Signaling and Regulation

Downregulation of Filamin A Interacting Protein 1-Like is Associated with Promoter Methylation and Induces an Invasive Phenotype in Ovarian Cancer

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Abstract

Ovarian cancer is the most lethal gynecologic malignancy with a five-year survival rate below 25% for patients with stages III and IV disease. Identifying key mediators of ovarian cancer invasion and metastasis is critical to the development of more effective therapeutic interventions. We previously identified Filamin A interacting protein 1-like (FILIP1L) as an important mediator of cell proliferation and migration. In addition, targeted expression of FILIP1L in tumors inhibited tumor growth in vivo. In our present study, we confirmed that both mRNA and protein expression of FILIP1L were downregulated in ovarian cancer cells compared with normal ovarian epithelial cells. FILIP1L expression was inversely correlated with the invasive potential of ovarian cancer cell lines and clinical ovarian cancer specimens. We also provide evidence that DNA methylation is a mechanism by which FILIP1L is downregulated in ovarian cancer. The CpG island in the FILIP1L promoter was heavily methylated in ovarian cancer cells. Methylation status of the FILIP1L promoter was inversely correlated with FILIP1L expression in ovarian cell lines and clinical ovarian specimens. Reduced methylation in the FILIP1L promoter following treatment with a DNA demethylating agent was associated with restoration of FILIP1L expression in ovarian cancer cells. A transcription activator, cAMP-responsive element binding protein (CREB) was shown to bind to the CREB/ATF site in the CpG island of the FILIP1L promoter. Overall, these findings suggest that downregulation of FILIP1L associated with DNA methylation is related with the invasive phenotype in ovarian cancer and that modulation of FILIP1L expression has the potential to be a target for ovarian cancer therapy. Mol Cancer Res; 9(8); 1–13. ©2011 AACR.

Introduction

Ovarian cancer is the second most common gynecologic malignancy but the most common cause of gynecologic cancer-related death. Most patients are diagnosed with advanced stage disease, and the 5-year survival rate is below 25% for stages III and IV disease (1). The standard cytotoxic systemic therapies following surgical cytoreduction are carboplatin and paclitaxel (www.nccn.org). Cure rates for this approach are less than 40% with overall survival averaging 50 months. Recurrences are common and, therefore, gynecologic oncologists often enroll patients in clinical trials for platinum-resistant disease recurrence. Researchers are making modest advances in the development of targeted therapy to improve the overall survival in ovarian cancer (2). A better understanding of the pathways involved in ovarian cancer invasion and spread are critical to the development of more effective therapies, especially for patients with advanced or refractory disease.

We identified Filamin A interacting protein 1-like (FILIP1L; previously known as downregulated in ovarian cancer 1) as a mediator of cellular proliferation and migration in both normal and tumor cells (3, 4). We showed that overexpression of FILIP1L resulted in inhibition of cell proliferation and migration and increased apoptosis (5). Overall, these findings suggested that the novel protein FILIP1L may be an important mediator of cell growth and migration and may play a role in defining the invasive potential of tumor cells.
**Molecular Cancer Research**

**Materials and Methods**

**Cell culture**

Normal ovaries were obtained from the operating room under an Institutional Review Board (IRB) exemption at Montefiore Medical Center, NY, and HOSE cells were cultured following a published protocol (12). Immortalized normal ovarian surface epithelial (IOSE) cells including IOSE 144, 523, and 385 were provided by the Canadian Ovarian Tissue Bank and cultured in M199:MCDB105 (1:1) containing 5% FBS. Human ovarian cancer cell lines including ES2, SKOV3, and OV90 were purchased from the American Type Culture Collection. ES2 and SKOV3 cells were cultured in McCoy’s 5a containing 10% FBS, and OVCA8 was cultured in RPMI 1640 containing 10% FBS. OVCA429 was provided by Dr. Barbara Vanderhyden, University of Ottawa, Ontario and was cultured in MEM containing 10% FBS and 1% nonessential amino acids.

