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SPARC Ameliorates Ovarian Cancer–Associated Inflammation[1]

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Abstract

We have recently identified that the role of secreted protein acidic and rich in cysteine (SPARC) in amelioration of peritoneal ovarian carcinomatosis is mediated, at least in part, through mesothelial cell/lysophosphatidic acid–induced inflammatory response in ovarian cancer cells. The aim of this study was to elucidate the molecular mechanisms of the interactions between tumor cells and the cellular components of the ovarian cancer peritoneal microenvironment, specifically, mesothelial cells and macrophages. We found that SPARC not only significantly reduced macrophage chemoattractant protein-1 production and its macrophage chemotactic effect, but also attenuated the response of ovarian cancer cells to the mitogenic and proinvasive effects of macrophage chemoattractant protein-1 and decreased macrophage-induced cancer cell invasiveness. Overexpression of SPARC in ovarian cancer cells significantly attenuated macrophage- and mesothelial cell–induced production and activity of interleukin-6, prostanoids (prostaglandins E2 and 8-isoprostanes) as well as matrix metalloproteinases and urokinase plasminogen activator. Moreover, the effects of SPARC overexpression in ovarian cancer cells were mediated, in part, through inhibition of nuclear factor-κB promoter activation. These results indicate, for the first time, that the effects of tumor SPARC as a negative regulator of ovarian cancer are mediated through decreased recruitment of macrophages and downregulation of the associated inflammation.

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Introduction

The importance of reactive tumor microenvironment was emphasized as early as 1889 by Paget's "Seed and Soil" explanation of preferential tumor metastasis [1]. In peritoneal ovarian carcinomatosis, peritoneal mesothelial cells provide soil for shed ovarian cancer cells, whereas ascitic fluid represents the reactive tumor microenvironment that provides an efficient exchange of soluble factors among cancer, mesothelial, and inflammatory cells to support tumor growth and invasion [2]. The positive correlation between the density of tumor-associated and tumor-infiltrating macrophages (TAM and TIM) and the poor prognosis of ovarian cancer has long been established [3,4]. Macrophages are recruited to the ovarian tumors by a tumor-derived chemotactic factor, macrophage chemoattractant protein-1 (MCP-1) [5], which in turn directly stimulates cancer cell growth and induces angiogenesis [6–8]. MCP-1, together with other inflammatory cytokines and growth factors, is produced by ovarian cancer cells stimulated by lysophosphatidic acid (LPA), which is constitutively produced by peritoneal mesothelial cells [9,10]. LPA has been reported to activate macrophages and cancer cells through multiple pathways that ultimately lead to upregulation of vascular endothelial growth factor (VEGF), urokinase plasminogen activator (uPA), matrix metalloproteinases (MMPs), interleukin (IL)-6, and prostaglandin E (PGEs) [8,11,12]. These high levels of VEGF, proteases, and PGE2 in turn promote monocyte recruitment and favor their differentiation into macrophages, rather than antigen processing dendritic cells [6,9,13,14].

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SPARC (secreted protein acidic and rich in cysteine) is a matricellular glycoprotein that has been implicated in modulating tumor-host interactions. The role of SPARC in the pathogenesis of different tumors appears to be highly contextual and attributed to whether this protein is produced by tumor or by neighboring stromal cells and to the concerted effect of the protein on these cells [15–17]. High expression of SPARC in tumor cells has been correlated with enhanced tumor growth, metastasis, and poor survival in various malignancies [18–22]. Stromal, or host-derived SPARC, has been reported to exert a dual function on tumor growth. A positive effect of stromal SPARC on the organization of the extracellular matrix (ECM) and inhibition of angiogenesis has been shown to limit tumor growth [15,23]. Conversely, high levels of stromal expression of SPARC in tumor cells with epigenetic inactivation of *Sparc* promoter have been positively correlated with poor prognosis in various cancers [24–28] or may establish differential interactions with the surrounding stroma by recruiting immune cells and favoring proteolysis of stromal proteins, resulting in either promotion or regression of tumor growth [29].

The results of *in vitro* and *in vivo* studies have identified SPARC as a novel ovarian cancer suppressor that functions primarily by virtue of its de-adhesive ability [30] and antiproliferative and proapoptotic effects [30–32]. It has also been reported that SPARC expression in ovarian cancer cells is inversely correlated with the degree of malignancy [31–33]. In agreement with these findings, SPARC promoter has been found to be hypermethylated in human ovarian cancer cell lines and tissues, leading to loss of its production and secretion (Socha and Motamed, unpublished data). We have also reported the ability of SPARC to modulate ovarian cancer cell interaction with the ECM components, the production and the activity of specific growth factors, cytokines, proteases, and bioactive lipids [10,34]. Moreover, we found that in the immunocompetent $SP^{-/-}$ mice, the enhanced peritoneal ovarian carcinomatosis was concomitant with massive recruitment of macrophages and was positively correlated with the augmented levels and biological activity of ascitic fluid and its constituents, namely, VEGF, MMPs, MCP-1, IL-6, prostanoids, and bioactive lipids [34]. The latter results suggested that host SPARC suppresses peritoneal ovarian cancer through a negative effect on the interplay between ovarian cancer cells, mesothelial cells, and macrophages.

To assess the role of tumor SPARC in modulating the ovarian cancer microenvironment, we used *in vitro* systems to dissect the molecular mechanisms of the interactions between human ovarian cancer cells, mesothelial cells, and macrophages. Our results strongly indicate that restoration of SPARC expression in ovarian cancer cells disrupts the interplay between these three key players, resulting in significantly attenuated macrophage recruitment and expression of known markers of inflammation.

Materials and Methods

Cell Lines and Reagents

Human ovarian carcinoma cell lines (SKOV3 and OVCAR3) and human peritoneal mesothelial cell line (Meso301) were obtained and maintained as described previously [10,30]. Human monocytoid cell line (U937) was purchased from ATCC (Manassas, VA) and was maintained in RPMI 1640 containing 10% FBS (Atlanta Biologicals, Norcross, GA). Bovine and human osteonectin were purchased from Haematologic Technologies (Essex, VT). Reduced growth factor Matrigel was from BD Biosciences (Bedford, MA). PGE2 was from Cayman Inc. (Ann Arbor, MI). Recombinant human MCP-1 was from Peprotech (Rocky Hill, NJ). Rabbit polyclonal antibodies against MCP-1 were purchased from Chemicon (Temicula, CA). Unless otherwise stated, all other chemicals and culture media were purchased from Sigma (St. Louis, MO) and Fisher Scientific (Fairlawn, NJ).

Adenoviruses and Plasmids

Replication-deficient adenoviruses expressing either SPARC or green fluorescent protein (GFP) under the control of the cytomegalovirus promoter were generated as described previously [10]. For preparation of SPARC plasmid (pSPARC), human SPARC open reading frame under the control of the cytomegalovirus promoter was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) using the TOPO sites. Plasmids of nuclear factor (NF)-κB that contained two copies of the wild-type NF-κB (WT-pNF-κB-Luc) or the mutated NF-κB (Mut-pNF-κB-Luc) binding sites ligated to luciferase reporters were kindly provided by Dr. Jinsong Liu (The University of Texas M. D. Anderson Cancer Center, Houston, TX). uPA promoter plasmid was kindly provided by Dr. Shuang Huang (Medical College of Georgia, Augusta, GA).

For adenoviral gene transfer, ovarian cancer cell lines were transduced for 24 hours with adenovirus gene encoding GFP (Ad-GFP) and GFP and SPARC (Ad-GFP-SPARC) in complete growth medium at a multiplicity of infection of 15 to 25 as described previously [10]. SPARC protein was detected in cell lysates and conditioned medium of SKOV3 and OVCAR3 ovarian cancer cell lines, after Ad-GFP-SPARC transduction, but not GFP-transduced controls. Cells were then serum-starved for 18 to 24 hours before stimulation with LPA (10 to 50 μM), MCP-1 (1 to 50 ng/ml), PGE2 (0.5 to 20 nM) or use in coculture experiments.

Microinvasion Assays

Invasion assays were performed as described previously [30]. Harvested cells were added to the upper chamber of polycarbonate inserts (8-μm pore size, Corning Costar, Corning, NY) coated on ice with Matrigel diluted 1:3 in serum-free medium (SFM). To test the effect of ovarian cancer cell invasiveness, SKOV3 and OVCAR3 cells, transduced or not with Ad-GFP and Ad-GFP-SPARC, were seeded into the upper well of the chamber. To test the effect of macrophages on ovarian cancer cell invasion, invasion assays were carried out in the presence of U937 macrophages seeded in the lower chambers. The concentrations of U937 macrophages used in the present study recapitulated those reported in patients with ovarian cancer and were consistent with previously reported *in vitro* studies [35,36]. Assays were carried out for 6 hours at 37°C in a 5% CO_2 humidified incubator. The migrated cells were counted in six fields after staining with hemacolor 3 stain, using an inverted microscope equipped with a DFC 320 digital camera (Leica Microsystems, Wetzlar, Germany) under 200× magnification. Macrophage chemotaxis assays were performed using 3-μm pore-size polycarbonate inserts (Corning Costar). Ovarian cancer cells, grown to 90% confluence in 24-well plates, were transduced with Ad-GFP and Ad-GFP-SPARC as described earlier and were co-incubated with Meso301 and U937 macrophages as described in the figure legends. After incubation at 37°C for 90 minutes, the contents of the upper chamber were aspirated, washed with PBS, and stained with DAPI (Sigma). U937 migration was assessed by counting the number of cells attached to the lower surface of the membrane in six high-power fields per well, using a

fluorescent microscope equipped with a Q-imaging digital camera (Leica Microsystems).

