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# Aberrant Promoter Methylation of *Sparc* in Ovarian Cancer[1,2]

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## Abstract

Epigenetic silencing of tumor suppressor genes is a new focus of investigation in the generation and proliferation of carcinomas. Secreted protein acidic and rich in cysteine (SPARC) is reportedly detrimental to the growth of ovarian cancer cells and has been shown to be epigenetically silenced in several cancers. We hypothesized that SPARC is downregulated in ovarian cancer through aberrant promoter hypermethylation. To that end, we analyzed SPARC expression in ovarian cancer cell lines and investigated the methylation status of the *Sparc* promoter using methylation-specific polymerase chain reaction. Our results show that SPARC mRNA expression is decreased in three (33%) and absent in four (44%) of the nine ovarian cancer cell lines studied, which correlated with hypermethylation of the *Sparc* promoter. Treatment with the demethylating agent 5-aza-2′-deoxycytidine rescued SPARC mRNA and protein expression. Addition of exogenous SPARC, as well as ectopic expression by an adenoviral vector, resulted in decreased proliferation of ovarian cancer cell lines. Investigation of primary tumors revealed that the *Sparc* promoter is methylated in 68% of primary ovarian tumors and that the levels of SPARC protein decrease as the disease progresses from low to high grade. Lastly, *de novo* methylation of *Sparc* promoter was shown to be mediated by DNA methyltransferase 3a. These results implicate *Sparc* promoter methylation as an important factor in the genesis and survival of ovarian carcinomas and provide new insights into the potential use of SPARC as a novel biomarker and/or treatment modality for this disease.

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## Introduction

With more than 21,000 new cases and more than 15,000 deaths expected in 2008, ovarian cancer ranks as the fifth leading cause of cancer deaths in women (http://www.cancer.org). More than 90% of ovarian cancers in humans arise from the surface epithelial cells of the ovaries. Recent studies have shown that several tumor suppressor genes are methylated in ovarian cancer [1–8]. Epigenetic silencing of tumor suppressor genes by methylation is rapidly becoming an area of interest in cancer research. Methylation most often occurs at cytosines that are 5′ to guanosine (known as the CpG dinucleotide) [9]. Stretches of DNA that are rich in CpG dinucleotides are known as CpG islands and can be found proximal to the promoter region of approximately 50% of human genes [9]. Methylation of these normally protected CpG islands results in the silencing of the alleles downstream of the promoter regions [10].

The secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin, BM-40, and 43K protein, is a membrane-associated

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glycoprotein that belongs to the matricellular family of proteins [11]. SPARC is predominantly expressed in tissues undergoing remodeling or repair, and it also plays an important role in normal development [12]. In addition to its antiproliferative and de-adhesive properties, SPARC is known to play a role in angiogenesis and extracellular matrix production [13]. The role of SPARC in tumor development and metastasis varies owing to the contextuality of its functions [14]. SPARC is significantly downregulated in ovarian cancer cells, and restoring its expression leads to decreased tumor growth and apoptosis [15]. Our laboratory has previously shown that SPARC ameliorates peritoneal carcinomatosis by inhibiting tumor cell adhesion and invasion during metastasis [16]. We have also identified roles for SPARC in ovarian cancer integrin–mediated adhesion and lysophosphatidic acid–induced mesothelial–ovarian cancer cell cross talk [17,18]. Therefore, SPARC plays a pivotal role in controlling malignancy of ovarian carcinoma.

In this study, we have attempted to elucidate the mechanism by which ovarian carcinoma cells downregulate SPARC expression. We hypothesized that epigenetic silencing of *Sparc* by aberrant methylation during ovarian carcinogenesis is responsible for the down-regulation of SPARC. To that end, we examined the *in vitro* methylation status of the *Sparc* promoter in ovarian cancer cell lines, immortalized control cell lines, and nonimmortalized, normal human ovarian surface epithelial (NHOSE) primary cells. The *in vivo* correlate studies were performed in tumor tissue samples from patients with serous ovarian cancer in varying stages and grades. Our results revealed that the down-regulation of SPARC in ovarian cancer cells is indeed caused by the hypermethylation of the *Sparc* promoter and that the loss of SPARC protein expression is inversely correlated with tumor grade. Moreover, demethylation of the CpG islands by 5-aza-2′-deoxycytidine (5-Aza-CdR) resulted in the restored expression of SPARC, and chromatin immunoprecipitation (ChIP) studies revealed DNA methyltransferase 3a (DNMT3a) as the major DNMT responsible for *de novo* methylation of *Sparc* promoter in ovarian cancer cells.

## Materials and Methods

### *Cell Culture and Tumor Tissue Samples*

We studied nine human ovarian cancer cell lines: SKOV3, TOV112, OVCAR3, OVCA 420, OVCA 429, DOV 13, OV-90, TOV21, and PA-1. OVCA 420, OVCA 429, and DOV 13 were prepared as previously described [6]. The remaining cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). SKOV3 was grown in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO) supplemented with 15% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) as well as penicillin, streptomycin, and amphotericin B (PSA; Sigma). OVCAR3 was grown in RPMI 1640 (ATCC), 15% FBS, PSA, and 50 μg/ml bovine insulin (Sigma). PA-1 was grown in Dulbecco's modified Eagle's medium (Sigma), 10% FBS, PSA. OVCA 420, OVCA 429, and DOV 13 were all grown in a mixture of MCDB105 (Sigma) and M199 (Sigma) media (1:1), 10% FBS, PSA. TOV112, OV-90, and TOV21 were all grown in MCDB105/M199 media (1:1), 15% FBS, PSA. Control cell lines Meso 301 (an immortalized mesothelial cell line), HOSE 1-15 (an immortalized human ovarian surface epithelial cell line), and three nonimmortalized, NHOSE primary cultures (preparations 56, 58, and 59) were obtained as previously described [6,19] and grown in MCDB105/M199 media (1:1), 10% FBS, PSA. All cell lines were incubated in 5% $CO_2$ at 37°C. Highly undifferentiated (grade III) serous-type ovarian carcinoma tumor samples from patients with varying disease stages (FIGO stages I-IV) were obtained from an in-house tumor tissue bank (Department of Pathology, Medical College of Georgia) with an approved institutional review board protocol. Samples were embedded in paraffin blocks and sectioned for microdissection. Ovarian cancer tumor cells were selectively microdissected from sections at our in-house laser capture microdissection (LCM) core facility and were stored at 4°C for later analysis.