**Clinical specimens**

Formalin-fixed paraffin-embedded (FFPE) tissue blocks of ovarian serous borderline tumor \( n = 10 \) and ovarian serous carcinoma \( n = 17 \) were obtained from Pathology at Montefiore Medical Center, under our IRB exemption. The criteria for noninvasive serous borderline tumor were tumor composed of arborizing papillary structures lined by a proliferating population of serous epithelial cells, which show stratification, cytologic atypia, mitotic activity, and tufting of cells (destructive invasion is not present). The criteria for invasive serous carcinoma were tumors that showed all of the above with destructive or frank stromal invasion and usually with marked cytologic atypia. Three different regions containing more than 90% tumor cells were scraped from the tissue section of each specimen (7 μm). Genomic DNA and total RNA were purified from the same scraped tissue by using the AllPrep DNA/RNA FFPE Kit (Qiagen). Real-time reverse transcriptase PCR (RT-PCR) and Sequenom EpiTYPER Mass Array were carried out as described in the following sections.

**5-Aza-2'-deoxycytidine and trichostatin A treatment**

Ovarian cancer cells were seeded in 6-well plates at a density of \( 1 \times 10^5 \) cells per well 16 hours before treatment. Cells were treated with 5-aza-2'-deoxycytidine (DAC; Sigma–Aldrich) daily for 72 hours or with Trichostatin A (TSA; Sigma–Aldrich) once for 24 hours.

**Transfection of cells with FILIP1L plasmids**

Cloning of **FILIP1LAC103** (amino acid 1–790) was described previously (5). Plasmids were purified by using Endo-free maxiprep kit (Qiagen). ES2 and SKOV3 cells were transfected with equimolar amounts of either control empty plasmid or plasmid encoding **FILIP1LAC103** using X-fect solution following the manufacturer’s protocols (Clontech). Twenty-four hours after transfection, the cells were subjected to cell invasion assay and Western blot analysis.

**Quantitative real-time RT-PCR**

HOSE cells, IOSE cells, and ovarian cancer cells, either untreated or DAC- and TSA-treated, were cultured and harvested at approximately 80% confluence. Total RNA was prepared by RNeasy kit (Qiagen), and cDNA was prepared by Superscript II reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was carried out by using ABI 7900HT SDS real-time PCR instrument as per manufacturer (Applied Biosystems). Expression of the **FILIP1L** gene was normalized to **hRPL7** gene expression. The primers used were 5'-ACGGCTGTATACGCTGCAA-3' and 5'-ATCTCTGACCTGCTCCTCATT-3' for **FILIP1L** (probe A); 5'-AAGAAGCGAATTGCTTTGACAGA-3' and 5'-AACGCTGGTATCATGGCTGAA-3' for **hRPL7**. For FFPE tissue specimens, another set of **FILIP1L** probe (probe B; 5'-GGACATTTGAGATGATGATC-3') was also used.

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**FILIP1L** mRNA was originally characterized by its presence in human ovarian surface epithelial (HOSE) cells and its absence in ovarian carcinoma cells (6). By using cDNA microarray analysis, **FILIP1L** was shown to be preferentially expressed in HOSE cells as opposed to ovarian carcinoma cells from patients with stage IIC and IV disease (7). Additionally, differential gene expression analysis revealed that the **FILIP1L** gene in ovarian cancer has several tagging single nucleotide polymorphisms (8). **FILIP1L** was shown to be one of 9 genes associated with functional suppression of tumorigenicity in ovarian cancer cell lines (9).

On the basis of these observations, we asked whether **FILIP1L** expression was inversely correlated with the degree of invasive potential or aggressive histologic morphology and behavior of ovarian cancer. Further, because epigenetic aberrations are intimately associated with ovarian tumorigenesis (10, 11), we examined whether or not the control of **FILIP1L** expression was mediated through epigenetic mechanisms.