Cell Proliferation Assay

CellTiter^{96} kit (Promega) was used according to the manufacturer's instructions. The number of proliferating cells was determined colorimetrically by measuring the absorbance at 590 nm (A_{590}) of the dissolved formazan product after the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) for 3 hours.

Immunoblotting and Gelatin Zymography

Subconfluent monolayers of ovarian cancer cells, transduced or not with Ad-GFP and Ad-GFP-SPARC, were grown in six-well plates and serum-starved in SFM overnight. Cells were then cocultured with U937 (2×10^6 cells/ml SFM) and added to 0.22-μm transwell chambers (Corning, Costar) without direct cell-cell contact for an additional 24 hours. Cell culture conditioned media (CM) were collected and cleared by centrifugation. Cells in upper and lower chambers were harvested in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% sodium deoxycholate, 1% NP-40, 1 mM Na_3VO_4, and 1× protease inhibitor cocktail mixture). Lysates were cleared by centrifugation at 12,000*g* for 20 minutes at 4°C, and protein concentrations were determined by BCA assay (Pierce). CM were collected and concentrated five-fold using Centricon centrifugal filters (Millipore, Bedford, MA). CM equivalent to 200 μg of ovarian cancer cells, and/or U937 cells and analyzed by gelatin zymography as described previously [30]. Concomitantly, cell lysates (50 μg) of SKOV3, OVCAR3, and/or U937 cells were resolved by 10% SDS-PAGE, transferred onto polyvinylidene fluoride membranes (BioRad, Hercules, CA), and probed with monoclonal antibody against β-tubulin to ensure equal protein loading. Protein detection was carried out using HRP-conjugated secondary antibodies and a SuperSignal West Dura Chemiluminescence kit (Pierce).

Luciferase Assays

Ovarian cancer cell lines (5×10^4/well of 24-well plate) were transfected with 0.5 μg of pSPARC, 0.5 μg of pNF-κB-Luc, 0.5 μg of mutant-pNF-κB-Luc, 1.5 μg of u-PA-Luc promoter, plus 0.2 μg of reference plasmid β-galactosidase as an internal control (pβgal), using Lipofectamine 2000 (Invitrogen) for 24 hours, and then starved for an additional 24 hours. Cells were then stimulated by LPA and PGE2 for 6 hours or cocultured with U937 cells (in 0.22-μm inserts) before lysis (Glo Lysis Buffer, Promega, Madison, WI). Luciferase activity (Luciferase Assay Solution, Bright Glo, Promega) was measured according to the manufacturer's instructions. β-Galactosidase activity (Galacto-Star System, Applied Biosystems, Bedford, MA) was also measured to normalize the luciferase activity. Transfection efficiency of ovarian cancer cells ranged between 60% and 70% (data not shown). The concentration of pSPARC used herein was sufficient to induce SPARC expression in ovarian cancer cells at levels comparable to that of exogenous SPARC concentration used herein, as confirmed by Western blot analysis of SPARC expression in cell lysates and CM (data not shown).

Determination of MCP-1, IL-6, PGE2, and 8-isoprostane

CM of SKOV3, OVCAR3, Meso301, and U937 from the experimental conditions described in the figure legends were collected and stored at −80°C. Commercial ELISA kits were used to determine the concentrations of IL-6 and MCP-1 (RayBioTech. Inc., Norcross, GA). Enzyme immunoassay kits for 8-isoprostane and PGE2 (Cayman Inc.) were used according to the manufacturer's instructions.

Determination of uPA Activity

CM from ovarian cancer cells from different experimental conditions described in the figure legends were collected, centrifuged, stored at −80°C, and assayed within 48 hours. uPA activity was measured by a colorimetric assay kit (Chemicon), according to the supplier's guidelines. The assay is sensitive over a range of 0.05 to 50 units of u-PA activity, which cleaves a chromogenic substrate, a tripeptide with pNA group, forming a colored product with detectable absorbance at 405 nm (A_{405}).

Statistical Analysis

Data were statistically analyzed by Student's *t* test and one-way analysis of variance, using GraphPad Prism version 3.1 (GraphPad Software, San Diego CA). Differences were considered significant at $P < .05$.

Results

Effect of SPARC on Production and Activity of MCP-1 from Ovarian Cancer Cells Cultured with Mesothelial Cells

Using cytokine arrays, we have previously shown that exogenous SPARC inhibits basal and LPA induced MCP-1 production by ovarian cancer cells [10]. *In vivo*, ascitic fluid of $SP^{-/-}$ mice with peritoneal ovarian carcinomatosis had significantly higher levels of MCP-1 concomitant with increased TAM and TIM [34]. In the present study, we show significant time-dependent increase of MCP-1 production by ovarian cancer cell lines SKOV3 and OVCAR3, after LPA stimulation (approximately three- and nine-fold increase after 24 and 48 hours, respectively). Exogenous SPARC, as well as adenoviral expression of SPARC in SKOV3 and OVCAR3 cell lines, significantly inhibited both basal (54% to 64%) and LPA-induced MCP-1 production (54% and 43% inhibition in SKOV3 at 24 and 48 hours, respectively; 59% and 30% inhibition in OVCAR3 at 24 and 48 hours, respectively (Figure 1, *A* and *B*). Coculture of peritoneal mesothelial cells, Meso301, with ovarian cancer cells further augmented MCP-1 production in a time-dependent manner. The increase was 16- to 21-fold with SKOV3 at 24 and 48 hours, respectively, and 14- to 20-fold with OVCAR3 at 24 and 48 hours, respectively. Overexpression of SPARC significantly attenuated the augmented MCP-1 production in the cocultures by ~26% to 36% in SKOV3 at 24 and 48 hours, respectively, and 36% to 28% in OVCAR3 at 24 and 48 hours, respectively. At the functional level, monolayers of SKOV3 and OVCAR3 attracted U937 and stimulated migration toward tumor cells. This chemotactic effect was significantly attenuated in SKOV3 and OVCAR3 cells overexpressing SPARC (47% and 49%, respectively; Figure 1, *C* and *D*). To mimic the *in vivo* human disease in which the adhesion of ovarian cancer cells to mesothelial cells triggers the cascade of peritoneal ovarian carcinomatosis, we used a triple-culture system where ovarian cancer cells, transduced or not with Ad-SPARC, were seeded onto monolayers of mesothelial cells in the bottom chamber of the transwells and tested their chemotactic effect on U937 macrophages placed in the top chamber of the transwells. In this system, coculture of ovarian cancer-mesothelial cells increased chemotactic effect on macrophages by five-fold, relative to that exerted