### *Reagents and Antibodies*

SPARC from mouse parietal yolk sac was purchased from Sigma. Human and bovine osteonectin were purchased from Haematologic Technologies Inc. (Essex, VT). Anti-human SPARC antibodies were purchased from Haematologic Technologies Inc. and Santa Cruz Biotechnology (Santa Cruz, CA). Anti-DNMT antibodies were obtained from Epigentek (Brooklyn, NY). 5-Aza-CdR and trichostatin A (TSA) were purchased from Sigma-Aldrich and MP Biomedicals (Irvine, CA), respectively.

### *Reverse Transcription–Polymerase Chain Reaction*

Reverse transcription–polymerase chain reaction (RT-PCR) was used to assess the levels of SPARC mRNA expression in the cell lines. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol and further purified with the RNeasy isolation kit (Qiagen, Valencia, CA). Two micrograms of RNA was reverse-transcribed using oligo-(dT) primer and Improm-II Reverse Transcriptase (Promega, Madison, WI) into cDNA for subsequent PCR analysis. Polymerase chain reaction amplification was performed using JumpStart TAQ (Sigma) with the following general protocol: initial denaturation at 95°C for 4 minutes, followed by a variable number of cycles of 95°C for 30 seconds, specific annealing temperature for 45 seconds, 72°C for 1 minute, and a final elongation at 72°C for 8 minutes. Primer sequences, annealing temperatures, and cycle numbers can be found in Table 1. PCR products were visualized on 2% agarose gels containing ethidium bromide and photographed using a Kodak Gel Logic 100 imaging system and Kodak 1-D 3.6 software (Eastman Kodak, Rochester, NY).

### *Real-time Quantitative PCR*

The real-time quantitative PCR (qPCR) analyses were performed with primers designed from human *Sparc* promoter sequences (GenBank Accession No. U65081) using a Bio-Rad iQ5 cycler (Hercules, CA). Sense primer (32-52): 5′-GGA TCA TGA GGT CAG GCA TT-3′. Antisense primer (210-230): 5′-GCT GGA GTG CAG TGG TAT GA-3′. The qPCR reaction cocktail, PerfeCTa SYBR Green SuperMix for iQ, was purchased from Quanta BioSciences (Gaithersburg, MD). The qPCR was performed in 25-μl reaction volumes with an initial cycle at 95°C for 3 minutes, followed by 40 cycles at 95°C for 10 seconds, and at 60°C for 30 seconds. Because equal amounts of the same input were used in all samples, the ratio between positive ChIP and negative ChIP (Positive IP/Negative IP) was used to determine the ChIP efficiency. Relative occupancy of the immunoprecipitated factor at *Sparc* locus was calculated using the following equation: Relative occupancy = $2^{[Ct\ (\text{Negative control}) - Ct(\text{Target})]}$, where $C_t$ (Negative control) and $C_t$ (Target) are mean threshold cycles of PCR done in triplicates on DNA samples from negative control ChIP

**Table 1.** Polymerase Chain Reaction Primer Sequences, Annealing Temperatures, and Cycle Numbers Used in RT-PCR and MSP Experiments.

| Gene | Forward Primer | Reverse Primer | Annealing Temperature (°C) | Cycle Number |
|---|---|---|---|---|
| RT-PCR primers | | | | |
| *SPARC* | 5′-AAGATCCATGAGAATGACAAG-3′ | 5′-AAAAGCGGGTGGTGCAATG-3′ | 65 | 30 |
| *DNMT1* | 5′-GGGACGACGGGAAGACCTACT-3′ | 5′-CTCGCTGGAGTGGACTTGTG-3′ | 60 | 40 |
| *DNMT3a* | 5′-ACGACGACGACGGCTACCAGT-3′ | 5′-TTGCGAGCAGGGTTGACGAT-3′ | 61 | 40 |
| *DNMT3b* | 5′-AAAGCCCAGCTGTCCGAACTC-3′ | 5′-GAACTTGCCATCGCCAAACC-3′ | 59 | 40 |
| *GAPDH* | 5′-CACTGGCGTCTT CACCACCATG-3′ | 5′-GCTTCACCACCTTCTTGATGTCA-3′ | 56 | 30 |
| MSP primers | | | | |
| Methylated *Sparc* | 5′-GAGAGCGCGTTTTGTTTGTC-3′ | 5′-AACGACGTAAACGAAAATATCG-3′ | 62 | 40 |
| Unmethylated *SPARC* | 5′-TTTTTTAGATTG TTTGGAGAGTG-3′ | 5′-AACTAACAACATAAACAAAAATATC-3′ | 52 | 40 |

(using nonimmune IgG) and targeted ChIP (using specific DNMT isoform-specific antibodies), respectively.

### *Immunoblot Analysis*

Protein lysates were prepared for Western blot analysis from cultured cells. The cells were washed with cold Dulbecco's phosphate-buffered saline (PBS; Sigma), lysed in RIPA buffer containing sodium orthovanadate and a cocktail of protease inhibitors, and sonicated as previously described [16]. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Lysates were mixed with 1× SDS sample buffer, boiled for 5 minutes, and analyzed by SDS-PAGE. Proteins were then transferred to Immun-Blot PVDF Membrane (Bio-Rad) and blocked with 5% BSA in PBS. The membranes were washed with PBS containing 0.2% Tween 20 (PBS-T) and incubated with anti-human SPARC overnight. After washing with PBS-T, the membranes were incubated with horseradish peroxidase–conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 hour. Signals were visualized using the SuperSignal West Dura Extended Duration Substrate (Pierce). Photography and densitometry measurements were performed using a Kodak Gel Logic 100 imaging system and Kodak 1-D 3.6 software (Eastman Kodak).

### *Methylation-Specific PCR*

Genomic DNA was isolated from cell lines and LCM tumor cells from tissue sections using DNAzol Reagent (Invitrogen) according to the protocol recommended by the manufacturer. Methylation patterns were analyzed using methylation-specific PCR (MSP) adopted from Herman et al. [20]. Genomic DNA (1 μg, or 500 ng when sample was not large enough) was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the protocol recommended by the manufacturer. Two microliters of the final eluant was used for subsequent PCR amplification. The primer sequences as well as annealing temperatures and cycle numbers were derived from a published study and can be found in Table 1 [21]. Polymerase chain reaction products were visualized on 2% agarose gels containing ethidium bromide and were photographed using a Kodak Gel Logic 100 imaging system and Kodak 1-D 3.6 software (Eastman Kodak).

