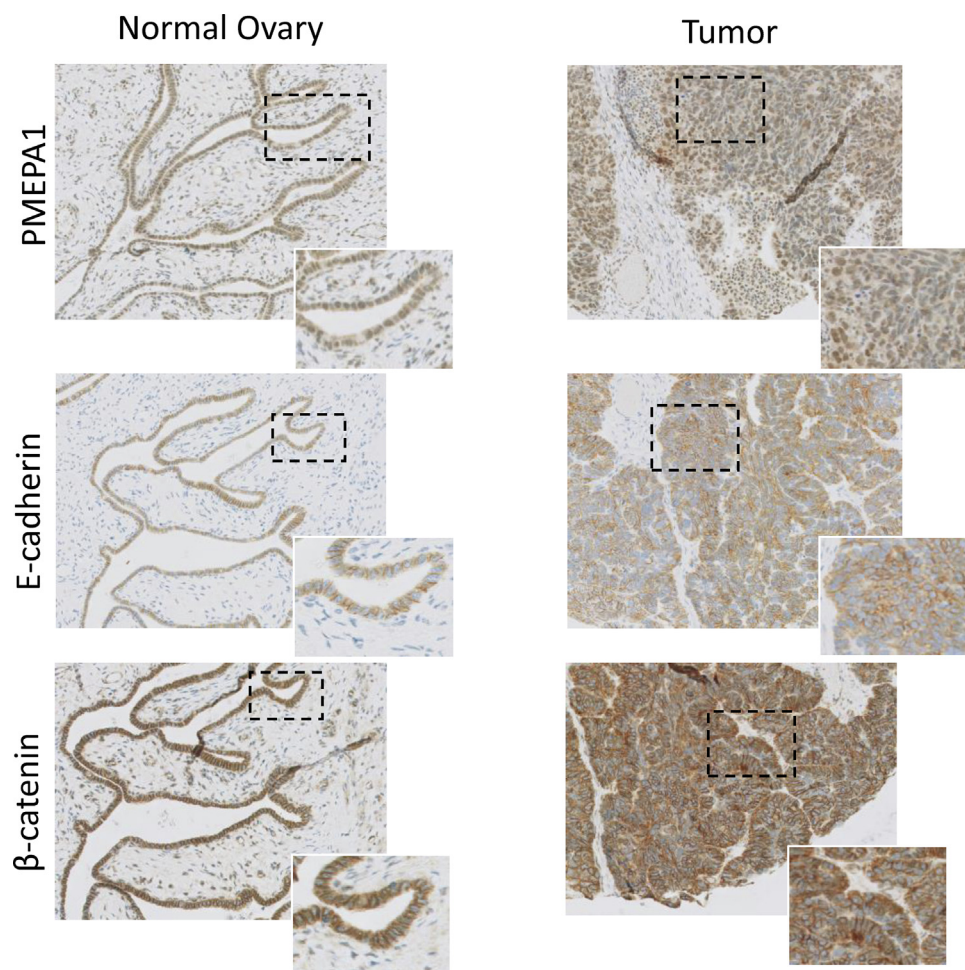
We then analyzed PMEPA1 transcriptional activity, using two luciferase reporter constructs: the PMEPA1 promoter fragment 1972PMEPA1-luc and PMEPA1 first intron pGL3ti850 [32]. In SKOV3 cells, pGL3ti850 activity was stimulated by TGF treatment but not by fluprostenol alone, although it showed a strong synergistic activity with TGF. On the other hand, 1972PMEPA1-luc was only activated by the combination of fluprostenol and TGF (Figure 2E). These results would indicate that SMADs and NFAT cooperatively stimulate PMEPA1 mRNA expression, while the PGF_{2α} induction of PMEPA1 could be also partially due to TGFB1 induction.

We searched the GTRD ChIPseq data and identified multiple NFATc1 binding sites, Site IDs 2498230711, in the promoter and the first intron regulatory region of PMEPA1. Interestingly, we found several SMAD2, SMAD3 binding sites adjacent to the NFATc1 ones (Suppl. Figure S4).

All the above suggest a cooperation of NFAT with SMADs for PMEPA1 induction, pointing to is a positive feedback loop between PGF_{2α}/NFAT and TGF for PMEPA1 expression. TGF induced PMEPA1 expression can be further potentiated by the PGF_{2α}/Ca²⁺/Ca²⁺ pathway, both through its main promoter as by the first intron enhancer.

PMEPA1 overexpression in cancer cells enhances cell growth

To investigate the biological effects of PMEPA1 in tumor cells, we generated ovarian cancer cell lines (Skov3, OVCAR8, A2780 and TOV112D), stably overexpressing PMEPA1 and compared them with control cells carrying the empty vector (EV). Additionally, 5 PMEPA1 knockdown SKOV3Luc cell lines were generated. Knockdown or overexpression were confirmed by RTqPCR, WB and immunocytochemistry of



	Normal	Primary tumor	Relapse	Totals
Diffuse	14	10	8	32
Strong	5	41	29	75
Totals	19	51	37	107

Chi-Square: 21.16 , df = 2 , $P < 0.0001$
Cramer's V = 0.4447

Figure 1. PMEPA1 expression is widely expressed in ovarian cancer. Representative images of PMEPA1 (top), E-cadherin (middle), and beta-catenin (down) expression by immunohistochemistry in normal A, and primary ovarian tumors B. Magnification 40x, inset 63x. The magnification areas are highlighted in the square. C. Quantitative analysis of PMEPA expression in normal, primary tumor and metastasis/relapse ovarian samples. The significance of the differences observed between the different groups was determined with a Chi-Square test $P < 0.0001$.

thin layer preparations (Suppl. Figure S5). Interestingly, SKOV3 cells had a higher proliferative rate than SKOV3EV cells (Figure 3A). Similar results were obtained in PMEPA1 overexpressing A2780 and TOV112D (Figure 3B). PCNA protein levels increased accordingly, in agreement to this increased proliferation rate (Figure 4B). However, no difference in growth was observed in OVCAR8 cells (not shown). In contrast, proliferation rates of PMEPA1 knockdown cells were much lower compared to scrambled (SCR) control cells (Figure 3A). Anchorage independent survival revealed that PMEPA1 overexpressing cells survived better and formed bigger clusters that grew faster than EV cells (Figure 3C and D).