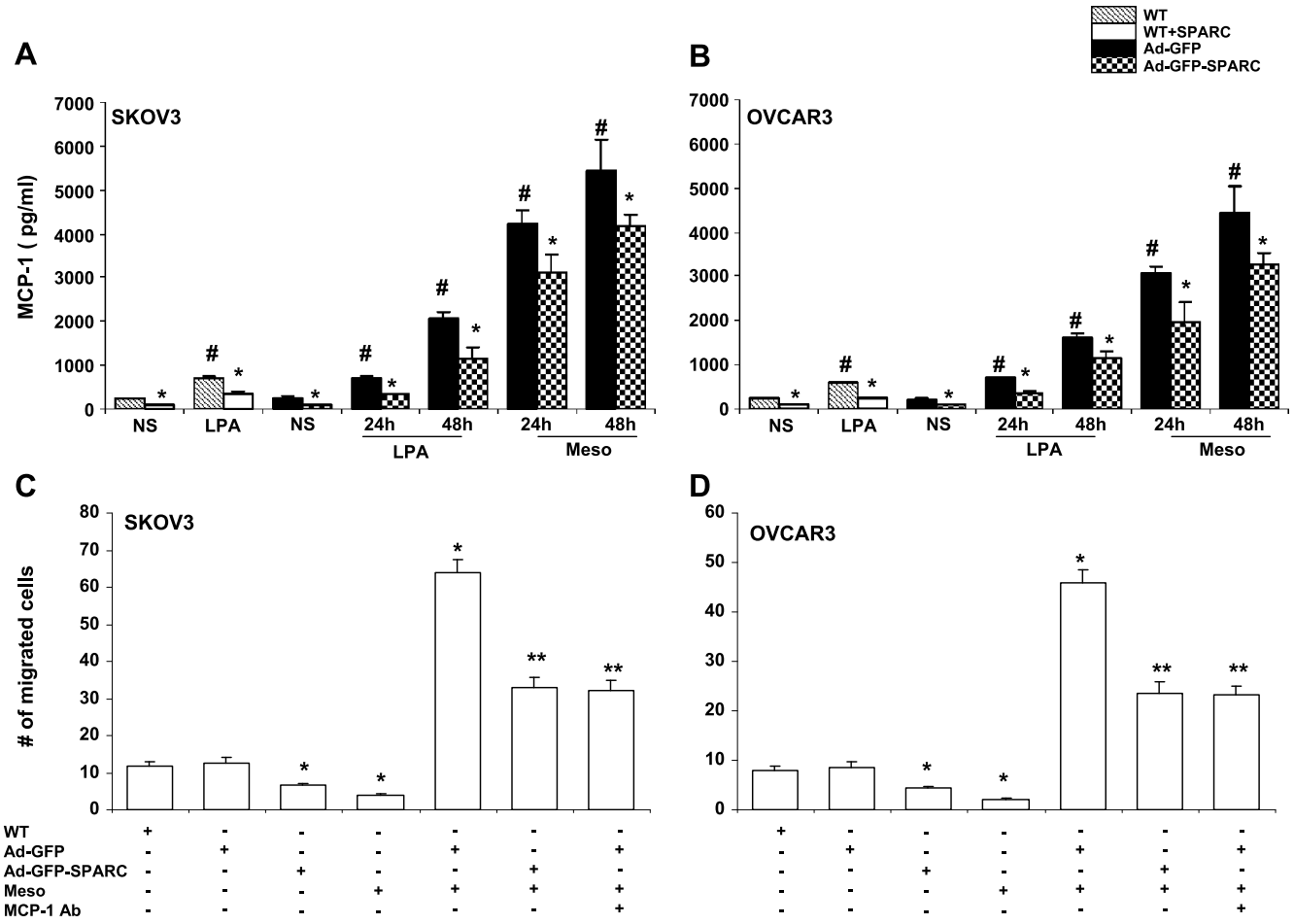

Figure 1. Effect of SPARC on production and activity of MCP-1 in ovarian cancer cells. Confluent monolayers (~1 × 10^6 cells, serum-starved overnight) of wild type (WT), SKOV3, and OVCAR3, were stimulated with 50 μM LPA for 24 hours in the presence and absence of SPARC (10 μg/ml). Similarly, adenovirus-transduced SKOV3 and OVCAR3 were stimulated with 50 μM LPA for 24 to 48 hours. Alternatively, adenovirus-transduced SKOV3 and OVCAR3 were seeded onto confluent monolayers or cocultured with Meso301 for 24 to 48 hours. MCP-1 protein secreted in the CM of SKOV3 (A) and OVCAR3 (B) was determined by ELISA. Controls included cells that were not stimulated (NS) with LPA. *$P < .05$, compared to matched nonstimulated or Ad-GFP condition. #$P < .05$, compared to nonstimulated WT or Ad-GFP cells. Results shown are mean ± SEM of a representative of three independent experiments, each performed in duplicate. Overexpression of SPARC in SKOV3 and OVCAR3 abrogated MCP-1–mediated chemotaxis of U937 macrophages. Twenty-four-hour–transduced SKOV3 (C), and OVCAR3 (D) cells (2 × 10^5 cells per 600 μl SFM per well) were grown to confluence in 24-well plates or were seeded onto confluent monolayers of Meso301. U937 macrophages (1 × 10^5 cells per 100 μl SFM) were added to the top chamber of the transwells. In some experiments, neutralizing anti–MCP-1 antibody (MCP-1 Ab, 25 μg/ml) was included in Ad-GFP–transduced ovarian cancer cell-Meso301 cocultures, Results shown are mean ± SEM of a representative of three independent experiments, each performed in triplicate. *$P < .05$, compared to nonstimulated (NS) or Ad-GFP–transduced cells. **$P < .05$, compared to cocultures of Meso301 and Ad-GFP–transduced ovarian cancer cells.

by ovarian cancer cells alone. It is noteworthy that mesothelial cell monolayers had a minor chemotactic effect on macrophages. The chemotactic activity was significantly abrogated (48%) by overexpression of SPARC in ovarian cancer cells, an inhibitory effect comparable to that of MCP-1 neutralizing antibody.

SPARC Attenuates the Mitogenic and Proinvasive Effect of MCP-1 on Ovarian Cancer Cells

MCP-1 has been reported not only to attract macrophages and inflammatory cells, but also to promote tumorigenesis through its proliferative and proinvasive effect on a variety of cancer cells as well as endothelial cells [15,16,37–40]. Herein, we report that MCP-1 exerts a concentration-dependent stimulatory effect on SKOV3 and OVCAR3 cell proliferation, an effect that was observed at a concentration range of 10 to 50 ng/ml (Figure 2, *A* and *B*). Similar concentrations of MCP-1 also significantly stimulated SKOV3 and OVCAR3 cell invasiveness (Figure 2, *C* and *D*). Overexpression of SPARC in ovarian cancer cells abrogated their responses to the mitogenic (17% to 23% and 15% to 27% inhibition for SKOV3 and OVCAR3, respectively) and the proinvasive (15% to 28% and 30% to 43% inhibition for SKOV3 and OVCAR3, respectively) effects of MCP-1 at all the tested concentrations. These data indicate that MCP-1 secretion by ovarian cancer cells is augmented on LPA stimulation and in cocultures with peritoneal mesothelial cells. MCP-1 not only mediated ovarian cancer cell–induced macrophage chemotaxis, but also exerted a potent mitogenic and a less pronounced proinvasive effect on ovarian cancer cells themselves. Overexpression of SPARC in ovarian cancer cells attenuated MCP-1

production and resulted in a partial, although significant, inhibition of its biological activity.

SPARC Suppresses Macrophage-Induced Ovarian Cancer Cell Invasion

In patients with ovarian cancer, tumor burden has been positively correlated with ascitic fluid macrophage content. TIM and TAM have been reported to enhance tumor dissemination by increasing tumor cell adhesion molecules on the peritoneal mesothelium [41]. TAM purified from ascites of patients with ovarian cancer revealed the presence of a macrophage-derived factor that promoted tumor neovascularization, growth, and metastasis. Ovarian tumor macrophage products include growth, angiogenic, and survival factors such as IL-6 and VEGF [41]. *In vitro*, coculture of macrophages with ovarian tumor cells resulted in the induction of a tumor-promoting phenotype [35,36]. Recently, in an *in vivo* study using immunocompromised as well as immunocompetent animal models, an overall protumor effect of macrophages in ovarian cancer dissemination has been reported [42]. Interestingly, using a syngeneic ovarian cancer model in $SP^{-/-}$ mice, we have recently reported that TAM and TIM were positively correlated with tumor burden, ascitic fluid volume, and the levels of IL-6, VEGF, and MMPs [34]. These observations led us to investigate the effect of tumor-derived SPARC on the interaction of macrophages with ovarian cancer cells. We found that cocultures of U937 macrophages with tumor cells significantly increased the invasiveness of SKOV3 (~50%) and OVCAR3 (approximately three-fold) cell lines, over single-cell cultures (Figure 3*A*). Restoring SPARC expression in ovarian cancer cell lines significantly abrogated macrophage-induced invasiveness (48% to 50%).

We next sought to identify the soluble factors implicated in macrophage-induced tumor cell invasiveness and the effect of SPARC on these factors. We found that in cocultures of ovarian cancer cells and macrophages, IL-6 levels were significantly augmented over the basal secretion by SKOV3 (17-fold), OVCAR3 (17.6-fold), or U937 (25- to 28-fold). Restoring SPARC expression in ovarian cancer cells significantly decreased IL-6 production in cocultures