### *5-Aza-CdR and TSA Treatments*

Nine ovarian cancer cell lines (SKOV3, TOV112, OVCAR3, OVCA 420, OVCA 429, DOV 13, OV-90, TOV21, and PA-1) at 65% confluence were treated with the global genomic DNA demethylating agent 5-Aza-CdR or the histone deacetylase inhibitor TSA. For the 5-Aza-CdR treatment, cells were seeded in six-well culture dishes and incubated overnight in growth media. The normal growth media were replaced with growth media supplemented with 5-Aza-CdR (6 μM final concentration) and was replenished daily for 5 days. For the TSA treatment, cells were seeded in 60-mm culture dishes and incubated in their respective growth media containing TSA (500 ng/ml) for 24 hours. At the conclusion of either treatment, cells were harvested for RNA, genomic DNA, and protein as described above.

### *Immunohistochemistry*

SPARC levels in ovarian cancer tissue was analyzed by immunohistochemistry using ovarian cancer tissue arrays OV801 and OV2001 (US Biomax, Rockville, MD) containing 133 serous papillary adenocarcinoma samples of varying TNM stages and grades (T1 N*x* M*x*, $n$ = 118; T2 N*x* M*x*, $n$ = 13; T3 N*x* M*x*, $n$ = 2; grade I, $n$ = 17; grade II, $n$ = 38; grade III, $n$ = 78). For immunostaining, slides were deparaffinized and rehydrated before being incubated in 0.3% $H_2O_2$ in methanol to block endogenous peroxidases. Slides were then treated with AutoZyme (BioMeda Corp, Foster City, CA) before incubating with anti-human SPARC overnight. After washing with PBS-T, the slides were incubated with a horseradish peroxidase–conjugated secondary antibody for 1 hour before being developed with Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and stable 3,3′-diaminobenzidine (ResGen, Huntsville, AL). Slides were then counterstained with hematoxylin and mounted. Images were acquired with a Leica microscope (DM5000) equipped with a Q-Imaging digital camera (Leica Microsystems, Wetzlar, Germany). To quantify the SPARC staining, images of each section were assigned random numbers and scored by three observers blinded to the study on a scale of 0 to 4 for SPARC protein in the tumor and stroma.

### *Cell Proliferation Assays and Doubling Time Measurements*

*Bromodeoxyuridine proliferation assay.* Proliferation was measured using bromodeoxyuridine (BrdU) incorporation as a marker of DNA synthesis. The Cell Proliferation Biotrak ELISA System (Amersham Biosciences, Piscataway, NJ) was used according to the manufacturer's protocol. A total of 2500 cells in 100 μl of growth media were seeded in a 96-well plate in triplicate. After being allowed to adhere overnight, the media were switched to the respective experimental conditions (with or without exogenous SPARC), and the cells were incubated for 48 hours. After 48 hours, the cells were labeled with 100 μM BrdU for 3 hours at 37°C and 5% $CO_2$ in a humidified incubator. Cells were then fixed, incubated in blocking buffer, and treated with anti-BrdU antibody for 90 minutes. The cells

were washed extensively with the provided wash buffer, developed with tetramethylbenzidine substrate for 20 minutes and stopped with 1 M sulfuric acid, and the absorbance was read at 450 nm.

*Calculation of doubling time.* Ovarian cancer cell lines were transduced with an adenovirus-overexpressing SPARC to assess the effect of SPARC expression on doubling times. Generation of replication-deficient adenoviruses expressing either green fluorescent protein (GFP) or SPARC-GFP constructs under the control of the cytomegalovirus promoter was reported previously [18]. After viral infection, the ovarian cancer cells were seeded in 24-well plates for 96 hours in three groups: wild type (WT) untransduced cells, cells transduced with GFP construct (GFP), and cells transduced with SPARC-GFP construct (SPARC-GFP). Cells from each group were trypsinized and counted to determine the doubling time. The experiments were performed in quadruplicates, and the results are expressed as the mean ± SEM.

*Nonradioactive cell proliferation assay.* Cell proliferation was measure using a CellTiter$^{96}$ kit (Promega) as previously described [18]. Previously, adenovirus-infected cells (described above) were seeded in 96-well plates and allowed to adhere overnight in growth media. The next day, the number of proliferating cells was determined colorimetrically by measuring the absorbance at 590 nm 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, as recommended by the manufacturer. The experiments were performed in triplicates, and the results are expressed as the mean ± SEM.

### *Chromatin Immunoprecipitation*

Chromatin immunoprecipitation studies were performed with Imprint ChIP kit (Sigma). SKOV3 cells ($1 \times 10^7$) were cross-linked with 1% formaldehyde (Pierce), and the cell nuclei were isolated, incubated with cell lysis buffer, and sonicated with 15 × 5-second pulses. Equal amounts of sonicated chromatin were immunoprecipitated with 1 μg of each of the following antibodies: mouse IgG, RNA polymerase II (provided in the ChIP kit), DNMT1, DNMT3a, and DNMT3b (Epigentek). The chromatin was washed, and the cross-links were hydrolyzed. The DNA was then purified through DNA binding column and subjected to qPCR analysis. The PCR primers were derived from a 1.3-kb region upstream of *Sparc* translation start site, the sequences of which contain CpG islands.

### *Statistical Analysis*

Scoring of the arrays was analyzed for statistical significance using a one-way analysis of variance followed by a Bonferroni correction. Doubling times and proliferation data are presented as mean ± SEM. To determine statistical differences between groups in the proliferation assays and ChIP assays, statistical differences were determined by 2-tailed *t* tests. A *P* value of <.05 was considered statistically significant for all data.

## Results

### *Decreased Expression of SPARC in Ovarian Cancer Is Caused by Aberrant Methylation*

SPARC expression was examined by RT-PCR in nine ovarian cancer cell lines, two immortalized control lines, and three nonimmortalized HOSE primary cultures. *Sparc* mRNA expression was present in HOSE 1-15 and Meso 301 (data not shown) cell lines as well as in all three of the NHOSE samples (Figure 1*A*). However, expression was decreased in three (33%) and absent in four (44%) of the nine ovarian cancer cell lines investigated. Only DOV 13 and PA-1 showed *Sparc* mRNA levels comparable to those of the positive controls. Western blot analysis of the cell lines confirmed that SPARC protein was present in HOSE 1-15 and Meso 301 cell lines, as well as in all three of the NHOSE samples (Figure 1*B*). Conversely, SPARC protein was absent in the lysates from seven (77%) of the ovarian cancer cell lines. Only DOV 13 and PA-1 exhibited SPARC protein expression, albeit at a significantly reduced level compared to positive controls. This discrepancy can be attributed, at least in part, to differences in posttranscriptional regulation of SPARC (e.g., differences in the rates of translation, RNA/protein stability, and/or expression of microRNAs) in normal *versus* malignant cells. Investigation by MSP revealed that in the cell lines lacking expression of SPARC, the *Sparc* gene was methylated (Figure 1*C*). Conversely, DNA from normal samples, including Meso 301, HOSE 1-15, and three NHOSE samples, showed no methylation of the *Sparc* gene.