PMEPA1 overexpression favors a partially epithelial phenotype

SKOV3 ovarian cells have been classified as intermediate mesenchymal phenotype cells [33], in agreement with the fibroblastlike morphology the cells have in culture, and TOV112D and A2780 as mesenchymal whereas OVCAR8 cells are classified as epithelial. Once SKOV3 cells were transfected with the PMEPA1 expression vector, morphological changes began to be noticeable towards a more epithelial phenotype (Figure 4A). We observed similar changes to an epithelial phenotype in A2780 and TOV112D upon PMEPA1 transfection while but no changes in transfected already epithelial OVCAR8 cells. The intercellular junction

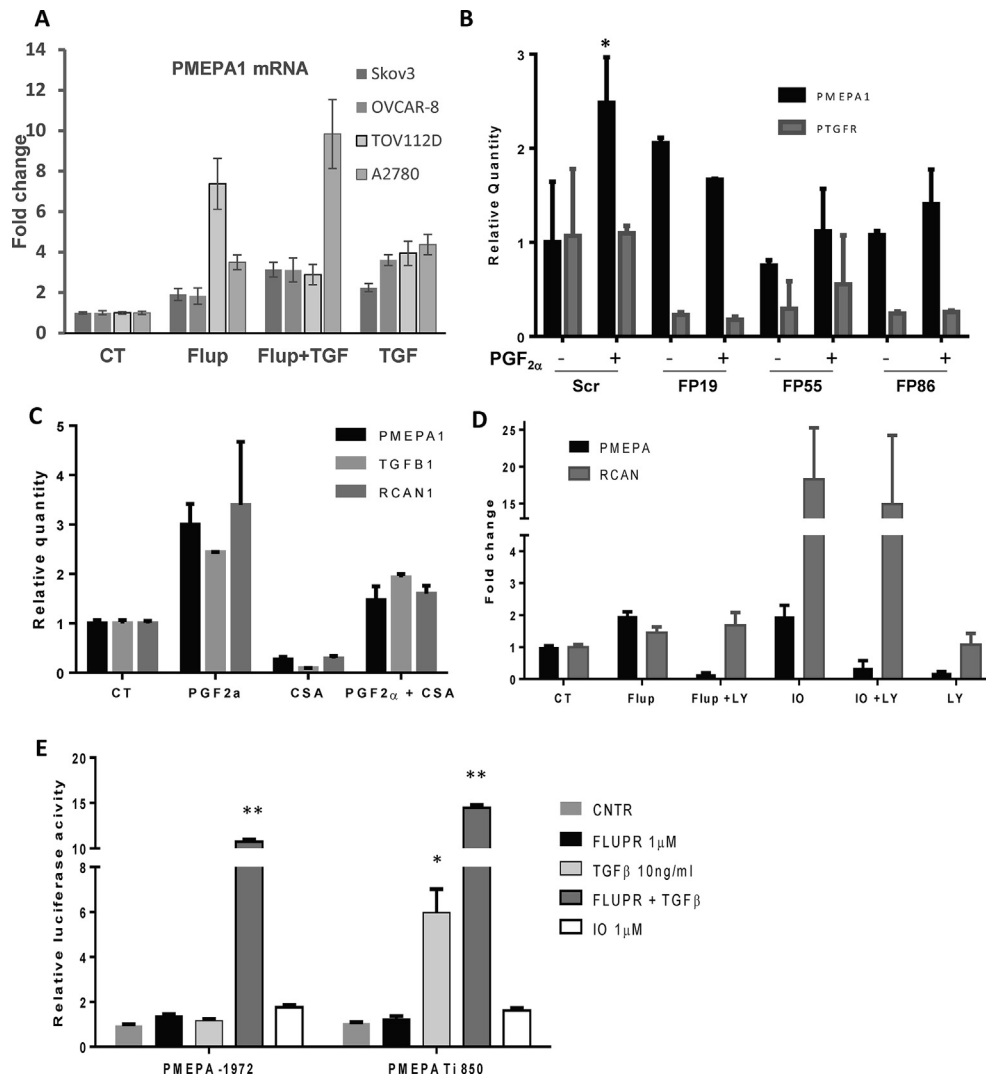


Figure 2. PMEPA1 is upregulated by cooperative action of TGF and $PGF_{2\alpha}$. **A.** PMEPA1 mRNA levels quantification in the cells indicated after 24 h of treatment with vehicle (CT), 1 M fluprostenol (flup), 5 ng/ml TGF, and the combination of the two (Flup + TGF). **B.** PMEPA1 and PTGFR mRNA levels quantification in SKOV3 derived cell lines stably expressing a scrambled shRNA (scr) or three PTGFR specific shRNAs (FP19, FP55, FP86) after 24 h of treatment with vehicle (CT) or 1 M $PGF_{2\alpha}$. Only scr cells were able to significantly increase (*: $p < 0.05$) PMEPA1 mRNA with $PGF_{2\alpha}$ treatment. **C.** PMEPA1, TGFB1 and RCAN1 mRNA levels quantification in SKOV3 cells after 24 h of treatment with vehicle (CT), 1 M $PGF_{2\alpha}$, 100 ng/ml cyclosporine A (CSA) or combination of the two. CSA significantly reduced the levels of the three genes below the control levels ($p < 0.05$). $PGF_{2\alpha}$ significantly increased the three genes levels, while the combination of the two reduced the levels of the three genes, although in the case of TGFB1, the levels were significantly different from CT as well as from $PGF_{2\alpha}$. **D.** PMEPA1 and RCAN1 mRNA levels quantification in SKOV3 cells after 24 h of treatment with vehicle (CT), 1 M Flup, 1 mM A23187 Ca^{2+} ionophore (IO), 10 M LY2109761 (LY) or combinations. Both genes are induced by Flup or IO ($p < 0.05$). LY treatment only affects PMEPA1 levels reducing them below CT levels, even in combined treatments ($p < 0.05$). **E.** Luciferase reporter assays with SKOV3 cells transfected with the mentioned reporter constructs of the PMEPA1 promoter, and treated as shown for 24. TGF- treatment only activated the PMEPA1 Ti 850 reporter ($p < 0.05$), while the combination of Flup and TGF- activated both reporters ($p < 0.001$). IO or Flup alone did not have any activating effect.

Ecadherin expression was higher in PMEPA1 overexpressing cells (Figure 4B). This is in agreement with our finding that most ovarian tumors tested express both PMEPA1 and Ecadherin (Figure 1) and supported by the fact that *CDH1* is 40fold more expressed in ovarian tumors as compared to normal ovarian tissue (Suppl. Figure S7).

The interaction between the Ecadherin and the WNT signaling pathway member leads to the retention of catenin in the cell membrane proximity [34]. Thus, we next studied catenin protein subcellular localization in PMEPA1 overexpressing cells. catenin localized both in the cytoplasm and nucleus of SKOV3EV cells, while in PMEPA1 overexpressing cells it

had a perinuclear localization as well as in cellcell contacts, where Ecadherin is localized (Figure 4C, D). As expected, TGF treatment induced translocation of catenin to the nucleus in SKOV3 cells, while this effect was absent when PMEPA1 was overexpressed (Figure 4C, D). Both the mesenchymal phenotype and catenin dissociation from the membrane of SKOV3 cells could be due to autocrine or paracrine TGF signaling, since a TGF blocking antibody (Figure 4C) or the LY2109761 TGFRI inhibitor (not shown) also caused catenin membrane localization and a change to epitheliallike morphology. Surprisingly, Ecadherin expression was not affected by TGF treatment in PMEPA1 cells (Figure 4D). On