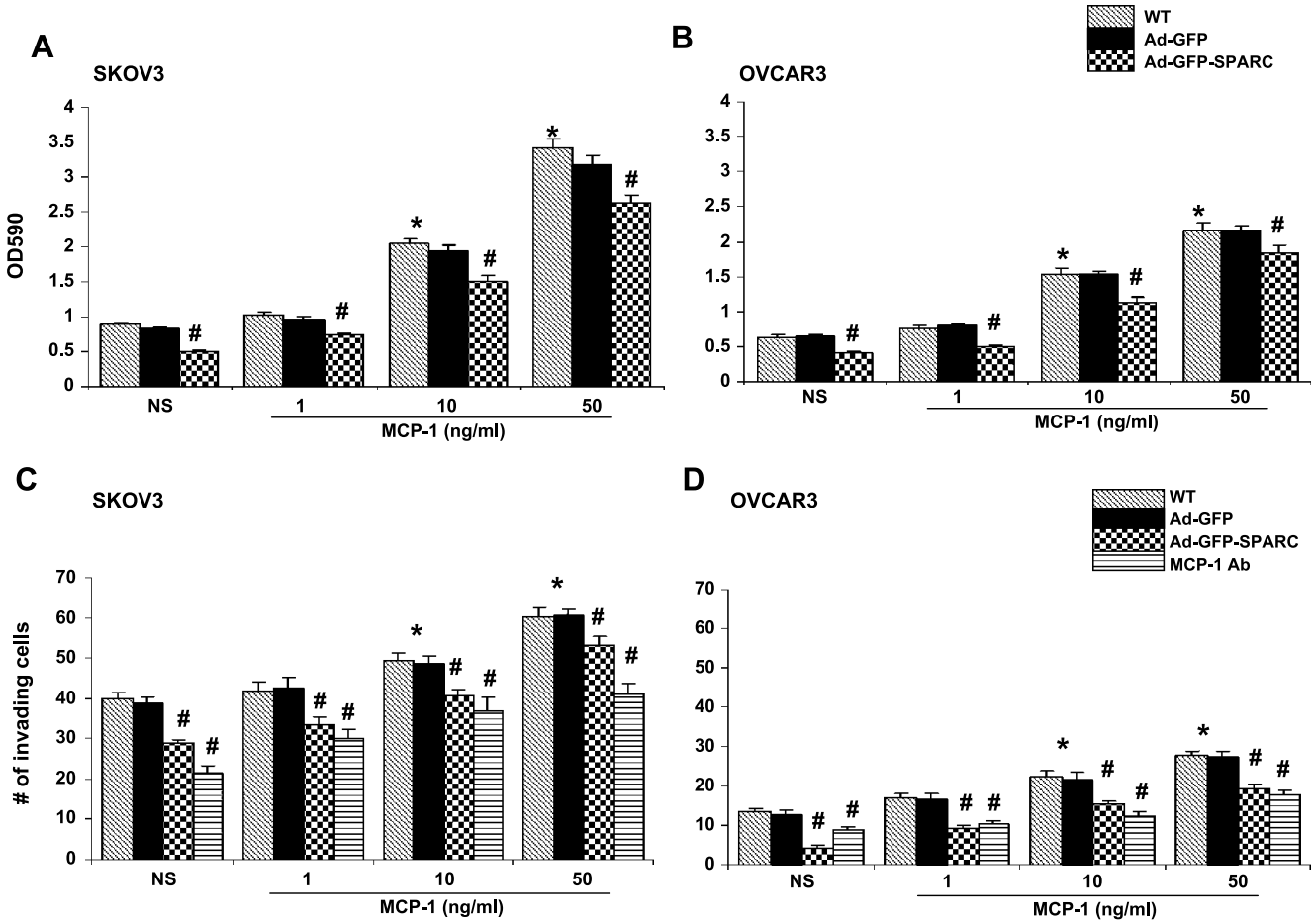

Figure 2. Effect of SPARC on regulation of mitogenic and proinvasive functions of MCP-1 in ovarian cancer cells. Proliferation of SKOV3 (A) and OVCAR3 (B) transduced or not with Ad-GFP and Ad-GFP-SPARC, in response to indicated concentrations of MCP-1, was assessed by MTS assays. Results shown are expressed as the mean ± SEM of three independent experiments performed in quadruplicate. $^{*}P < .05$, compared to NS cells. $^{\#}P < .05$, compared to the matched untransduced WT or Ad-GFP–transduced cells. The proinvasive activity of MCP-1 was assessed by Matrigel-invasion assays. SKOV3 (C) and OVCAR3 (D), transduced or not with Ad-GFP and Ad-GFP-SPARC (1×10^5 cells per 100 μl SFM), were added to the upper chamber of Matrigel-coated inserts in the presence and absence of the indicated concentrations of MCP-1. In some experiments, cells were pretreated with MCP-1 antibody (Ab) or its isotype control (50 μg/ml) for 30 minutes before stimulation, as described earlier. The bottom chambers contained complete growth medium. Results shown are the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < .05$, compared to control NS cells. $^{\#}P < .01$, compared to matched WT or Ad-GFP–transduced cells.

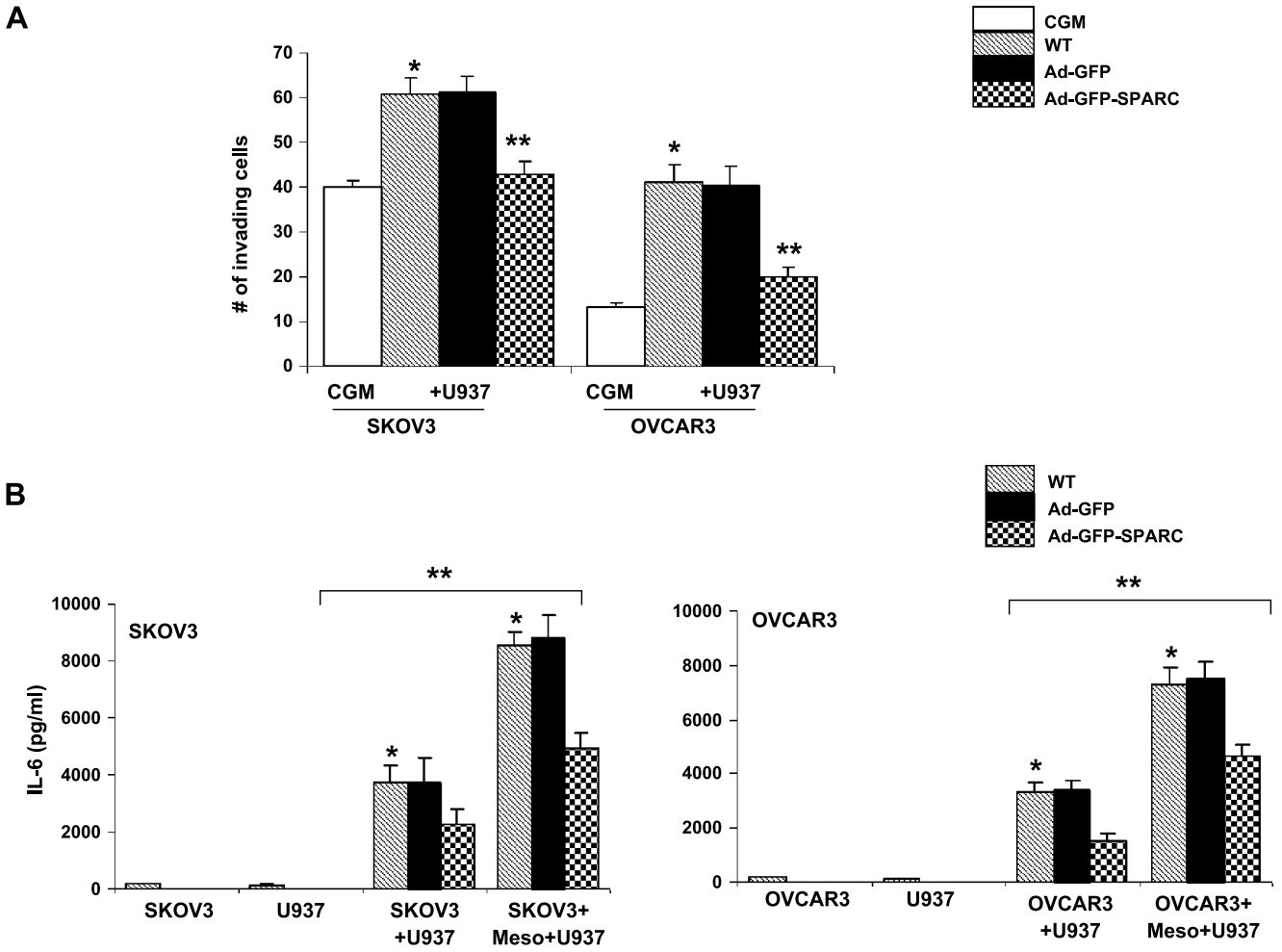

Figure 3. Regulation of macrophage-induced ovarian cancer cell invasiveness by SPARC. SKOV3 and OVCAR3 cells, transduced or not with Ad-GFP and Ad-GFP-SPARC, were seeded into the upper chambers of Matrigel-coated inserts (1×10^5 cells per 100 μl SFM per well). U937 macrophages (2×10^6 cells per 600 μl SFM) were seeded in the lower chambers. The number of invading cells was determined as reported earlier (A). Results shown are the mean ± SEM of three independent experiments performed in triplicate. *$P < .05$, compared to control SKOV3 and OVCAR3 cells in single-cell cultures, using complete growth medium (CGM) in the bottom chamber. IL-6 production in the CM of U937-ovarian cancer cocultures or triple cultures with Meso301 cells were determined by ELISA (B). Cells were treated as described in the legend of Figure 1, *A* and *B*, and 24-hour CM were used for IL-6 determination by ELISA. Bars represent the mean ± SEM of one of three independent experiments, each performed in duplicates. *$P < .05$, compared to matched WT or Ad-GFP. **$P < .05$, compared to double cultures in the absence of mesothelial cells.

with SKOV3 and OVCAR3 by 39% and 55%, respectively (Figure 3*B*). Interestingly, incorporating Meso301 in the triple cultures resulted in a significant increase in IL-6 production (~2.5-fold over U937-SKOV3 and U937-OVCAR3 cocultures). This increase was attenuated by restoring SPARC expression in SKOV3 and OVCAR3 cells (44% and 28%, respectively). Neither exogenous SPARC nor overexpression of SPARC in U937 had any effect on their IL-6 levels. We have recently reported that SPARC partially, but significantly, attenuated the mitogenic and motogenic effects of IL-6 on human ovarian cancer cells *in vitro* as well as *in vivo*, in a syngeneic murine ovarian cancer model [10,34].