### *Rescue of SPARC Expression by 5-Aza-CdR Treatment*

To confirm that methylation of the *Sparc* gene is responsible for the loss of SPARC protein expression, the ovarian cancer cells lines that were positive for *Sparc* methylation were treated with the demethylating agent 5-Aza-CdR. The 5-Aza-CdR treatment was able to restore SPARC mRNA expression in the cell lines that did not originally express SPARC (SKOV3, TOV112, OVCAR3) but had no significant effect on HOSE 1-15 or Meso 301 (Figure 2*A*). Moreover, protein expression was restored in five (71.4%) of the seven cell lines previously lacking SPARC expression (Figure 2*B*). OV-90 and TOV21 showed no significant increase in SPARC protein expression. Conversely, PA-1 responded well to 5-Aza-CdR treatment regardless of its relatively high basal levels of SPARC protein, suggesting that this cell line has hemimethylation of the *Sparc* promoter (one allele methylated, one unmethylated). We also investigated whether histone acetylation played a role in the regulation of *Sparc* transcription. Treatment of the ovarian cancer cell lines with the histone deacetylase inhibitor TSA did not significantly enhance *Sparc* mRNA expression, except for OV-90 (Figure 2*C*). These results suggest that other epigenetic mechanisms, bedsides methylation, may account for the regulation of SPARC in a minor subpopulation of epithelial ovarian carcinoma cell lines.

### Sparc *Is Aberrantly Methylated in Primary Ovarian Tumors*

In an effort to show that the methylation of *Sparc* is not an artifact found only in cultured cells, we obtained 22 high-grade, primary serous ovarian tumor samples for analysis. The samples were microdissected by LCM to ensure minimal stromal cell contamination of the tumor samples. Methylation-specific PCR of the tumors showed that of the 22, 15 (68.2%) had full methylation of the SPARC promoter, 5 (22.7%) showed hemimethylation, and only 2 (9.1%) showed no methylation. A representative MSP analysis of these tumors is shown in Figure 3*A*. In addition to looking at the methylation status of *Sparc*, we obtained two ovarian cancer tumor arrays containing a total of 133 samples of serous ovarian carcinoma at varying disease stages (TNM stages 1-3) and grades (I-III) to investigate the levels of SPARC protein in primary tumors by immunohistochemistry (T1 N*x* M*x*, $n = 118$; T2 N*x* M*x*, $n = 13$; T3 N*x* M*x*, $n = 2$; grade I, $n = 17$; grade II, $n = 38$; grade III, $n = 78$). The results showed that significantly lower levels of

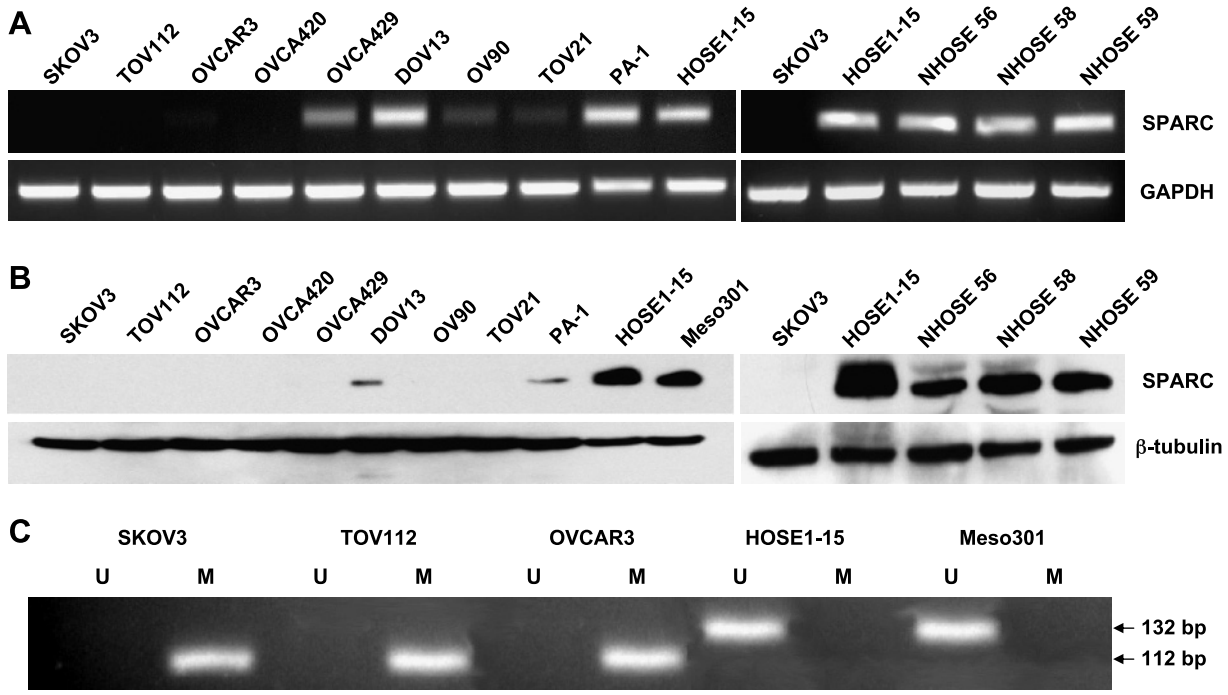


**Figure 1.** *SPARC* expression is reduced in ovarian cancer cell lines caused by aberrant methylation. (A) Expression levels of SPARC measured by RT-PCR in nine ovarian cancer cell lines, one immortalized control cell line (HOSE 1-15), and three primary cell lines (NHOSE 56, 58, and 59). Pictures shown are representative of three independent experiments. (B) Expression of SPARC protein from nine ovarian cancer cell lines, two immortalized control cell lines (HOSE 1-15 and Meso 301), and three primary cell lines measured by Western blot. Blots shown are representative of three independent experiments. (C) Representative examples of MSP of the *Sparc* promoter in three ovarian cancer cell lines and two immortalized control cell lines. Results shown are representative of three independent experiments.