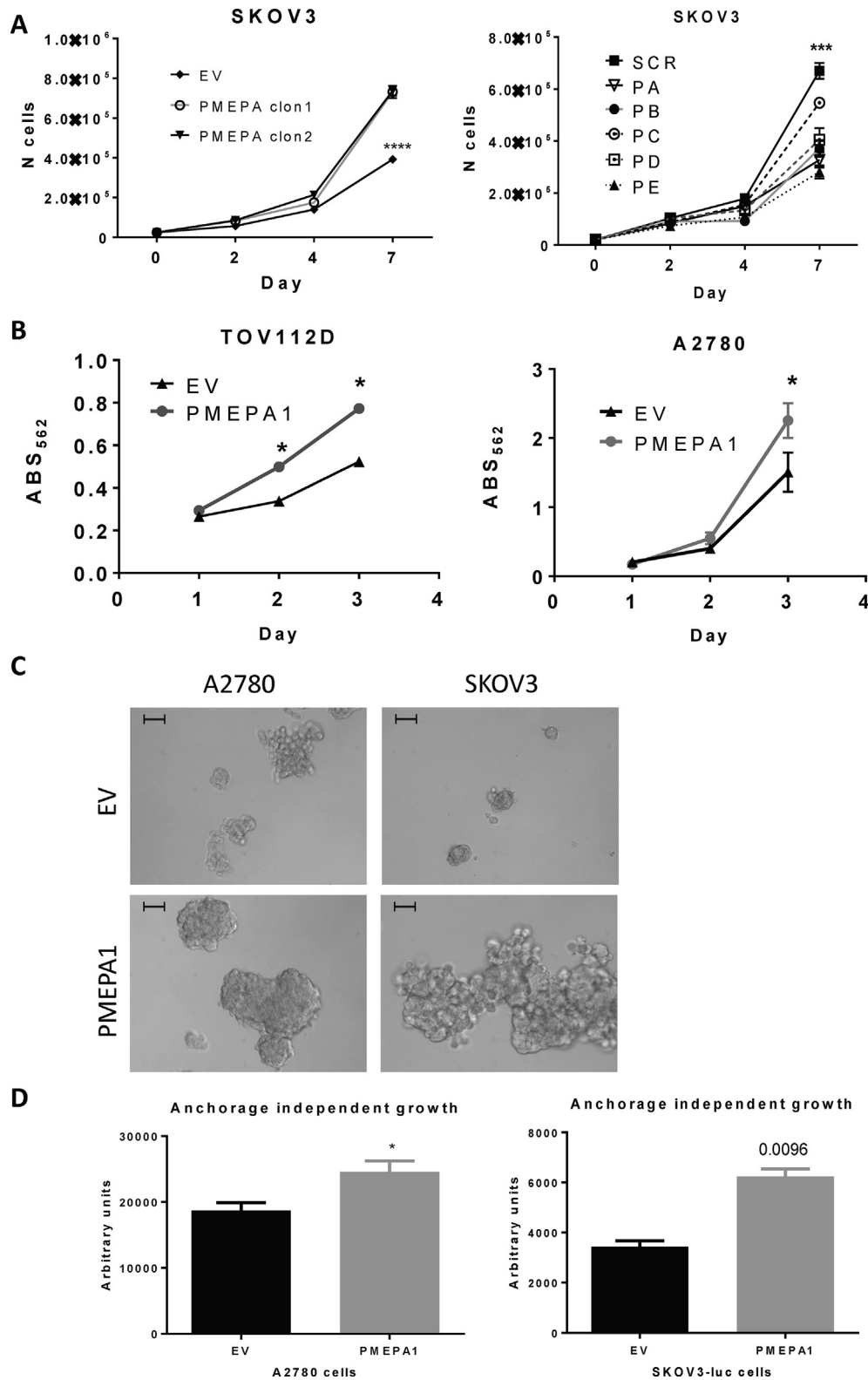


Figure 3. PMEPA1 overexpression in ovarian cancer cells enhances cell proliferation. A. Cell proliferation of 2 PMEPA1 overexpressing clonal SKOV3 cell lines (*left panel*) and 5 knockdown (*right panel*) SKOV3 cell lines was estimated by cell counting using a hemocytometer. Average SEM are shown, $n = 5$. Growth curves were significantly different, $p = 0.025$ for overexpressing vs control and $P < 0.001$ for knockdown vs SCR. These results were also confirmed by crystal violet stain quantification of SKOV3 (not shown), A2780 and TOV112D EV and PMEPA1 cells (B). C. Anchorage independent growth assays were performed with PMEPA1 overexpressing and control A2780 and SKOV3 cells that were monitored by light microscopy. Representative photos of the cells at 72 h after seeding are shown. Bars: 50 μ m. D. Cell survival and proliferation in the assays in (C) was estimated at 96 h after plating by Alamar blue assay. Stain reduction quantification is shown for A2780 and Skov3Luc. Average SEM are shown, $n = 3$.