Coculture of Ovarian Cancer Cells with Mesothelial Cells and Macrophages Induces Increased MMP Secretion by Macrophages

Coculture of macrophages with tumor cells has been shown to induce MMP expression in both macrophages and tumor cells, as well as increased MMP activity in the coculture supernatants [43]. Furthermore, MMP-2 and -9 have been colocalized with TAM in lung cancer specimens as well as in cocultures [21]. We have recently reported that the augmented proteolytic activity of ascitic fluid as well as the transcriptional and posttranscriptional upregulation of MMP-2 and -9 in tumor-bearing $SP^{-/-}$ mice was concomitant with a marked increase in macrophage infiltration in tumors and the ascitic fluid of the $SP^{-/-}$ compared with the $SP^{+/+}$ counterparts [30,34]. We next determined which cells contributed to MMP production and the possible mechanisms of regulation by SPARC. Whereas CM of OVCAR3 had barely detectable basal activity of pro- and active MMP-9, that of SKOV3 showed a significant activity of pro- and active MMP-9 as well as pro-MMP-2. There was no difference in the enzymatic activity of these gelatinases between WT and Ad-GFP–transduced ovarian cancer cells. Ad-GFP-SPARC transduction decreased levels of pro- and active MMP-9 in OVCAR3, whereas in SKOV3, it decreased both MMP-2 and

MMP-9 activity without affecting either pro-MMP-2 or MMP-9 levels (Figure 4*A*).

LPA stimulation significantly increased the levels of active MMP-9 in OVCAR3 and that of pro- and active MMP-9 in SKOV3. Ad-GFP-SPARC transduction markedly decreased LPA-induced MMP-9 activity in both OVCAR3 and SKOV3. Interestingly, coculture of either ovarian cancer cell line with Meso301 resulted in the most significant increase in the levels and activity of the pro- and active forms of MMP-2 and MMP-9 (Figure 4*A*). To determine the contribution of macrophages to production of MMPs, we found that the CM of U937 exhibited the highest basal activity level of MMP-2 and MMP-9 compared with either SKOV3 or OVCAR3 (Figure 4*B*). LPA significantly increased MMP-2 and MMP-9 activity. Exogenous SPARC (20 μg/ml) markedly decreased LPA-induced activity of both MMP-2 and MMP-9. Coculture of U937 with either SKOV3 or OVCAR3 resulted in a marked increase in the levels of

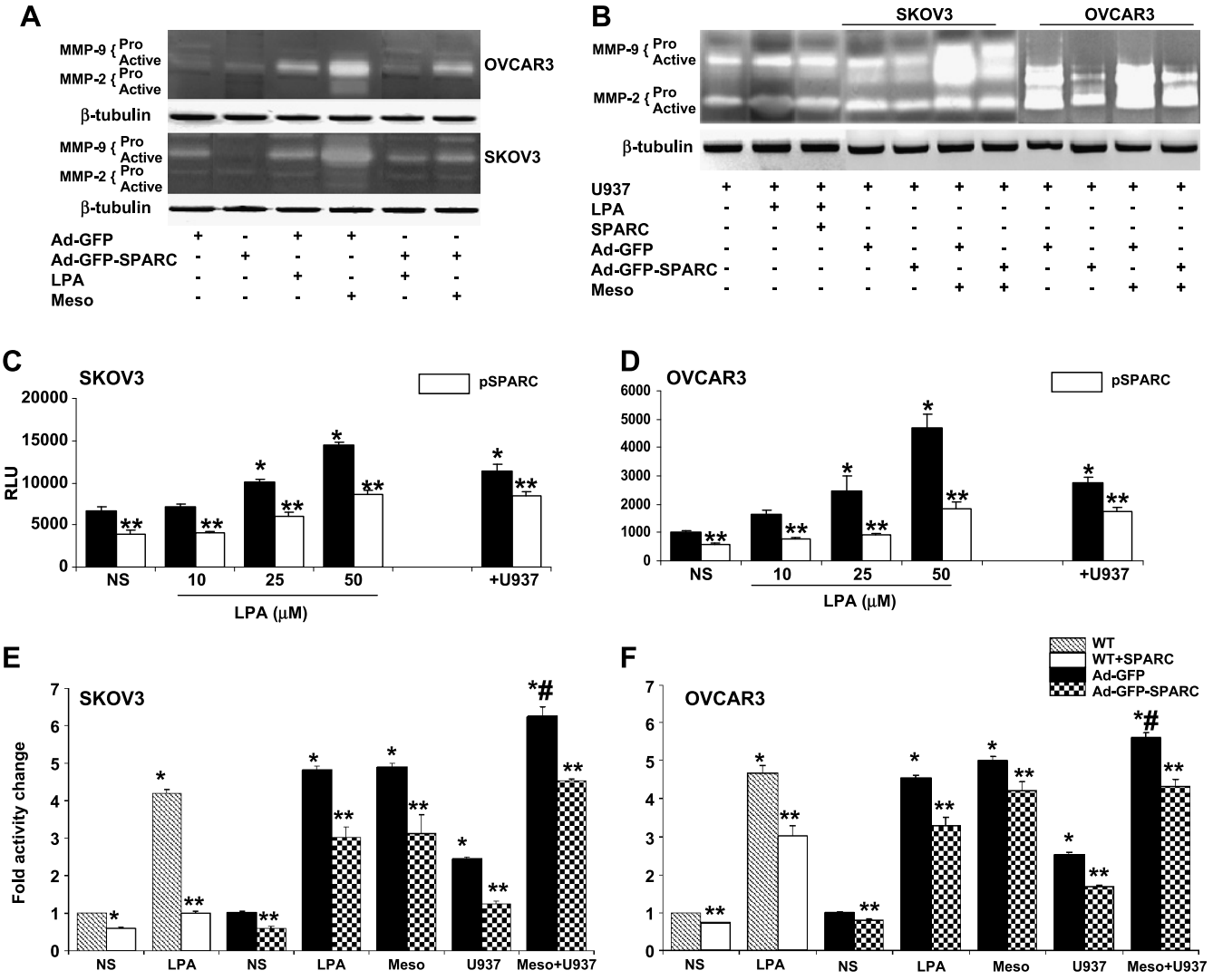

Figure 4. Differential effect of SPARC on the proteolytic activity of ovarian cancer cells and macrophages. The gelatinolytic activity of MMP-2 and -9 in CM of SKOV3 and OVCAR3 cells, transduced or not with Ad-GFP-SPARC, was determined after LPA stimulation or coculture with Meso301 monolayers (A). The gelatinolytic activity of MMP-2 and -9 in CM of U937 stimulated with LPA (50 μM) or cocultured with SKOV3 and OVCAR3, with and without Meso301 (B). Exogenous SPARC (20 μl) was included in some experiments. SKOV3 (C) and OVCAR3 (D) cells were cotransfected with a uPA promoter luciferase reporter plasmid and a mammalian expression vector encoding SPARC (pSPARC) and relative luciferase units (RLU) was determined after LPA (10 to 50 μM) stimulation for 6 hours or after coculture with U937 cells. Bars are the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < .05$, compared to NS controls. $^{**}P < .05$, compared to matched experimental condition without pSPARC cotransfection. uPA activity in CM of confluent monolayers serum-starved overnight (~1 × 10^6 cells) of SKOV3 (E) and OVCAR3 (F) were measured in WT cells stimulated for 24 hours with LPA (50 μM) in the presence and absence of SPARC (10 μg/ml). Similarly, adenovirus-transduced SKOV3 and OVCAR3 were stimulated with LPA. Alternatively, adenovirus-transduced SKOV3 and OVCAR3 were either cocultured with Meso301 monolayers, U937 macrophages (added in 0.22-μm transwell inserts), or in triple-culture systems. uPA activity is expressed as fold change from control WT SKOV3 or OVCAR3 that were not stimulated (NS), assigned a value of 1. Results shown are mean ± SEM of a representative of three independent experiments, each performed in duplicate. $^{*}P < .05$, compared to nonstimulated WT or Ad-GFP cells. $^{**}P < .05$, compared to matched WT or Ad-GFP condition. $^{\#}P < .05$, compared to coculture of ovarian cancer cells with either Meso301 or U937 in two-cell culture systems.

pro- and active MMP-9 and MMP-2. Both were significantly attenuated by Ad-SPARC transduction of ovarian cancer cells. As anticipated, CM from triple cultures including Meso301 cells exhibited a pronounced increase in the levels and activity of pro- and active MMP-2 and MMP-9. The inhibitory effect of Ad-SPARC transduction was more pronounced on MMP-9 levels and activity than on MMP-2 in SKOV3 and OVCAR3. It is noteworthy that CM from Meso301 alone had undetectable gelatinolytic activity (data not shown). These results indicate that the proteolytic activity of MMP-2 and MMP-9 in ovarian cancer is attributed not only to the constitutive production by ovarian cancer cells and macrophages, but more importantly, to their activation by interaction of ovarian cancer cells with mesothelial cells and macrophages. Although these interactions differentially regulated the levels and activity of both MMP-2 and MMP-9 in SKOV3, OVCAR3, and U937 macrophages, changes in MMP-9 were more pronounced both in response to the stimulatory effects of LPA in cocultures and to the inhibitory effect of SPARC adenoviral gene transfer. Furthermore, the pattern of induction of MMP-2 and MMP-9 activity and their inhibition by Ad-SPARC observed in the present study suggest a possible role for SPARC in downregulation of the activity levels of these MMPs.