SPARC protein are present in the tumors compared to the surrounding normal stroma, suggesting that the tumor production of SPARC is diminished (Figure 3*B*). In addition, our results indicate that there is a clear decrease in the levels of SPARC protein in both the tumor and the surrounding stromal cells as the tumor becomes more undifferentiated (higher grade).

### *Ectopic Overexpression or Exogenous Addition of SPARC Inhibits Proliferation of Ovarian Cancer Cells*

To determine the significance behind the methylation of *Sparc* in ovarian cancer, we investigated the effects of both addition of exogenous SPARC and restoring SPARC expression on the ovarian cancer cell lines lacking SPARC (Figure W1). Measurement of proliferation rates by BrdU incorporation after the addition of exogenous SPARC protein to culture media of ovarian cancer cells showed a significant decrease in proliferation (Figure 4*A*). Proliferation rates decreased by 39.9% for SKOV3, 26.6% for TOV112, 37.2% for OVCAR3, and 14.1% for OVCA 420. In addition, inducing the ovarian cancer cells to express SPARC through the use of an adenovirus resulted in both an increase in their doubling time (SKOV3, 103.6%; TOV112, 215.7%; OVCAR3, 55.4%; OVCA 420, 156.0%; DOV 13, 128.9%; TOV21, 55.0%; Figure 4*B*) and a decrease in their rate of proliferation as measured by an MTS assay (SKOV3, 26.0%; TOV112, 32.1%; OVCAR3, 25.3%; OVCA 420, 37.5%; DOV 13, 38.3%; TOV21, 24.8%; Figure 4*C*).

### *Association of DNMT3a with* Sparc *Promoter in SKOV3 Cells*

In an effort to determine if the aberrant methylation of *Sparc* promoter was the result of changes in DNMT expression, we examined the expression of the DMNT isoforms DNMT (1, 3a, and 3b) through RT-PCR. DNMT1 is a maintenance methyltransferase, responsible for adding methyl groups to hemimethylated DNA during replication, whereas DNMT3a and DNMT3b are *de novo* methyltransferases, adding methyl groups to CpG islands that are initially unmethylated [22]. Whereas DNMT1 and DNMT3b showed no significant differences in mRNA expression levels (data not shown), DNMT3a expression levels were found to be elevated in the nine ovarian cancer cell lines, relative to the normal HOSE controls (Figure 5*A*). Comparison of basal levels of DNMT protein levels in SKOV3 and OVCAR3 ovarian cancer cell lines revealed abundant levels of DNMT3a, low levels of DNMT1, and undetectable levels of DNMT3b (Figure 5, *B* and *C*). It has been reported that 5-Aza-CdR is a potent inhibitor of DNMT1 and DNMT3a but not DNMT3b in colon cancer cells [23]. Our results showed that 5-Aza-CdR treatment significantly suppressed protein levels of DNMT3a in both SKOV3 and OVCAR3, whereas levels of DNMT1 were only significantly reduced in SKOV3 cells. As expected, expression of SPARC was increased in both cell lines.

To determine the mechanism by which the methylation of *Sparc* promoter is regulated, we used a ChIP assay to identify which DNMT associates with *Sparc* promoter in SKOV3 cells. Our results showed that DNMT3a antibody precipitated *Sparc* promoter DNA detected by qPCR (approximately four-fold over IgG control), suggesting that *Sparc* promoter in SKOV3 is associated with DNMT3a but not DNMT1 or DNMT3b (Figure 5*D*). As a positive control, RNA polymerase II was shown to be associated with *Sparc* promoter (14-fold over IgG control). Furthermore, 5-Aza-CdR treatment resulted in a significant (~1.5-fold) decrease in the association of DNMT3a with