In the present study, we show that cellular invasion and aggressive histology and behavior are inversely correlated with **FILIP1L** gene and protein expression in ovarian cell lines and clinical ovarian samples. We observed that overexpression of **FILIP1L** inhibited the invasive potential of an aggressive ovarian cancer cell line. We conclude that DNA methylation is a mechanism by which **FILIP1L** is downregulated in ovarian cancer and that the DNA methylation status of the **FILIP1L** promoter is inversely correlated with **FILIP1L** expression in ovarian cell lines as well as clinical ovarian specimens. Taken together, these data suggest that the degree of **FILIP1L** expression may be a predictor of ovarian cancer behavior and further, the modulation of **FILIP1L** expression in ovarian cancer may be a useful target for the development of novel ovarian cancer therapies.

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Western blot
Whole cell lysates were prepared from radioimmunoprecipitation assay buffer, separated on SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blotted with antibodies against FILIP1L (S) and GAPDH (Chemicon) followed by incubation with anti-mouse antibody conjugated to horseradish peroxidase. The signal was detected using chemiluminescence (Millipore).

Pull-down assay
Nuclear extracts were prepared from IOSE523 cells by using NE-PER nuclear protein extraction kit (Thermo Scientific) as recommended by the manufacturer. Custom-synthesized biotinylated oligos were purchased from Operon. Nuclear extracts (200 µg) were incubated with or without biotinylated oligo (4 µg) in the presence of streptavidin-sepharose (Cell Signaling Technology) at 4°C for 2 hours. The precipitated immune complex was subject to immunoblot analysis with anti-c–AMP-responsive element binding protein (CREB) antibody (86B10; Cell Signaling Technology).

Methylation analysis
Genomic DNA was extracted by using Wizard SV Genomic DNA purification kit (Promega). Bisulphite modification was carried out using EZ DNA Methylation kit (Zymo Research) following manufacturer’s instructions. Bisulphite-modified DNA was subjected to nested PCR by using HotStar Taq DNA Polymerase kit (Qiagen) following manufacturer’s instructions. Primers were selected with MethPrimer software (http://www.urogene.org/methprimer/). The nested PCR primers used were 5'-aggagaagagGGATGTGTATTGAAGTTTTTGAAGTAA-3' and 5'-cagtaatacgactcactatagggagaaggctTCTAACAACAATAC-3' for the first reaction; 5'-aggagaagagAGTTTTAGGGATTTAGAGGGAAAAA-3' and 5'-cagtaatacgactcactatagggagaaggctTCTAACAACAATAC-CCCTTAAATAAA-3' for the second reaction [forward primer sequences contain a 10-bp tag at their 5' ends (aggaagag); reverse primer sequences contain a 31-bp tag at their 5' ends (cagtaatacgactcactatagggagaagct)]. Quantitative DNA methylation was analyzed by Sequenom Epityper Mass Array (13–16). The assays were conducted using the company’s standard protocol through Genomics Shared Facility at Albert Einstein College of Medicine, New York, NY. Matched peak data were exported using Epityper software and analyzed quantitatively.

Immunohistochemistry
FFPE tissue blocks of ovarian serous borderline tumor and ovarian serous carcinoma were obtained as described above. FFPE tissue sections (n = 10 each) were deparaffinized and hydrated in xylene and serial alcohol solutions, respectively. Endogenous peroxidase was blocked by incubation in 3% H2O2 for 10 minutes. Antigen retrieval was carried out in a steam pressure cooker with prewarmed antigen retrieval buffer, pH 9 (DakoCytomation) at 95°C for 20 minutes. To minimize nonspecific staining, the section was incubated with protein block (DakoCytomation) for 10 minutes. After washing with a buffer containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween-20, the specimen was incubated with 7 µg/mL anti-FILIP1L antibodies at room temperature for 2 hours. Antigen–antibody reactions were detected with DAKO Flex+ linker (Dako). The staining was visualized by using 3,3'-diaminobenzidine plus (Dako). The staining was lightly counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. Images were acquired by AxioImager microscope (Zeiss). A second pathologist scored the staining under blinded conditions. FILIP1L cytoplasmic staining was scored according to the staining intensity [categorized as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong)].