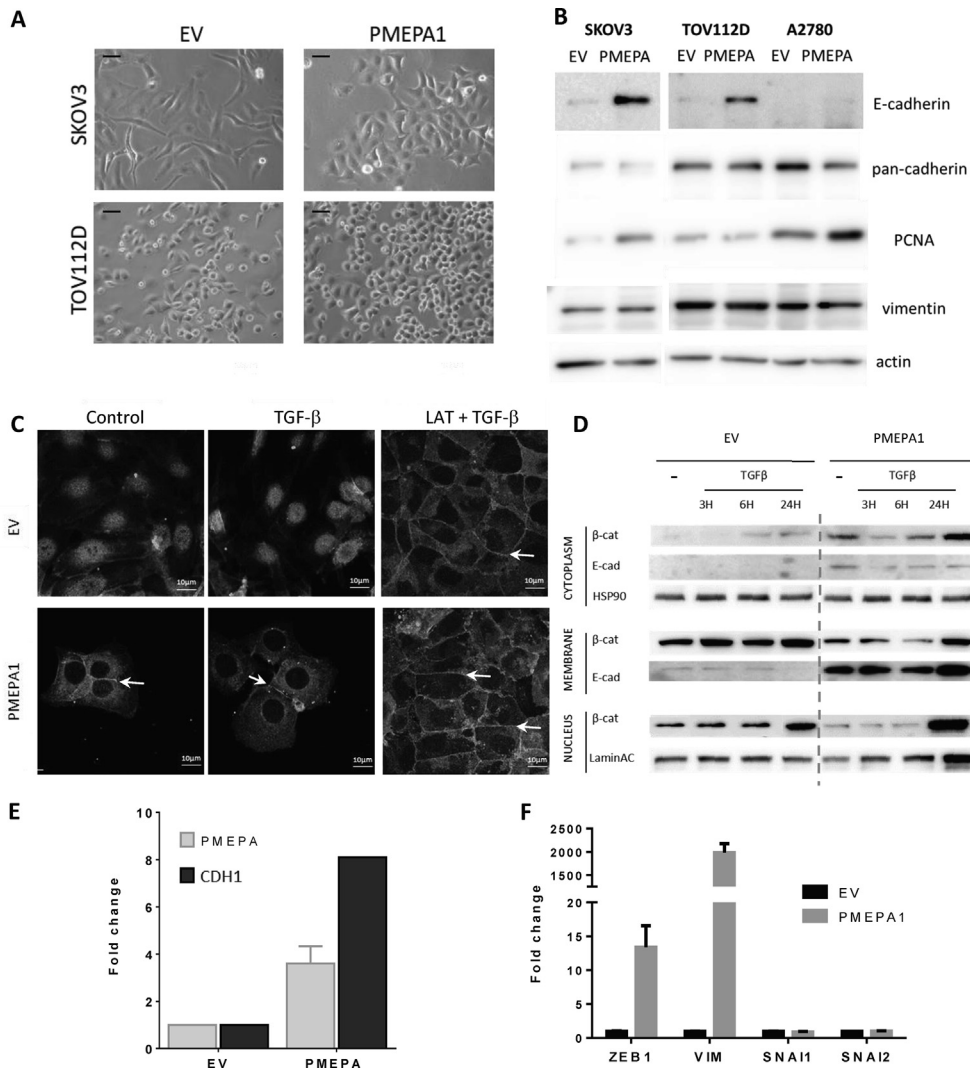


Figure 4. PMEPA1 overexpression favors a partially epithelial phenotype. A. Light microscopy images of SKOV3 and TOV112D -EV and PMEPA1 cells in culture. Bars: 50 μ m. B. WB for the indicated proteins in SKOV3, A2780 and TOV112D -PMEPA1 and -EV cells. C. Immunofluorescence for -catenin of SKOV3-PMEPA1 and SKOV3-EV (EV) cells. Cells were treated with 5 ng/ml of TGF- or combination of 10 ng/ml of recombinant Latency Associated Peptide (LAT-TGF-) and 5 ng/ml of TGF- for 24 h. Arrows indicate -catenin localization in cell-cell contacts. D. WB to detect the indicated proteins in nuclear, membrane and cytoplasmic extracts of the SKOV3-lucD6 EV and SKOV3-PMEPA1 cells. E. qRT-PCR analysis of relative mRNA levels of *PMEPA1* and *CDH1* (E-cadherin) in SKOV3-EV and PMEPA1 overexpressing cells. F. qRT-PCR analysis of relative mRNA levels of *ZEB1*, *VIM*, *SNAI1* and *SNAI2* (coding for Zinc Finger E-Box Binding Homeobox 1, vimentin, SNAIL and SLUG respectively) in SKOV3-EV and PMEPA1 overexpressing cells.