SPARC Attenuates LPA-Induced uPA Activity in Ovarian Cancer Cells

uPA is a serine proteinase that catalyzes the conversion of plasminogen to plasmin, a broad-spectrum serine protease that not only degrades numerous components of the ECM, but has also been implicated in activation of other MMPs as well as uPA itself. High levels of uPA have been associated with MMP-2 and -9 in the ascitic fluids and tumors of patients with stages I to IV ovarian cancer and were correlated with the increased invasiveness of ovarian cancer cells [14,44]. Furthermore, the expression of uPA in ovarian cancer has been shown to be upregulated by a myriad of stimuli, of which LPA is the most putative [45]. Therefore, we determined whether SPARC plays a role in regulation of uPA expression and activity. First, we studied the effect of SPARC on uPA promoter activity. We found that transient transfection of ovarian cancer cells with pSPARC significantly decreased the basal activity of uPA promoter in both SKOV3 (42%, Figure 4*C*) and OVCAR3 (~43%, Figure 4*D*). LPA stimulation of uPA promoter activity was concentration dependent in both SKOV3 (6% to 115%) and OVCAR3 (38% to 325%). Cotransfection with pSPARC significantly attenuated LPA-induced uPA promoter activity in SKOV3 (40% to 43% inhibition) and OVCAR3 (51% to 63%). Interestingly, coculture of either SKOV3 or OVCAR3 cells with U937 macrophages resulted in significant stimulation of uPA promoter activity (69% and 170% increase in SKOV3 and OVCAR3, respectively); an increase that was partially, although significantly, attenuated by pSPARC cotransfection (26% and 36% inhibition in SKOV3 and OVCAR3, respectively). Using a colorimetric assay for uPA activity analysis in CM, we found that the constitutive uPA activity in CM of SKOV3 (Figure 4*E*) and OVCAR3 (Figure 4*F*) was partially but significantly attenuated by either exogenous SPARC or Ad-SPARC transduction (40% and 29% in SKOV3 and OVCAR3, respectively). Exogenous SPARC significantly inhibited LPA-induced uPA activity in SKOV3 (76%) and OVCAR3 (36%). Although LPA induced a 4- to 4.5-fold increase in uPA activity in untransduced WT and Ad-GFP–transduced SKOV3 and OVCAR3 controls, respectively, the inhibitory effect of Ad-SPARC transduction was significant (38% and 28% inhibition, for SKOV3 and OVCAR3, respectively), albeit less pronounced than that of exogenous SPARC for both cell lines. Coculture of either SKOV3 or OVCAR3 with Meso301 resulted in an increase in uPA activity in their CM, comparable to that of LPA stimulation. Ad-SPARC transduction resulted in a modest yet significant decrease in uPA activity by ~160% and ~60% in SKOV3 and OVCAR3, respectively. Furthermore, cocultures of U937 with either SKOV3 or OVCAR3 increased uPA activity in CM by 1.7- to 2-fold that was attenuated by Ad-SPARC transduction of SKOV3 (~110%) and OVCAR3 (~70%). Moreover, triple cultures, including Meso301 with ovarian cancer cells and U937 macrophages, resulted in a significantly augmented uPA activity in their CM (6.2- and 5.6-fold for SKOV3 and OVCAR3, respectively) and was shown to be attenuated by Ad-SPARC transduction in SKOV3 (~160%) and OVCAR3 (~120%).

SPARC Attenuates PGE2 Production and Activity in Culture Supernatants of Ovarian Cancer Cells with Mesothelial Cells and/or Macrophages

PGE2, the most common prostanoid, is involved in tumor progression by inducing angiogenesis, invasion, and metastasis in several solid tumors [11,46,47]. *In vitro* PGE2 treatment stimulated proliferation of ovarian cancer cells and reduced apoptosis [48] as well as inhibition of immune surveillance [13]. PGE2 participates in these complex mechanisms by stimulating VEGF secretion [49], cell migration, and expression and activation of MMPs [11]. The increase in eicosanoids, including PGE2 observed in patients with cancer, has been variably attributed to active secretion by tumor tissues, the products of coagulation and inflammation, and active secretion by macrophages [11,49]. Having shown the different effects of SPARC in modulating tumor cell interactions with mesothelial cells and macrophages, as well as LPA stimulation, we sought to determine whether SPARC exerts an effect on prostanoids as well. We found that the baseline production of PGE2 by serum-starved SKOV3 (~7 pg/ml) and OVCAR3 (~5 pg/ml) was decreased by both exogenous and Ad-SPARC transduction by up to 30% to 50%, respectively (Figure 5, *A* and *B*). LPA stimulation of SKOV3 and OVCAR resulted in ~14-fold increase in PGE2 production, which was suppressed 40% to 55% by exogenous or Ad-SPARC transduction of either cell line, respectively. PGE2 production was augmented to 22- to 30-fold by coculture of Meso301 with SKOV3 and OVCAR3, respectively. Transduction of SKOV3 and OVCAR3 with Ad-SPARC resulted in significant (40%) decrease in PGE2 production. Similar augmentation of PGE2 production was observed in cocultures of SKOV3 or OVCAR3 with U937 macrophages. Ad-SPARC transduction resulted in a significant decrease in PGE2 production in cocultures involving SKOV3 (50%), but not with OVCAR3 (14%). Further amplification of PGE2 production (80- to 100-fold) was observed in triple cultures and was significantly attenuated by Ad-SPARC transduction of SKOV3 but not of OVCAR3. Next, we tested whether SPARC decreases the responsiveness of ovarian cancer cells to the mitogenic and proinvasive effects of PGE2. We found that PGE2-stimulated proliferation of SKOV3 (Figure 5*C*) and OVCAR3 (Figure 5*D*) was concentration dependent up to 10 nM. Ad-SPARC transduction partially attenuated the response of SKOV3 and OVCAR3 to PGE2-induced proliferation by 28% to 38% in SKOV3 and 32% to 49% in OVCAR3, respectively. Similarly, the proinvasive effect of PGE2 on SKOV3 (Figure 5*E*) and OVCAR3 (Figure 5*F*) was concentration dependent and was partially