Cell invasion assay
IOSE cells and ovarian cancer cells either untransfected or FILIP1LΔC103-transfected were cultured at approximately 80% confluence. Cells were starved in basal medium containing 0.2% bovine serum albumin for 16 hours. Matrigel invasion was measured by using the BD BioCoat Tumor Invasion System (BD Biosciences #354165) as recommended by the manufacturer. It consists of a BD FluoroBlok 24-multiwell insert plate with an 8.0-micron pore size polyethylene terephthalate membrane that is coated with BD Matrigel Matrix. A total of 4.5 × 104 of starved cells were seeded into the apical chambers, and a chemoattractant (10% FBS) was added to the basal chambers. After 20-hours incubation, quantification of cell invasion was achieved by postcell invasion labeling with a fluorescent dye, calcein AM (BD Biosciences), and measuring the fluorescence of invading cells of the underside of the membrane at 494/517 nm (excitation/emission). Because the BD FluoroBlok membrane effectively blocks the passage of light from 490 to 700 nm at more than 99% efficiency, fluorescently labeled cells that have not invaded are not detected by a bottom reading fluorescence plate reader. Synergy Mx microplate reader (BioTek) was used to measure fluorescence, and Gen5 software (BioTek) was used to analyzed the data.

Statistical analysis
Statistical analyses were conducted by using a 2-tailed Student’s t test (GraphPad Prism 3.0), and differences were considered statistically significant at a value of P < 0.05. The correlation of the FILIP1L mRNA expression with DNA methylation status of the CpG island in the FILIP1L promoter as well as that with invasiveness of the cells was estimated by Spearman’s rank correlation method (GraphPad Prism 3.0).

Results
Downregulation of FILIP1L in ovarian cancer cell lines
Although FILIP1L mRNA has been shown to be preferentially expressed in HOSE cells compared with ovarian carcinoma cells derived from patients, the quantitative expression of FILIP1L mRNA has not been previously investigated. To test the expression levels of FILIP1L in
Ovarian cells, we measured FILIP1L mRNA expression in HOSE cells as well as in ovarian cell lines by real-time RT-PCR. Three IOSE cell lines as well as various ovarian cancer lines were used. As shown in Figure 1A, mRNA expression of FILIP1L was highest in HOSE cells and higher in IOSE cells compared with ovarian cancer cell lines. To test if the protein expression of FILIP1L is correlated with its mRNA expression in ovarian cells, we measured the FILIP1L protein in these cells by Western blot analysis by using anti-FILIP1L antibody. As shown in Figure 1B, FILIP1L protein expression was much higher in HOSE and IOSE cells compared with ovarian cancer cell lines. In particular, FILIP1L protein was undetectable in ovarian cancer cell lines SKOV3, OVCA429, OCC1, and ES2. These data show that the protein expression of FILIP1L is correlated with its mRNA expression in ovarian cells. Interestingly, although the amount of the full-length FILIP1L protein was correlated with its mRNA expression, FILIP1L-high expressing cells also showed a substantial amount of detection of smaller sized FILIP1L proteins as shown in Supplementary Figure S1. Thus, these findings suggest that FILIP1L expression is downregulated in ovarian cancer cell lines compared with normal ovarian cells.