the other hand, PMEPA1 overexpression caused an 8fold induction in SKOV3 cells. This fact was not contrary to the strong induction of *ZEB1* and Vimentin mRNA in PMEPA1 overexpressing cells, while *SNAI1* and 2 mRNA levels do not vary. In the same sense, we found that PMEPA1 mRNA levels positively correlated with the levels of two ovarian cancer cell EMT signatures as calculated using the TCGA Ovarian Cancer data (Suppl. Figure 9). These results indicate that PMEPA1 overexpression can revert downregulation of Ecadherin in ovarian cancer cells and decrease nuclear catenin by TGF.

The relationship between PMEPA1, Ecadherin and catenin, is also supported by the finding that biopsies with high PMEPA1 levels, also express Ecadherin and catenin. catenin was in most cases detected in the plasma membrane and not nuclear (Figure 1). These results support *in vitro* experiments, indicating that PMEPA1 high expression coincides with Ecadherin expression and blocks catenin nuclear translocation in ovary patients tumors.

PMEPA1 overexpression affects the TGF- signaling pathway

Increased phosphorylation of SMAD1/5/8 and SMAD2/3 was observed in PMEPA1 overexpressing unstimulated cells while the fold increase in SMAD phosphorylation after TGF treatment was less pronounced in the case of SKOV3PMEPA1 cells (Figure 5A). On the other hand, we detected high levels of SMAD2/3 and PSMAD2/3 proteins in SKOV3shPMEPA1 compared to SCR and the opposite situation in the case of pSMAD1/5/8, observing lower phosphorylation levels in knock-down cells as compared to SCR (Figure 5B). For quantifications of the blots please see Suppl. Figure S8). Interestingly, reduced levels of SMAD2/3 in nuclear extracts were found in SKOV3PMEPA1 cells, while the membrane bound SMAD2/3 increased (Figure 5C). Besides, we tested if PMEPA1 affected SMADdependent transcription of TGFtarget genes. PMEPA1 overexpression decreased *EDN1* and *CDKN1A* mRNA levels (Figure 5D), while its knockdown showed a tendency to increase SER-