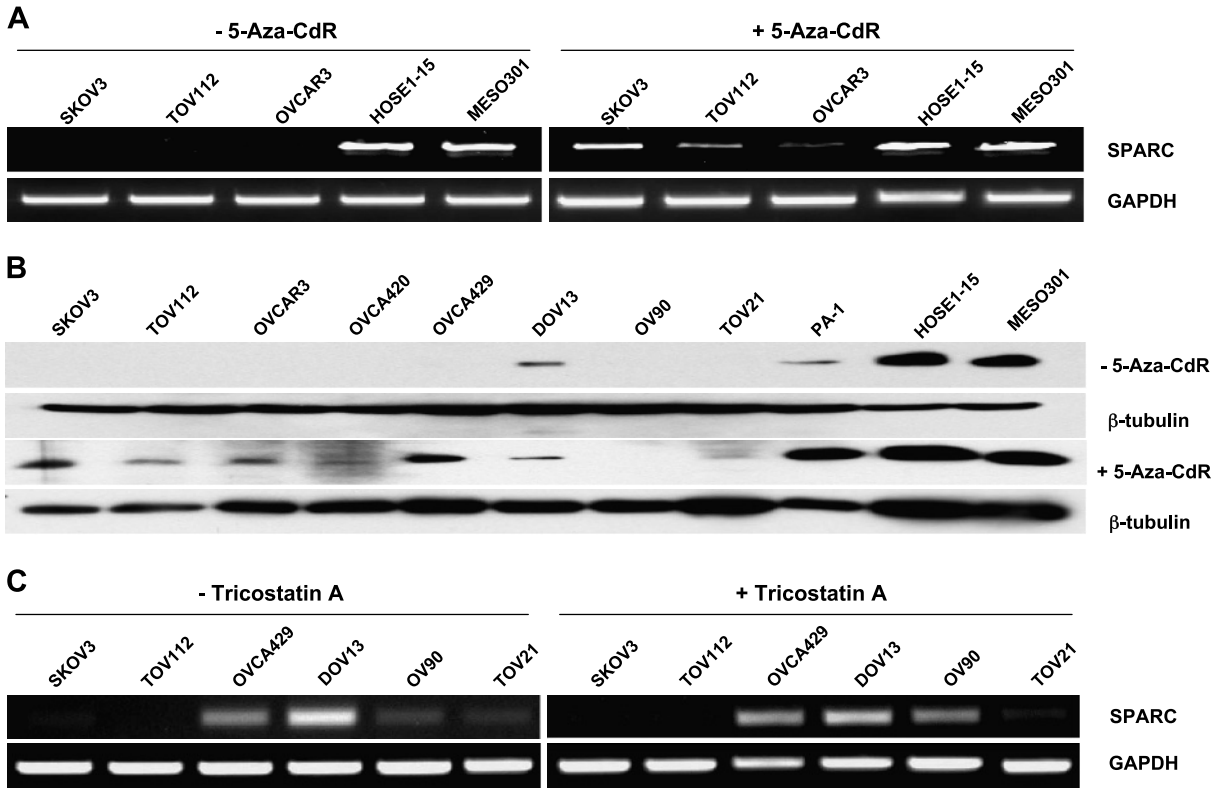


**Figure 2.** Restoration of *Sparc* mRNA and protein expression through global demethylation is independent of histone acetylation *in vitro*. (A) Expression of *Sparc* mRNA measured by RT-PCR from three representative ovarian cancer cell lines and two normal control cell lines before (right) and after (left) treatment with 5-Aza-CdR. Pictures shown are representative of three independent experiments. (B) Expression levels of SPARC protein measured by Western blot analysis in nine ovarian cancer cell lines and two normal control cell lines before (top) and after treatment (bottom) with 5-Aza-CdR. Blots shown are representative of three independent experiments. (C) Expression of SPARC mRNA as measured by RT-PCR from six representative ovarian cancer cell lines before (left) and after (right) treatment with TSA, a histone deacetylase inhibitor. These results confirm that, with the exception of DOV 13 cell line, histone acetylation seems to play no significant role in SPARC expression. Results shown are representative of three independent experiments.

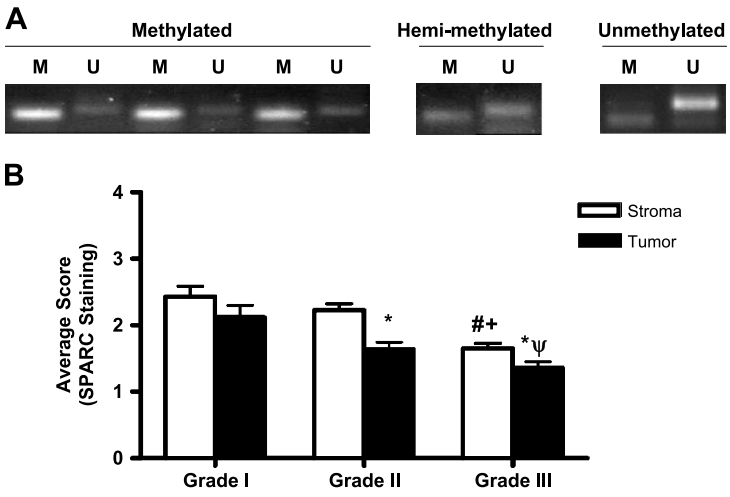


**Figure 3.** *Sparc* is aberrantly methylated in ovarian cancer tumor tissue, and this methylation results in an inverse correlation between disease stage and SPARC levels. (A) Methylation-specific PCR analysis of the *Sparc* promoter from laser-captured ovarian tumor samples. Images are representative of methylation, hemimethylation, and no methylation of the promoter. Pictures shown are representative of three independent experiments. (B) Graphical representation of the relative levels of SPARC protein present in tumor samples of varying grades. Images were blindly scored on a scale of 0 to 4 for SPARC staining intensity ($^{*}P < .05$ *vs* stroma; $^{\#}P < .05$ *vs* grade I stroma; $^{+}P < .05$ *vs* grade II stroma; $^{\Psi}p < .05$ *vs* grade I tumor).

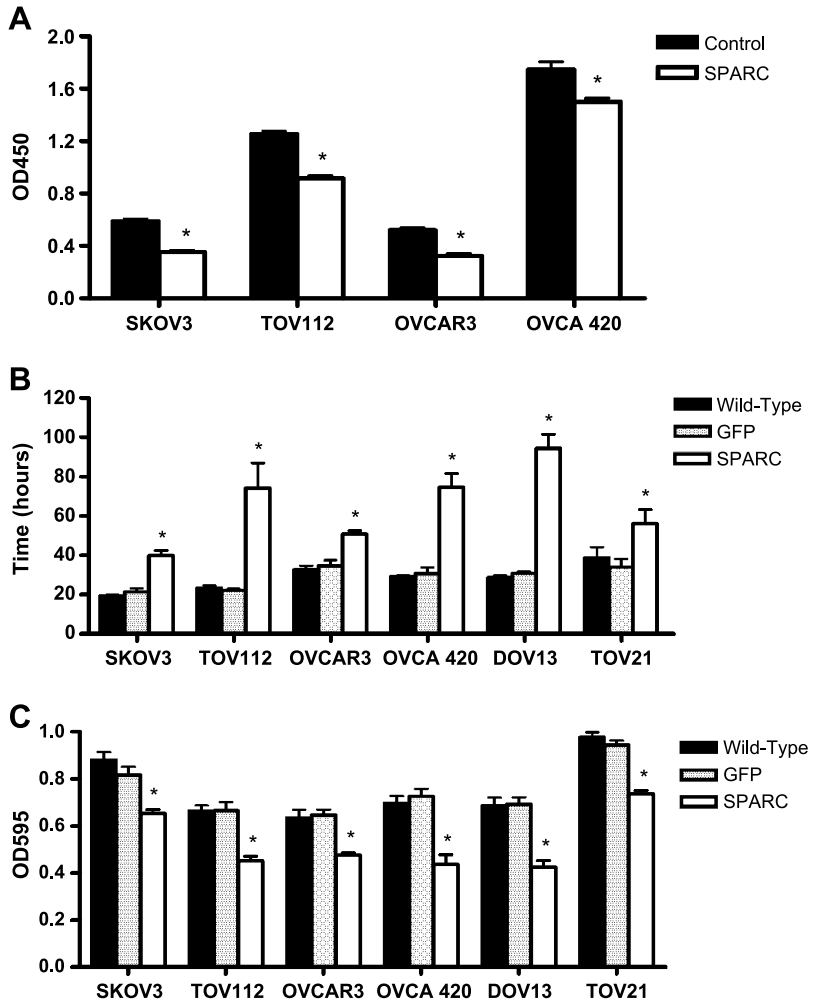


**Figure 4.** Exogenous SPARC inhibits proliferation of ovarian cancer *in vitro*. (A) BrdU incorporation in the absence or presence of exogenous SPARC (40 $\mu$g/ml) shows that in the four ovarian cancer cell lines, SPARC inhibits proliferation. Results shown are expressed as the mean ± SEM and were representative of two independent experiments performed in quadruplicates (*$P < .05$). (B) Measurements of the doubling time of six of the ovarian cancer cell lines showed significant increases after induction of SPARC production through an adenoviral vector (*$P < .05$). (C) MTS assay on the six ovarian cancer cell lines expressing SPARC after adenoviral infection shows a significant decrease in the proliferation rate of the cells. Results shown are expressed as the mean ± SEM and were representative of two independent experiments performed in triplicates (*$P < .05$).

the *Sparc* promoter (Figure 5*E*). Taken together, these results suggest that DNMT3a is mostly responsible for the aberrant *de novo* methylation of *Sparc* promoter, resulting in down-regulation of SPARC expression in ovarian cancer cell lines.