Inverse correlation of FILIP1L expression with the invasive potential of ovarian cell lines

We tested whether FILIP1L expression is inversely correlated with the invasive potential of ovarian cell lines. We examined the invasive activity for the same cell lines utilized in Figure 1 by Matrigel invasion assay. As shown in Figure 2A, all the cell lines that showed low FILIP1L expression invaded Matrigel significantly more than those that showed high FILIP1L expression. In addition, the FILIP1L mRNA expression showed a significant inverse correlation with invasiveness of the cells (Fig. 2B), suggesting that FILIP1L expression is inversely correlated with the invasive potential of ovarian cell lines. We then tested if overexpression of FILIP1L in FILIP1L-low expressing, highly invasive ovarian cancer cell lines resulted in inhibition of cell invasion. Our previous studies have shown that FILIP1LAC103 (COOH terminal truncation mutant 1-790) was more potent than wild-type FILIP1L in mediating antiproliferative activity in human umbilical vein endothelial cells (HUVEC; ref. 5). In addition, overexpression of FILIP1LAC103 in HUVECs as well as DU145 prostate cancer cells, leads to inhibition of cell migration (5). Thus, we transfected ES2 and SKOV3 cells with a plasmid encoding either control or FILIP1LAC103 cDNA and measured invasion. FILIP1LAC103 protein expression was confirmed by Western blot analysis using anti-FILIP1L antibody (Fig. 2C). As shown in Figure 2D, both cell lines transfected with FILIP1LAC103 cDNA invaded Matrigel significantly less than those transfected with control. We also transfected ES2 cells with a plasmid encoding wild-type FILIP1L cDNA and measured invasion. As shown in Supplementary Figure S3, ES2 cells transfected with wild-type FILIP1L cDNA invaded Matrigel significantly less than those transfected with control. Collectively, these data suggest that downregulation of FILIP1L is associated with an invasive phenotype in ovarian cancer cell lines and that this phenotype can be reversed by overexpression of FILIP1L.

Reduced expression of FILIP1L mRNA in invasive serous carcinomas compared with noninvasive serous borderline tumors

Having observed that FILIP1L expression is inversely correlated with invasive potential in cell lines, we examined if downregulation of FILIP1L expression is also associated with the invasive potential in clinical ovarian specimens. We chose to compare serous borderline tumors (low malignant potential tumors) with invasive serous carcinomas because the former are distinguished from carcinomas by their lack of stromal invasion (17). Total RNA was purified from FFPE tissue sections of ovarian cancer patient specimens and real-time RT-PCR was carried out. As shown in Figure 3A, FILIP1L mRNA expression was significantly lower in invasive serous carcinoma than in noninvasive serous borderline tumors. We confirmed that these data were not produced by artifacts resulting from the handling of FFPE tissues, by real-time RT-PCR using another probe spanning an interexon region of the FILIP1L genome. We observed that FILIP1L mRNA expression was also significantly lower in invasive serous carcinoma than in noninvasive serous borderline tumors using this second probe (Fig. 3B). We then examined FILIP1L protein expression in these tissues using immunohistochemical staining. As shown in Figure 3C and D, FILIP1L expression was significantly lower in invasive serous carcinoma than in noninvasive serous borderline tumors. These findings further suggest that
downregulation of FILIP1L is associated with an invasive phenotype in ovarian cancer.

Methylation in the CpG island of the FILIP1L promoter in ovarian cancer cells

Epigenetic modifications have been shown to be associated with ovarian tumorigenesis (10, 11). A number of genes including the tumor suppressor BRCA1 have been shown to be hypermethylated and downregulated in ovarian cancer. By using MethPrimer software (http://www.urogene.org/methprimer/), we found that the promoter region of FILIP1L has a CpG island of 407 base pairs (Fig. 4A). To investigate whether or not DNA methylation mediates FILIP1L downregulation in ovarian cancer cells, we tested whether the DNA methylation status at the FILIP1L promoter inversely correlates with FILIP1L expression. The percent methylation for each CG site in the CpG island of the FILIP1L promoter is shown in Figure 4B. The average overall methylation for all 21 CG sites is shown in Figure 4C. The analyzed CG sites out of total 59 CG sites in the CpG island of the FILIP1L promoter is shown in Supplementary Table S1. Most cytosines analyzed in the CpG island of the FILIP1L promoter showed similar percent methylation in each cell type (Fig. 4B). The average overall methylation showed that these CG sites were highly methylated in ovarian cancer cells such as ES2 (93.1% ± 0.3%) and OCC1 (88.9% ± 0.5%), whereas these sites were partially