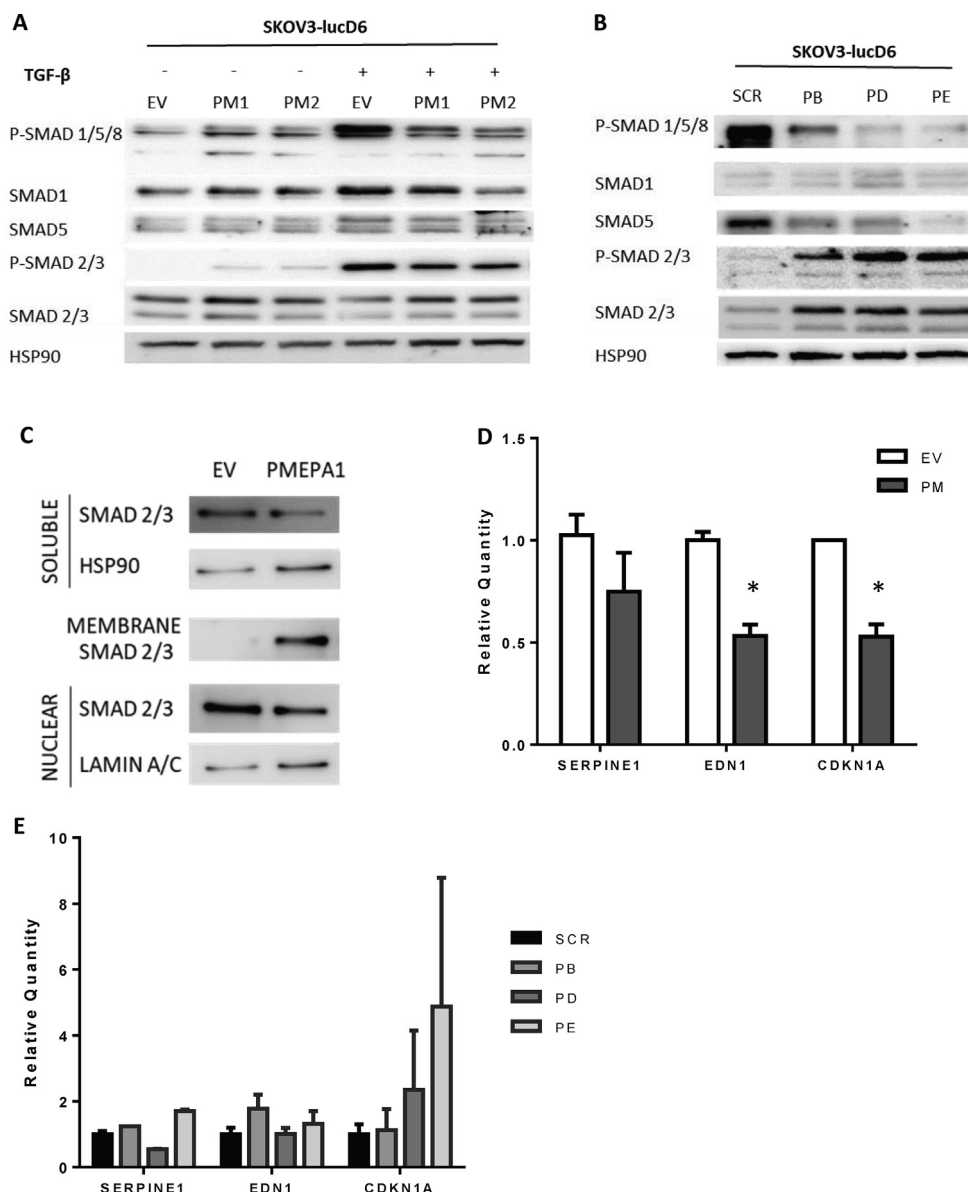


Figure 5. PMEPA1 overexpression affects the TGF signaling pathway. SMAD1/5/8 and SMAD2/3 phosphorylation state as detected by WB in overexpressing (A) and knockdown (B) SKOV3 cells and their appropriated controls treated or not with 5 ng/ml TGF- for 1 h. C. WB for SMAD2/3 protein levels in SKOV3-EV and -PMEPA1 cells nuclear, membrane and soluble extracts. Relative SERPINE1, EDN1 and CDKN1A (coding for PAI-1, Endothelin and p21^{waf1} respectively) mRNA levels in PMEPA1 overexpressing (D) and knockdown (E) SKOV3 cells and their corresponding controls. *: $p < 0.05$.

PINE1, EDN1 and CDKN1A (Figure 5E). These results indicate that PMEPA1 can modulate the TGF pathway output in ovarian cancer cells.

PMEPA1 promotes tumor growth *in vivo*

Finally, to confirm that PMEPA1 promotes ovarian tumor growth, we tested PMEPA1 overexpressing or knockdown cell lines growth in mice (Figure 6). We used several xenograft mouse models to address different aspects of ovarian cancer growth and metastasis. In the case of subcutaneous xenografts, SKOV3PMEPA1 tumor volume growth was much higher compared to SKOV3EV derived tumors that only became palpable around day 55 (Figure 6A). Monitoring tumor bioluminescence we found a growth advantage of SKOV3PMEPA1 over SKOV3EV xenografts from the beginning of the experiment (Figure 6B). At the end of the experi-

ment, we extracted tumors to corroborate the mentioned results (Suppl. Figure 10). SKOV3EV derived tumors were smaller; in some cases, we were not able to isolate a tumor mass, although we could detect bioluminescent signal. We confirmed these results, performing a similar experiment, of subcutaneous injection of TOV112D and A2780 control and PMEPA1 overexpressing cells, in the more permissive R2G2 mice. In the case of TOV112D cells, PMEPA1 overexpression duplicated tumor initiation rates comparing to EV cells, PMEPA1 tumors grew faster, reaching maximum allowed size at least a month earlier than the EV ones (Suppl. Figure 11). On the other hand, only A2780PMEPA1 cells, but not the EV, presented detectable growing tumors during a 15week monitoring period (Suppl. Figure 11).

To investigate the effects of PMEPA1 on tumor cell peritoneal dissemination, we injected intraperitoneally two SKOV3PMEPA1 cell lines and