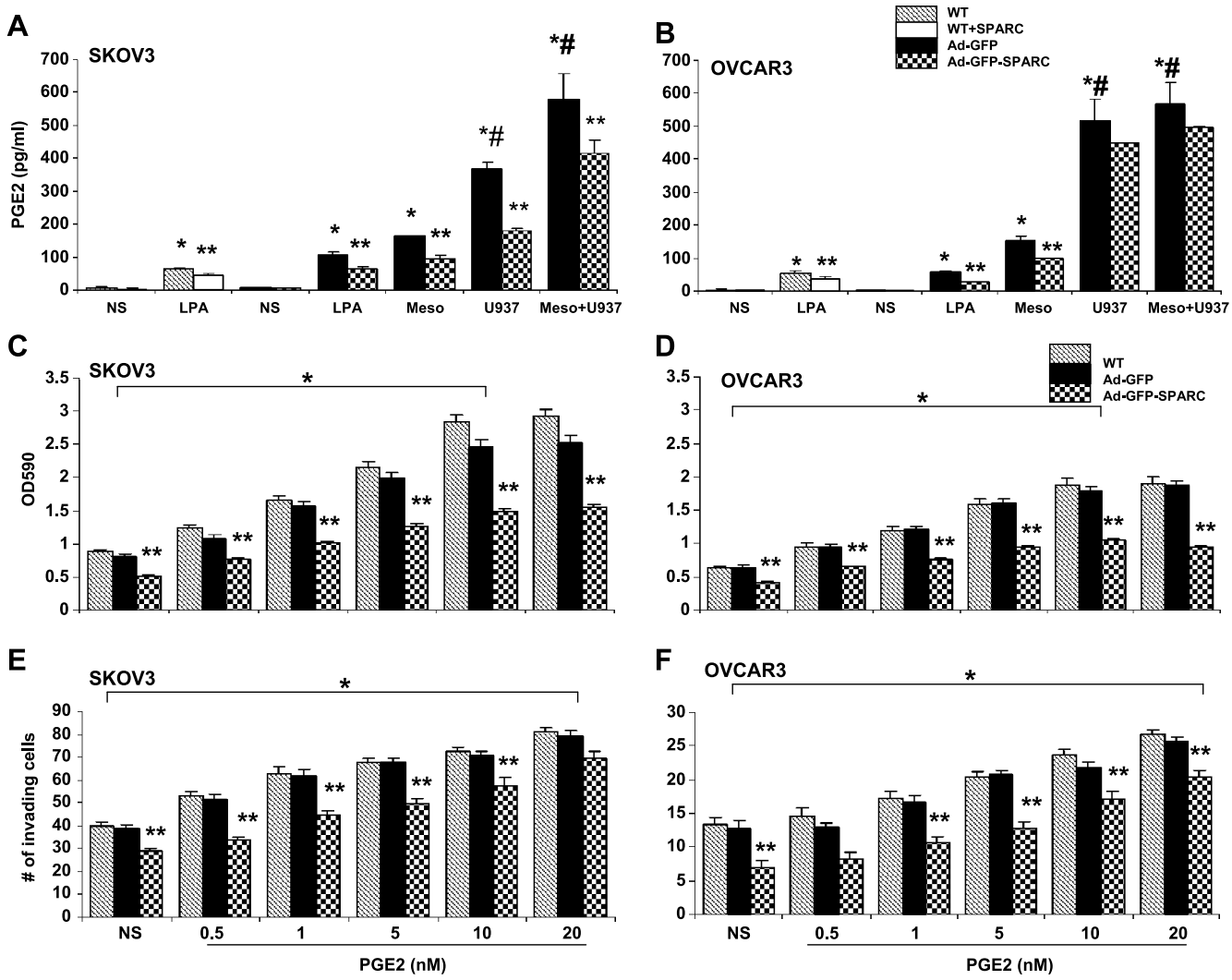

Figure 5. SPARC attenuates PGE2 production and activity in cocultures of ovarian cancer cells with mesothelial cells and/or macrophages. SKOV3 (A) and OVCAR3 (B) cells were treated as described in Figure 4, *A* and *B*, and PGE2 level was determined in the CM by enzyme immunoassay. $^{*}P < .05$, compared to nonstimulated WT or Ad-GFP cells. $^{**}P < .05$, compared to matched WT or Ad-GFP condition. $^{\#}P < .05$, compared to cocultures of ovarian cancer cells with either Meso301 or U937 in a two-cell culture system. Proliferation of SKOV3 (C) and OVCAR3 (D) cells, transduced or not with Ad-GFP and Ad-GFP-SPARC, in response to the indicated concentrations of PGE2, was assessed by MTS assays. Results shown are expressed as the mean ± SEM of three independent experiments performed in quadruplicate. $^{*}P < .05$, compared to the matched WT or Ad-GFP transduction. $^{**}P < .05$, compared to NS control cells. The proinvasive activity of PGE2 on SKOV3 (E) and OVCAR3 (F) was determined in Matrigel invasion assays. Cells were treated as described in Figure 2, *C* and *D*, and were treated with the indicated concentrations of PGE2. Results shown are the mean ± SEM of three independent experiments performed in triplicates. $^{**}P < .05$, compared to matched WT or Ad-GFP–transduced cells. $^{*}P < .05$, compared to NS controls.

but significantly inhibited by Ad-SPARC (12% to 35% and 20% to 45% in SKOV3 and OVCAR3, respectively).

Effect of SPARC on the Production of Reactive Oxygen Species

Isoprostanes are prostaglandin isomers produced from the peroxidation of cell membrane phospholipids, and have been used as an indirect measure of oxidative stress. The chronic presence of the proliferating tumor cells and thus chronic activation of inflammatory cells, results in increased production of reactive oxygen species (ROS). This is supported by the observation that patients with cancer showed signs of extensive activation of inflammatory cells, with massive elevation of plasma levels of 8-isoprostane [50]. Furthermore, tumor cells activate inflammatory cells through production of cytokines. IL-6 is one of the candidate cytokines that are elevated in the serum of patients with cancer, and stimulate the oxidative burst and ROS generation [7,51]. Moreover, 8-isoprostane has recently been linked to UV-induced inflammation and lipid peroxidation in basal cell and squamous cell carcinomas of the skin as well as in melanoma [45]. We have recently shown that the increased production of 8-isoprostane in murine peritoneal ovarian carcinomatosis was positively correlated with enhanced tumor growth, increased TAM and TIM, as well as augmented levels of mitogenic and inflammatory cytokines and growth factors in $SP^{-/-}$ mice compared with $SP^{+/+}$ counterparts [34]. In the present study, we show that the basal levels of ROS production, as measured by 8-isoprostane in serum-starved SKOV3 (52 pg/ml) and OVCAR3 (38 pg/ml), was decreased

by both exogenous SPARC and Ad-SPARC by 70% to 90%, respectively (Figure 6, *A* and *B*). LPA stimulation of SKOV3 and OVCAR resulted in an increase (~2.2- to 2.5-fold, respectively) in 8-isoprostane production, which was suppressed up to 26% to 55% by addition of exogenous SPARC or Ad-SPARC transduction of either cell line, respectively. The suppression of LPA-induced 8-isoprostane production by SPARC was significant only with OVACR3 cells, but not with SKOV3 cells. The production of 8-isoprostane was amplified to approximately seven- and four-fold in cocultures of Meso301 with SKOV3 and OVCAR, respectively. Transduction of SKOV3 and OVCAR3 with Ad-SPARC resulted in a significant inhibition of ROS by 48% and 74% in cocultures of Meso301 with SKOV3 and OVCAR3, respectively. Similarly, cocultures of SKOV3 or OVCAR3 with U937 macrophages resulted in amplification of 8-isoprostane production that was significantly decreased (~50%) by Ad-SPARC transduction of either ovarian cancer cell line. Further amplification of 8-isoprostane production (five- to eight-fold) was observed in triple cultures and was significantly attenuated by Ad-SPARC transduction of SKOV3 (45%) and OVCAR3 (52%).

Activation of NF-κB Promoter

The activation of NF-κB, which is seen in most cancer cells, plays a key role in tumor initiation, progression, metastasis, and chemoresistance by mediating the production of a large variety of proinflammatory cytokines, chemokines, growth factors, collagenases, and antiapoptotic proteins. Analysis of the molecular basis of the TAM phenotype has identified NF-κB as the master regulator of macrophage-tumor cell interactions [35,36,52]. *In vitro* data have also linked ROS to activation of NF-κB, which, in turn, regulates transcription of many proinflammatory mediators [53]. In the present study, we tested whether the modulation of macrophage-tumor cell interaction by SPARC is mediated through interfering with NF-κB activation. We found that cocultures of U937 macrophages with SKOV3 (Figure 7*A*) and OVCAR3 cells (Figure 7*B*) resulted in a significant increase in NF-κB activation compared with noncocultured ovarian cancer cells (~5- and 3.5-fold increase in SKOV3 and OVCAR3, respectively). Transient transfection of ovarian cancer cells with pSPARC attenuated the macrophage-induced NF-κB activation by 40% and 53% in SKOV3 and OVACR3, respectively. LPA-induced NF-κB activation (40% to 50%) in both SKOV3 (Figure 7*C*) and OVCAR3 (Figure 7*D*). Cotransfection of SKOV3 and OVCAR3 with pSPARC attenuated LPA-induced NF-κB activation by ~50% in both cell lines. Furthermore, we also found that PGE2 treatment of SKOV3 (Figure 7*E*) and OVCAR3 (Figure 7*F*), resulted in a concentration-dependent NF-κB activation (approximately three-fold) up to 5 nM. Cotransfection of ovarian cancer cells with pSPARC resulted in a significant (30% to 50%) attenuation of PGE2-induced NF-κB activation in both cell lines. It is noteworthy that transient transfection of either SKOV3 or OVCAR3 with pSPARC significantly decreased (40%) their basal activity of NF-κB.

Discussion

Recent studies have elucidated the importance of the fluid microenvironment of malignant ascites in influencing the malignancy of ovarian tumors. The formation and composition of ascitic fluid

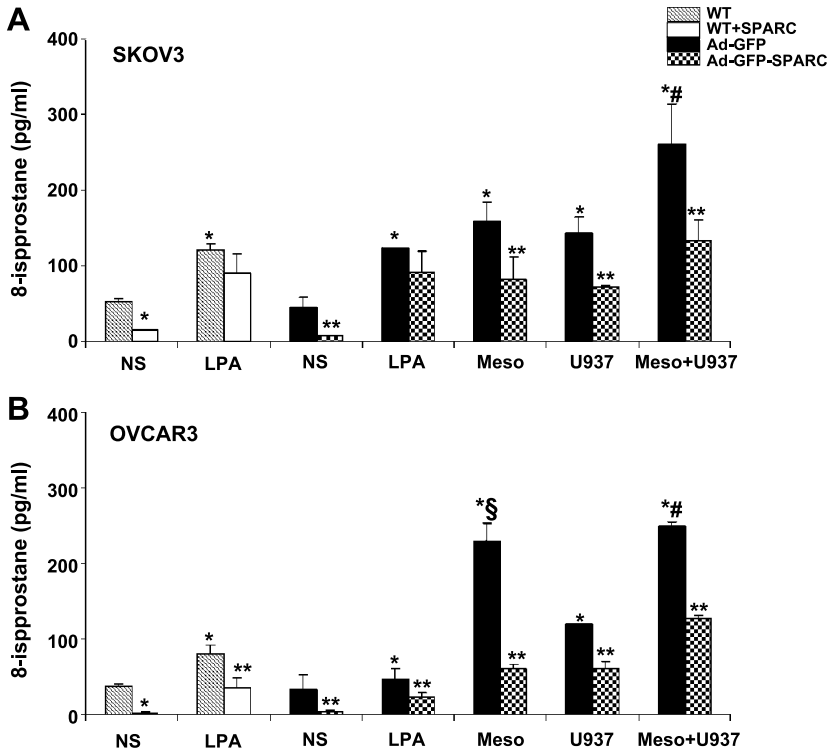

Figure 6. SPARC attenuates 8-isoprostane production in culture supernatants of ovarian cancer cells with mesothelial cells and/or macrophages. SKOV3 (A) and OVCAR3 (B) were treated as described in Figure 4, *A* and *B*, and the level of 8-isoprostane was determined in the CM by enzyme immunoassay. $^{*}P < .05$, compared to nonstimulated WT or Ad-GFP cells. $^{**}P < .05$, compared to matched WT or Ad-GFP condition. $^{\#}P < .05$, compared to coculture of ovarian cancer cells with either Meso301 or U937 in two-cell culture systems. $^{\S}P < .05$, between cocultures of OVCAR3-Meso and OVCAR3-U937.