Table 1. XXXX

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</tr>
<tr>
<td>ES2</td>
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methylated in OVCA429 (43.0% ± 0.9%) and SKOV3 (21.7% ± 2.3%; Fig. 4C). In contrast, these sites were unmethylated in normal HOSE (average of 10 HOSE cells; 6.4% ± 0.5%) and IOSE (average of 3 IOSE cell lines; 7.7% ± 0.5%) cells as well as some ovarian cancer cell lines such as OVCAR8 (5.7% ± 0.6%) and OV90 (3.6% ± 0.2%). We tested if the DNA methylation status of the CpG island of the FILIP1L promoter (Fig. 4C) inversely correlated with FILIP1L mRNA expression (Fig. 1A). As shown in Figure 4D, the DNA methylation status of the CpG island of the FILIP1L promoter displayed a significant inverse correlation with FILIP1L mRNA expression, suggesting that DNA methylation in the FILIP1L promoter may mediate FILIP1L downregulation in ovarian cancer cells.

Association of reduced methylation in the FILIP1L promoter with restoration of FILIP1L expression in ovarian cancer cells following treatment with a DNA demethylating agent

To further test if epigenetic regulation results in reexpression of FILIP1L, we treated ovarian cancer cells with either a DNA demethylating agent or histone deacetylase inhibitor. We chose to use ES2, OVCA429, and SKOV3 cells because they showed lower amounts of FILIP1L expression (Fig. 1A and B). Treatment of these cells with a DNA demethylating agent, 5-aza-2'-DAC, but not with the histone deacetylase inhibitor, TSA resulted in increased mRNA (Fig. 5A) and protein expression (Fig. 5B) of FILIP1L. FILIP1L mRNA expression in DAC- or TSA-treated cells was compared with that in DMSO control-treated cells. Significant increase in

Table 2. XXXX

<table>
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<td>OCC1</td>
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FILIP1L mRNA was observed in all 3 cell lines following DAC treatment. Induction of FILIP1L protein expression was observed in all 3 cell lines following DAC treatment. However, significant increases in FILIP1L mRNA and protein expression following TSA treatment were not observed. When a FILIP1L-high expressing cell such as OVCAR8 was treated with these reagents, we did not observe a significant increase in mRNA and protein expression (Fig. 5A and B). To examine whether reduced methylation in the FILIP1L promoter is associated with restoration of FILIP1L expression, we analyzed the methylation status of the FILIP1L promoter from HOSE, IOSE, and ovarian cancer cell lines was analyzed by Sequenom Epityper Mass Array. Mass Array results are shown as the average overall methylation for all 21 CG sites from the same cells used in (A). Error bars indicate SEM (n = 3 except for HOSE cells where 10 independent cultures were used). D, a significant inverse correlation of the DNA methylation status of the CpG island in the FILIP1L promoter with FILIP1L mRNA expression (P = 0.0098 by Spearman’s rank correlation method), y-axis; percent methylation of the average overall methylation for all 21 CG sites shown in (C) was used. x-axis; standardized FILIP1L mRNA expression shown in Figure 1A was used.
As shown in Figure 5D, ES2, OVCA429, and SKOV3 cells treated with DAC showed a significant decrease in methylation (from 93.1% ± 0.3% to 56.1% ± 2.6% in ES2 cells, from 43.0% ± 0.9% to 28.5% ± 1.4% in OVCA429 and from 21.7% ± 2.3% to 13.9% ± 0.3% in SKOV3 cells). However, DAC-treated OVCAR8 cells showed no changes in methylation throughout the cytosines in the CpG island (from 5.7% ± 0.6% to 4.2% ± 0.6%). These data show that DNA methylation in the FILIP1L promoter is a mechanism by which FILIP1L is downregulated in ovarian cancer.