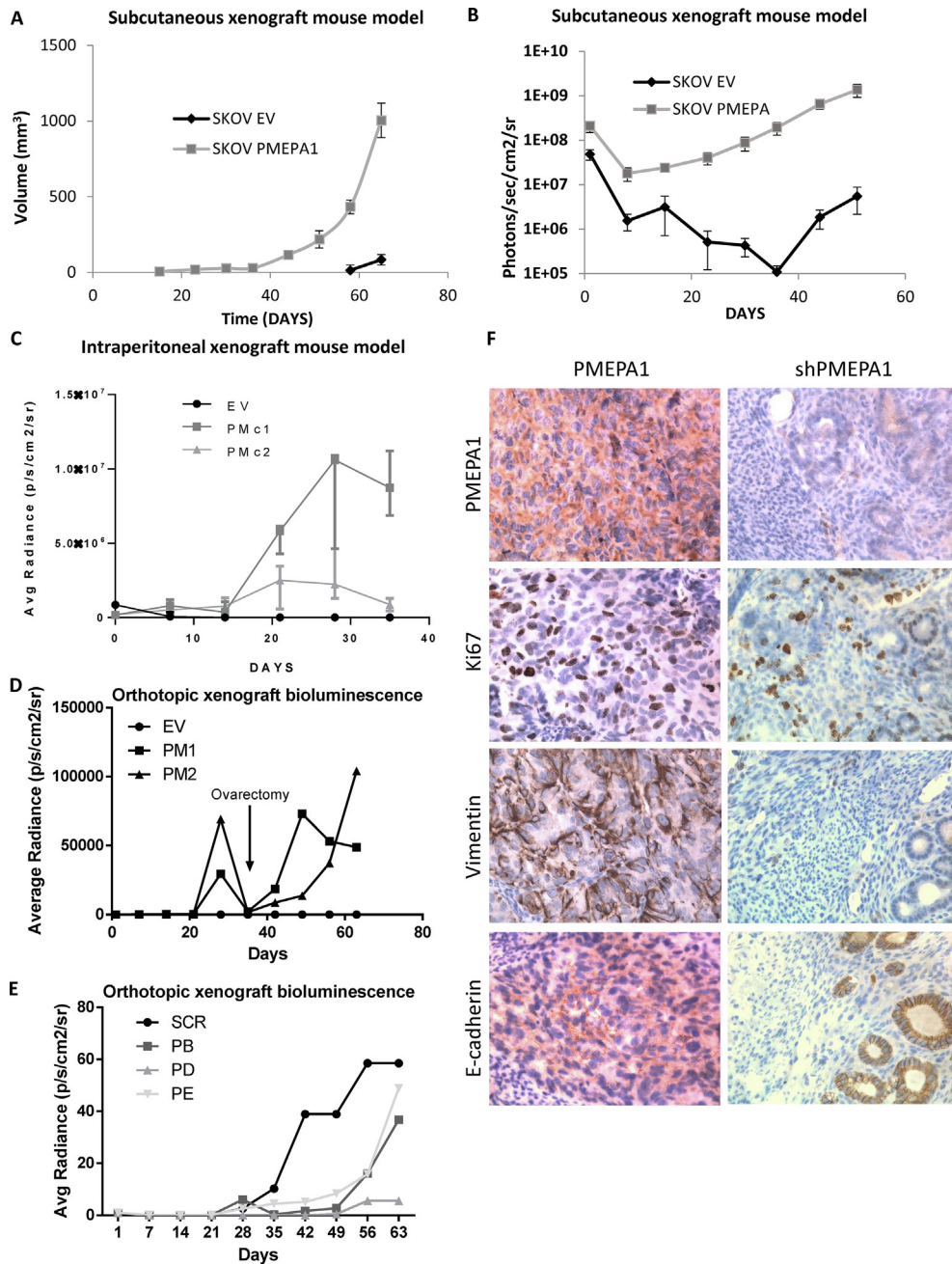


Figure 6. PMEPA1 promotes tumor growth *in vivo*. A. Subcutaneous xenograft Skov3Luc-EV and -PMEPA1 tumor volume, measured once a week, using a digital caliper. EV derived tumors became palpable around the 8th week post inoculation. B. Bioluminescence Imaging (BLI) quantification of the xenografts in (A). C. Growth of intraperitoneal injected cells was measure by bioluminescence quantification with IVIS Lumina Imaging system. Orthotopic tumor xenografts growth estimation through bioluminescence quantification of PMEPA1 overexpressing (D) and knockdown (E) SKOV3Luc derived cell lines. Tumors were removed at 35 dpi, but BLI monitorization continued until day 63 in both experiments. F. IHC of tumors derived from *PMEPA1* overexpressing or knockdown SKOV3 cells, stained for the indicated proteins. Magnification x400. B,C,D: Results are represented as logarithmic scale of Photons/sec/cm²/sr means SEM.

SKOV3EV cells. SKOV3PMEPA1 xenografts had an important proliferative advantage, even more remarkable than in the subcutaneous experiments (Figure 6C). To search for internal organ invasion, we extracted some organs (stomach, intestine, spleen, kidneys and pancreas) and peritoneal membrane. In mice that harbored SKOV3PMEPA1, bioluminescent signal could be detected in several internal organs, while in SKOV3EV mice, all organs were negative (Suppl. Figure S9). Besides, peritoneal walls of SKOV3PMEPA1 xenografts show localized lumines-

cence signals corresponding to established metastases (Suppl. Figure S12). These results suggest that intraperitoneal dissemination and metastasis is facilitated by PMEPA1 overexpression.

Finally, to recapitulate all the steps of ovarian cancer growth, dissemination and metastasis, cells were injected orthotopically into the ovary. SKOV3PMEPA1 xenografts showed a strong growth advantage compared with SKOV3EV, which could hardly form intraovary tumors (Figure 6D). More importantly, shPMEPA1 SKOV3 grew less, reaching lower BLI sig-

nal levels than SCR and much less than SKOV3PMEPA1 xenografts (Figure 6E) indicating that even the low PMEPA1 levels of control cells are important for tumor growth. Thus, the difference between overexpressing and silencing PMEPA1 in SKOV3 cells resulted in about 20,000 fold in growth as measured by bioluminescence intensity.

To investigate the potential for these cells to develop metastasis, ovaries were removed on day 35 (red arrow) and mice were monitored for bioluminescence, indicating metastatic tumor growth. A week after the operation, bioluminescence could be detected again, indicating cells had already metastasized (Figure 6E). Thus, PMEPA1 gives a clear advantage to ovarian tumor cells to adapt and grow in all the tested conditions. These *in vivo* results and the PMEPA1 expression in patient highgrade tumors strongly suggest that PMEPA1 could be an accurate biomarker of prognosis in ovarian cancer.

Discussion

PGF_{2 α} has only recently been associated with tumor progression in colorectal [5,35] and endometrial cancer [36]. Since it is naturally produced in the ovary and capable to produce strong effects on the epithelium [37] it would be logical to assume that it may have an important role also in ovarian cancer. Remarkably, although *PTGFR* levels are lower in ovarian tumors than in normal ovary, ovarian cancer patients with higher *PTGFR* mRNA levels exhibit lower survival probabilities than patients with lower *PTGFR* levels (Suppl. Figure S13). These findings make *PTGFR* a potentially interesting pharmacological target against ovarian cancer dissemination and metastasis.

The TGF superfamily plays an important role in ovarian function and pathogenesis [38] while, remarkably, mutations in genes of this pathway are infrequent in ovarian cancer [39]. Importantly, we observed *TGFBI* upregulation in ovarian cancer. Others and we have demonstrated the importance of TGF in ovarian cancer cell dissemination and metastasis in the peritoneal cavity [29,40], the primary metastatic site of this type of cancer [41]. We first describe here the induction of *TGFBI* by PGF_{2 α} in cancer cells and our observation supports one earlier publication observing this induction in healthy bovine *corpus luteum* [42]. Not surprisingly, we found a strong correlation between the expression of *TGFBI* and all the components of the COX2PGF_{2 α} PTGFRNFAT pathway tested. We are the first to report the association of Ca²⁺/Calcineurin/NFAT signaling with the transcriptional control of *TGFBI*. We thus offer data that show the convergence between two pathways of great importance in cancer progression. COX2 products, such as PGF_{2 α} , are able to induce *TGFBI* transcription. This could further contribute to explain many of the effects of COX2 and PGs in the tumor setting, such as EMT [43], metastasis and immune evasion [44].