## Discussion

In this study, we sought to determine the mechanism through which SPARC expression is diminished in human ovarian carcinoma. Our results indicate that hypermethylation of the promoter sequence is the primary mechanism through which SPARC is downregulated. Previous groups have demonstrated that in multiple neoplasms, including pancreatic, lung, colon, and endometrial carcinomas, the downregulation of SPARC expression was the result of epigenetic silencing through methylation of the *Sparc* promoter [21,24–26]. In concordance with these studies, we observed that the loss of SPARC expression in ovarian carcinoma correlated with increased methylation and that this loss of expression could be rescued in ovarian cancer cell lines on treatment with the demethylating agent 5-Aza-CdR. These findings indicate that although methylation may not be the only mechanism down-regulating SPARC, it is the primary mechanism in ovarian cancer.

It has been hypothesized that CpG island hypermethylation is simply a means to enable gene silencing through reorganization of chromatin into a silenced state through histone deacetylation [27]. This is supported by the evidence that inhibition of histone deacetylase restored the expression of methylated genes [28]. It has also been suggested that methylation and histone deacetylation act in synergy, as multiple layers of epigenetic gene silencing [29]. Our results using 5-Aza-CdR and TSA indicate that inhibition of DNA methylation, but not histone deacetylation, was sufficient to restore expression of SPARC in most of the cell lines investigated. However, only OV-90 cell line showed a significant increase in *Sparc* mRNA after TSA treatment but did not restore SPARC protein expression after 5-Aza-CdR treatment. These data suggest that in the OV-90 cell line, SPARC is most likely downregulated through histone deacetylation