**Increased methylation in the CpG island of the FILIP1L promoter is seen in invasive serous carcinomas compared with noninvasive serous borderline tumors**

Having observed the downregulation of FILIP1L expression associated with DNA methylation in the
FILIP1L promoter is related with invasive potential in ovarian cancer cell lines, we examined if downregulation of FILIP1L expression is associated with the degree of DNA methylation in the FILIP1L promoter in clinical ovarian tissue specimens. Genomic DNA was purified from the same scraped tissues used in Figure 3A and B for mRNA expression analysis, and DNA methylation was analyzed. Thirteen out of 21 CG sites analyzed in the CpG island of the FILIP1L promoter showed a significantly higher degree of methylation in invasive serous carcinomas than in noninvasive serous borderline tumors (Fig. 6A; \( P = 0.0204 \) for CG site 2; \( P = 0.0268 \) for CG site 3; \( P = 0.0063 \) for CG site 4; \( P = 0.0063 \) for CG site 6; \( P = 0.0204 \) for CG site 11; \( P = 0.0039 \) for CG site 12; \( P < 0.0001 \) for CG site 13; \( P = 0.0281 \) for CG site 14; \( P = 0.0053 \) for CG site 15; \( P = 0.0147 \) for CG site 16; \( P = 0.0426 \) for CG site 17; \( P = 0.0009 \) for CG site 19; \( P = 0.0254 \) for CG site 21). In Figure 6B, the average overall methylation for all 21 CG sites also showed a significantly higher methylation in invasive serous carcinomas than that in noninvasive serous borderline tumors. We then tested if the DNA methylation status of the CpG island of the FILIP1L promoter (Fig. 6B) inversely correlated with FILIP1L mRNA expression (Fig. 3A) in these clinical ovarian tissue specimens. As shown in Figure 6C, the DNA methylation status of the CpG island of the FILIP1L promoter displayed a significant inverse correlation with FILIP1L mRNA expression. These findings suggest that downregulation of FILIP1L associated with CpG island methylation of the FILIP1L promoter is related with the invasive phenotype of ovarian cancer.

**Binding of a transcription activator, CREB, to the CREB/ATF site in the CpG island of the FILIP1L promoter**

Inspection of the FILIP1L genomic sequence reveals that it has a CREB/activating transcription factor (ATF) site (TGACGTAA) 10 base pairs upstream of the transcription initiation site (–10) in the CpG island of its promoter region. CREB and a family of ATFs are transcription activators (18). Methylation of the CREB/ATF site in the CpG island of a promoter region results in suppression of CREB binding to the CREB/ATF site, which in turn suppresses gene expression (19–25). As shown in Figure 4B, the CpG in the CREB/ATF site in the CpG island of the FILIP1L promoter, CG site 12, is methylated in ovarian cancer cells (71.1% ± 2.8% for ES2, 75.3% ± 4.2% for OCC1, 25.3% ± 2.3% for OVCA429, and 22.2% ± 7.2% for SKOV3). In addition, the same CG site showed a significantly higher degree of methylation in invasive serous carcinomas than in the noninvasive serous borderline tumors (Figure 6A; 18.3% ± 2.8% for carcinomas; 4.3% ± 2.1% for borderline tumors; \( P = 0.0039 \)). Thus, we questioned whether the putative CREB/ATF site plays a role in mediating downregulation of FILIP1L in ovarian cancer. To determine if the CREB protein binds to this site, we carried out a pull-down assay as previously described (26). Biotinylated DNA oligos spanning the CREB/ATF site at -10 from the wild-type sequence were used to pull down the CREB complex. Three mutant biotinylated DNA oligos containing mutated nucleotides for the CREB/ATF binding site were constructed (Fig. 6D). As shown in Figure 6E, the wild-type oligo, but not mutant oligos, showed a pull down of the CREB complex. To
directly test the effect of DNA methylation on CREB binding activity, we carried out a pull-down assay by using methylated oligo. The oligo methylated at the cytosine in the CREB/ATF site failed to pull down the CREB complex (Fig. 6F). Thus, these data suggest that methylation of the CREB/ATF site in the FILIP1L promoter plays a role in mediating downregulation of FILIP1L in ovarian cancer.