As mentioned, ovarian tumors rarely acquire mutations in the TGF pathway, although this cytokine is upregulated in tumors over normal tissue. PMEPA1, already proposed to be a molecular switch that converts TGF, normally a tumor suppressor, to a tumor promoter [12] could be the responsible for this inconsistency. Indeed, we found PMEPA1 elevated in most ovarian tumors, its expression correlating with that of *TGFBI*. Remarkably, PMEPA1 is induced also by PGF_{2 α} /NFAT axis, as by TGF, reaching a synergistic effect when activating both pathways. PMEPA1 overexpression increased tumor cell growth both *in vitro* and *in vivo*, as we demonstrate using different cell lines and *in vivo* tumor models, while its knockdown had the opposite effect. Thus, silencing PMEPA1 resulted in reduced tumor growth *in vivo*. Our results also suggest that intraperitoneal dissemination and metastasis is strongly reduced by PMEPA1 knockdown. Given the fact that intracellular TGF signaling moderately decreased, switching from SMAD2/3 to SMAD1/5/8, we believe that PMEPA1 expressing cells are able to affect the tumor stroma without suffering the negative effects TGF could induce. Our data on the

protumoral effect of PMEPA1 in ovarian cancer are also supported by the fact that high PMEPA1 mRNA levels are associated with lower survival rate of ovarian cancer patients.

It has been proposed that mutations or loss of p53 modify TGF action in ovarian cancer [45], although this seems to be independent to the effect observed with PMEPA1, since this was found in both p53 wt (A2780) and null cell lines (SKOV3).

PMEPA1 overexpression altered cell morphology, prompting a more epithelial phenotype, although TGF production by the cells is increased. This altered morphology could be due to the upregulation of Ecadherin we observed. Indeed, PMEPA1 was able to elevate functional Ecadherin levels and thus concentrate part of the catenin in the intercellular junctions, while other epithelial markers failed to be induced. We demonstrate that catenin remains in a cytoplasmic and/or membrane localization in a PMEPA1 dependent manner, in agreement with a very recent publication by Amalia et al., showing how PMEPA1 inhibited Wnt signaling through catenin stability and nuclear localization regulation [46]. Ecadherin can be degraded or downregulated upon different stimuli, as TGF [47], and thus lose association with catenin, that could translocate to the nucleus. SKOV3EV cells show catenin nuclear localization probably due to basal autocrine TGF signaling [29] and absence of membrane Ecadherin. The effect of PMEPA may be explained, not only by a decrease in the suppression of *CDH1* by TGF, but potentially a decrease of Ecadherin endocytosis and degradation. This is also supported by the fact that we found the same epithelial morphology and catenin cytoplasmic localization when we treated cells with a latencyassociatedpeptide, inhibiting TGF or using a TGF inhibitor. Another plausible explanation to this phenomenon could be the fact that catenin can depend on SMAD3 to translocate to the nucleus [48]. Indeed, we also found that the overexpression of PMEPA1 reduced nuclear SMAD2/3, as already described in other cell systems [11,12,24].

It has been already shown that Ecadherin expression can have a positive effect on tumor aggressiveness and metastasis [49], which would perfectly agree with our results. Moreover, we observed that PMEPA1 overexpressing cells, not only had elevated growth capacity on substrate, but also anchorage independent growth, in accordance with previous reports attributing this role to Ecadherin [50].

It is well established that Ecadherin expression and decreased cell mobility are common epithelial cell characteristics, while upregulation of Ncadherin, vimentin and zincfinger domain proteins (SNAI1/SNAI2, SNAI2/SLUG), among others, are often linked to a mesenchymal phenotype [51]. A notable case is the ovarian surface epithelial (OSE) cells, in which overexpression of Ecadherin induces a number of epithelial characteristics and markers associated with malignant transformation and tumor progression [52]. Remarkably, both primary and metastatic ovarian carcinomas express Ecadherin, in contrast to normal ovarian surface epithelium, which rarely expresses Ecadherin [53,54]. Further work should be done to elucidate the detailed mechanisms through which PMEPA1 upregulates or avoids downregulation of Ecadherin and if these depend exclusively on TGF signaling. We believe this Ecadherin elevation is not due to the lack of *CDH1* suppression, since we were only able to overexpress Ecadherin in PMEPA1 overexpressing but not in EV cells (data not shown). On the other hand, although elevated Ecadherin expression was observed because of PMEPA1 expression, cells also induced Vimentin and ZEB1 at the same time. Moreover, *in silico* analysis of the TCGA Ovarian cancer cohort showed that PMEPA1 mRNA levels correlate both with *CDH1* levels as well as with EMT gene signatures scores. This indicates that PMEPA1 overexpressing cells could take advantage of both epithelial and mesenchymal characteristics, which could be an explanation of the aggressiveness of these cells. Interestingly, PMEPA1 effects on growth can be observed only in ovarian carcinoma cell lines with mesenchymal or intermediate mesenchymal phenotype but not in OVCAR8

cells that are classified as epithelial. This supports idea that the effects of PMEPA1 in favoring an epithelial phenotype are linked to those on growth advantages.

Using a highgrade ovarian tumor patient cohort, we found strong PMEPA1 expression in most tumors, perfectly correlating with Ecadherin and catenin high expression. Bigger cohorts, including lower grade primary tumor biopsies should be used to confirm if PMEPA1 is linked to metastasis and its prognostic value. All the above could indicate that PMEPA1 can tweak TGF signaling while making tumor cells produce more, upregulate Ecadherin protein levels and reduce catenin nuclear localization, thus increasing cell plasticity and proliferation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neo.2019.10.001>.

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