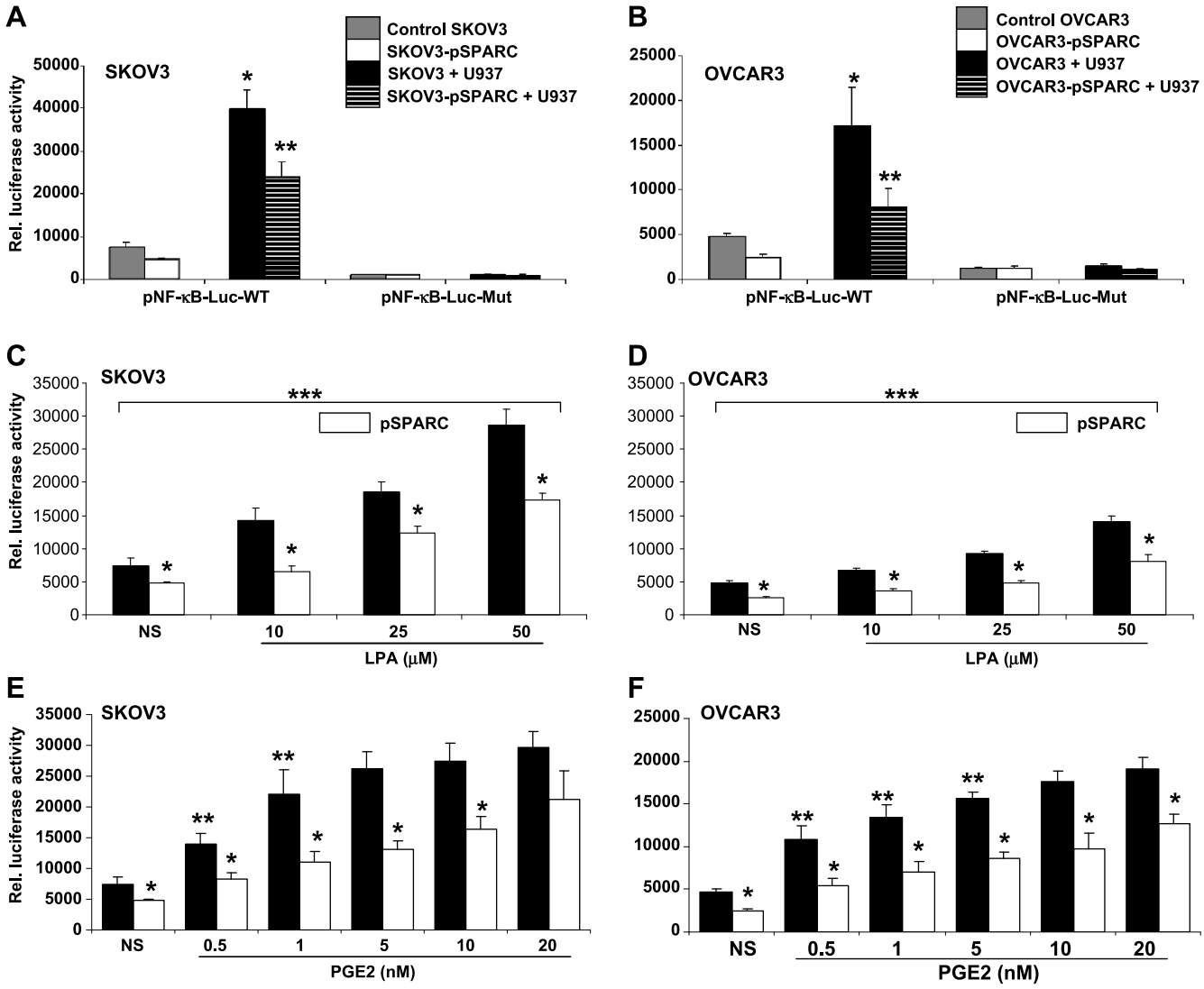

Figure 7. Effect of SPARC on NF-κB promoter activity. SKOV3 (A) and OVCAR3 (B) were transfected with pSPARC and either pNF-κB-Luc-WT or pNF-κB-Luc-Mut and were cocultured with U937 without direct cell-cell contact. Bars represent the mean ± SEM of the relative luciferase activity, corrected to β-gal activity, of three independent experiments performed in triplicates. *$P < .05$, compared to SKOV3 and OVCAR3 in single-cell culture. **$P < .05$, between cocultures without pSPARC transfection of ovarian cancer cells. SKOV3 (C) and OVCAR3 (D) were cotransfected as described earlier and stimulated with the indicated concentrations of LPA. Bars represent the mean ± SEM of relative luciferase activity, corrected to β-gal activity, of three independent experiments performed in triplicate. *$P < .05$, compared to matched NS or LPA-stimulated cells without pSPARC cotransfection. ***$P < .05$, compared to NS, and between each concentration of LPA stimulation. Similarly cotransfected SKOV3 (E) and OVCAR3 (F) cells were stimulated with the indicated concentrations of PGE2. Bars represent the mean ± SEM of three independent experiments performed in triplicate. *$P < .05$, compared to matched NS or PGE2-stimulated cells without pSPARC cotransfection. **$P < .05$, compared to NS and between the tested concentrations of PGE2.

not only represent the outcome of the tumor cell interactions with mesothelial and inflammatory cells, but also allow the efficient exchange of soluble factors among these cells, sustaining tumor cell growth and invasion [2]. Our recent *in vivo* approach allowed us to determine the effect of host SPARC in negative regulation of inflammation in a syngeneic model of murine ovarian cancer [30,34]. We found that ovarian tumor dissemination in the $SP^{-/-}$ peritoneum was accompanied by massive macrophage infiltration together with increased inflammatory mediators, which exerted mitogenic and motogenic effects on murine ovarian cancer cells and enhanced the angiogenic and proteolytic activity of the ascitic fluid. The current study was designed to determine whether tumor-derived SPARC affects the inflammatory responses that accompany the dissemination of ovarian tumors. Herein, we analyzed the effects of restoring SPARC expression in low- and high-metastatic ovarian cancer cell lines devoid of SPARC on their interaction with mesothelial cells and macrophages, the major cellular constituents of ovarian cancer microenvironment. Our results indicated that SPARC reexpression in ovarian cancer cells by adenovirus not only decreased their LPA- or mesothelial cell–induced macrophage chemotaxis, but also desensitized them to the mitogenic and motogenic effects of MCP-1.

The important role of macrophages in ovarian cancer cell invasion, proliferation, survival, and metastasis was attributed to the upregulation of VEGF and other proangiogenic and proteolytic factors in

response to hypoxia, with positive correlation with TIMs in ovarian cancer spheroids [35,42,43]. In the present study, we found that cocultures of macrophages with ovarian cancer cells significantly upregulated the expression of IL-6. This effect was further augmented in triple cultures, where ovarian cancer cells were overlaid onto monolayers of mesothelial cells. We have previously reported the effect of Ad-SPARC gene transfer in ovarian cancer cells on their response to the proinvasive as well as the mitogenic and survival signaling pathways induced by IL-6 [10]. There is a large body of experimental and clinical evidence that the proteolytic activity of ovarian cancer ascites is mediated through MMPs (-2 and -9) and uPA, which is induced by TAMs either directly or through overproduction of LPA, IL-6, and PGE2 [2,45,46,54,55]. Our results in the present study are consistent with those of the aforementioned reports. Furthermore, we found that reexpression of SPARC in ovarian cancer cells by adenovirus vectors markedly decreased the LPA-, mesothelial cell-, and macrophage-induced MMPs (-2 and -9) as well as the uPA activity. Consistent with these reports, PGE2 and 8-isoprostane production were significantly downregulated by Ad-SPARC. Moreover, we found that the effects of SPARC were mediated through decreased activation of the transcription factor NF-κB in ovarian cancer cells. It has been reported that exposure to malignant exudates or cocultures with activated macrophages enhances cancer cell proliferation and invasiveness. These events are all actively regulated by LPA and PGE2 [43,56].

Taken together, our results indicate that ectopic expression of SPARC in ovarian cancer cells negatively regulates the inflammatory responses elicited on interaction of tumor cells with mesothelial cells and/or macrophages. Therefore, it is plausible to suggest that restoring SPARC expression in ovarian tumors may not only impede the formation of ascites, but also decreases responsiveness of the ovarian cancer cells to the mitogenic and proinvasive stimuli in the ascitic fluid milieu.

SPARC-based gene or protein therapy has been shown to be a promising novel approach in regression of xenografted neuroblastoma as well as in chemo- and radiation therapy–refractory colorectal cancers [57,58]. Because ovarian cancer is confined mostly to the peritoneal cavity, it lends itself to intraperitoneal chemotherapy, which has fewer adverse effects compared with intravenous therapy [59–61]. Given our recent findings and the established antiproliferative, proapoptotic, and antimetastatic properties of SPARC in ovarian cancer, intraperitoneal delivery of SPARC by gene/protein therapy approaches in combination with traditional chemotherapy regimens could prove to be a novel, efficacious mode of therapy for late-stage peritoneal ovarian carcinomatosis.

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