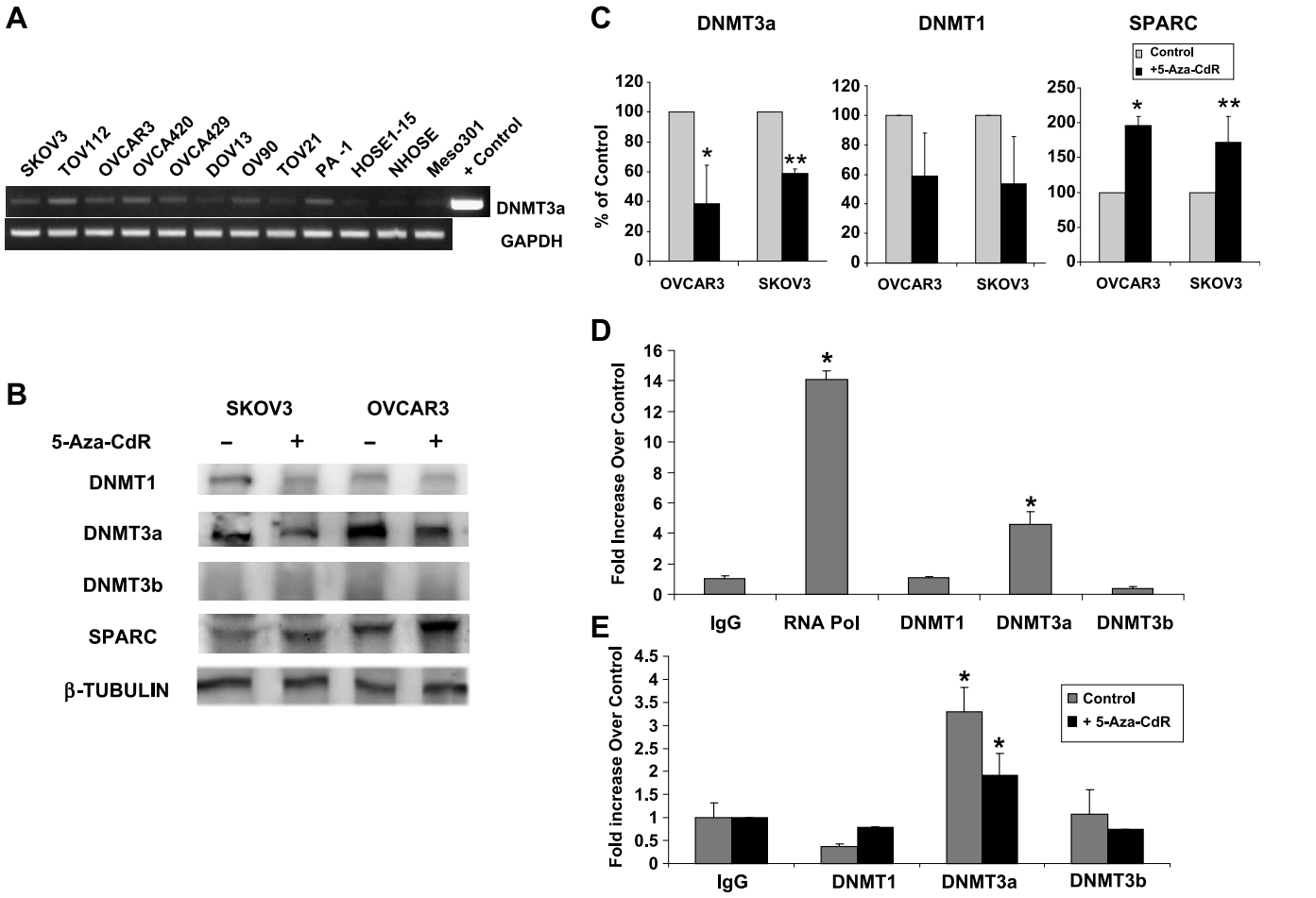


**Figure 5.** DNMT3a associates with *Sparc* promoter in ovarian cancer cells. (A) Expression of DNMT3a mRNA measured by RT-PCR in nine ovarian cancer cell lines and one immortalized control cell line show that this DNMT isoform is upregulated in ovarian cancer cells. The human breast cancer cell line MCF7, overexpressing the DNMT3a isoform, was used as a positive control. Pictures shown are representative of three independent experiments. (B) Basal and 5-Aza-CdR–treated protein levels of SPARC and DNMT isoforms in SKOV3 and OVCAR3 cell lines. 5-Aza-CdR treatment resulted in a significant increase in SPARC expression, but only significantly reduced DNMT3a levels. (C) Protein band intensities from three independent experiments were quantified by densitometry. Changes in the band intensity of DNMT1, DNMT3a, and SPARC in 5-Aza-CdR–treated samples, relative to untreated controls (set at 100%), are expressed as the mean ± SEM. DNMT3a, but not DNMT1, was reduced significantly, whereas SPARC was increased significantly in 5-Aza-CdR–treated OVCAR3 (*$P < .05$) and SKOV3 cell lines (**$P < .05$) relative to controls. (D) Chromatin immunoprecipitation analysis in SKOV3 cells revealed a significant DNMT3a association with *Sparc* promoter, relative to IgG-negative controls (set as 1). RNA polymerase II (RNA Poly) association was used as a positive control for the assay. (E) Chromatin immunoprecipitation analysis in SKOV3 cells in the absence or presence of 5-Aza-CdR revealed a significant decrease only in DNMT3a levels relative to respective IgG-negative controls (set as 1). Results shown are representative of two independent experiments performed in triplicates (*$P < .05$ relative to IgG controls).

but not promoter hypermethylation. However, the preponderance of evidence suggests that hypermethylation of the promoter region is the primary mechanism responsible for SPARC down-regulation.

In our present study, we investigated whether changes in DNMT expression were responsible for the aberrant methylation of the *Sparc* promoter. No differences were observed in the mRNA or protein expression levels of DNMT1 and DNMT3b mRNA, but a significant increase in that of DNMT3a in most of the ovarian cancer cell lines, compared to the immortalized HOSE controls. There is controversy regarding correlations between DNMT overexpression, CpG island hypermethylation, and neoplasm malignancy. Whereas some studies have shown a direct correlation between up-regulation of DNMTs and the development of neoplasms [30,31], others have reported no such associations [32,33]. Down-regulation of SPARC in ovarian carcinoma seems to enhance growth and metastasis of the neoplasm. We have previously shown that SPARC acts as a negative regulator of ovarian cancer metastasis in mice [16]. This suggests that the expression of SPARC could act to impair ovarian carcinoma growth. A previous study also concluded that SPARC is upregulated in reactive stroma, particularly at the tumor-stromal interface of the invading tumors [34]. Moreover, it is in accordance with our findings that both exogenous SPARC and ectopic SPARC expression inhibit the proliferation of ovarian cancer cells. Interestingly, a recent report revealed that transfer of normal human chromosome 3p fragments into ovarian cancer cells suppressed their tumorigenicity, which was correlated with the up-regulation of SPARC [35]. These studies, along with the results presented herein, persuasively implicate SPARC as a tumor suppressor in ovarian carcinoma.

The results of the ovarian cancer tumor tissue array indicated an inverse correlation between SPARC protein expression and tumor

grade. These results confirm an earlier study by Yiu et al. [19]. Scoring of >130 sections of serous ovarian tumor tissues on the tissue arrays revealed a clear inverse correlation between disease grade and SPARC protein expression. Furthermore, there was a statistically significant decrease in the stromal staining at grade III of the disease. These results are conclusive, owing to the high number of samples analyzed on the array. Consistent with the array data were our results on the methylation of the *Sparc* promoter in primary tumor tissues. Methylation-specific PCR results from highly undifferentiated (grade III) LCM tumor tissues confirmed that *Sparc* promoter was fully methylated in a high percentage of samples analyzed. However, owing to the small number of tumor tissue samples available for analysis, it was not possible to fully correlate these findings to those of our array results. Further study is needed to analyze a large number of tumor samples of various grades to confirm the association between high tumor grade, promoter methylation, and SPARC expression.

That exogenous SPARC and ectopic SPARC expression through adenovirus both impair ovarian cancer cell proliferation indicates that although the cells themselves do not produce SPARC, they retain responsiveness to SPARC. This supports the hypothesis of an as yet unidentified endogenous SPARC receptor. Support for this hypothesis can also be found in the previously mentioned studies, all of which show that neoplasms that do not express SPARC remain reactive to the protein [19,34,35]. Moreover, a recent report identified *Sparc* methylation as a possible biomarker of squamous intraepithelial lesions (SILs) and invasive cervical cancer [36]. In that study, methylation of *Sparc* observed in liquid Papanicolaou tests occurred more frequently in high-grade SIL than in low-grade SIL and nonmalignant intraepithelial lesions. These studies, along with our current data, support the hypothesis that the methylation status of *Sparc* in ovarian epithelial cells could potentially be used as a diagnostic tool as well as a therapeutic treatment of ovarian carcinoma to limit proliferation, induce apoptosis, and prevent metastasis.

In conclusion, we have demonstrated that SPARC expression is significantly downregulated in ovarian cancer cells mainly due to the aberrant methylation of its promoter region by DNMT3a. Furthermore, this down-regulation is necessary for the progression of ovarian cancer, as ectopic overexpression by adenovirus or addition of exogenous SPARC protein were shown to inhibit the proliferation of ovarian carcinoma cells. Taken together, these findings highlight a key role for the methylation of *Sparc* in the pathogenesis of ovarian cancer.

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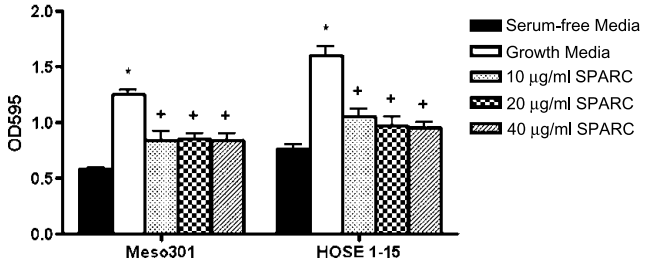


**Figure W1.** Exogenous SPARC inhibits proliferation of normal human ovarian cells and mesothelial cells. Addition of exogenous SPARC (10-40 $\mu$g/ml) to nonmalignant, immortalized human ovarian cell line HOSE 1-15 and human mesothelial cell line Meso 301 with high SPARC expression levels resulted in a significant decrease in the proliferation rate of the cells as determined by MTS assay. Results shown are expressed as the mean $\pm$ SEM and are representative of two independent experiments performed in triplicates ($^{*}P < .05$ *vs* serum-free media, $^{+}P < .05$ *vs* growth media).