**Discussion**

Epigenetic aberrations have been shown to be intimately associated with ovarian tumorigenesis (10, 11). A number of genes including the classic tumor suppressor, BRCA1, p16, MLH1, RASSF1A, ANGPTL2, ARHI, LOT1, ICAM1, HSulf-1, PALB2, and TUBB3 have been shown to be hypermethylated and downregulated in ovarian cancer.

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**Figure 6.** Increased methylation in the CpG island of the FILIP1L promoter in invasive serous carcinomas compared with noninvasive serous borderline tumors. A, DNA methylation status of the CpG island in the FILIP1L promoter from ovarian serous carcinomas and ovarian serous borderline tumors was analyzed by Sequenom Epityper Mass Array. Mass Array results are shown as described in Figure 4B (y-axis; n = 10 for serous borderline tumors and n = 17 for serous carcinoma). CG site 12 (*), the CG site in the putative CREB/ATF site is shown. B, Mass Array results are shown as described in Figure 4C from the same cells used in (A). Error bars indicate SEM (n = 10 for serous borderline tumors and n = 17 for serous carcinoma; P = 0.0003). C, a significant inverse correlation of the DNA methylation status of the CpG island in the FILIP1L promoter with FILIP1L mRNA expression in clinical ovarian tissue specimens (P = 0.0061 by Spearman’s rank correlation method). y-axis; percent methylation of the average overall methylation for all 21 CG sites shown in (B) was used. x-axis; standardized FILIP1L mRNA expression shown in Figure 3A was used. Data to plot the graph are presented in Supplementary Table S2. D, sequence of the oligos used in pull-down assay. The sequence from the FILIP1L promoter including CREB/ATF site is shown as wild type. Three mutant oligos containing the mutated sequence in CREB/ATF site are shown as mutant #1–3. The CREB/ATF site is shown in bold. Mutated nucleotides are shown as the underlined. E, a pull-down assay for CREB binding was carried out as described in Materials and Methods, and is shown as an immunoblot analysis for CREB. The far right lane shows the presence of CREB in the nuclear extracts (2.5 µg) from IOSE523 cells. The result is representative of 3 independent experiments. F, a pull-down assay for CREB binding was carried out as described in (E) except the oligo methylated at the cytosine in the CREB/ATF site was used. The result is representative of 3 independent experiments.
Downregulation of FILIP1L Induces an Invasive Phenotype in Ovarian Cancer

(11). In the present study, we showed that downregulation of FILIP1L (previously termed DOC1) is associated with DNA methylation in ovarian cancer. We have shown that both the mRNA and protein expression of FILIP1L are downregulated in ovarian cancer cells. FILIP1L expression is significantly lower in invasive serous carcinoma than in noninvasive serous borderline tumors. FILIP1L expression is inversely correlated with the invasive potential of ovarian cell lines as well as clinical ovarian specimens. The CpG island in the FILIP1L promoter is heavily methylated in ovarian cancer cells and almost completely nonmethylated in normal HOSE cells. The DNA methylation status of the FILIP1L promoter is inversely correlated with FILIP1L expression in ovarian cancer. Reduced methylation in the FILIP1L promoter following treatment with a DNA demethylating agent was associated with restoration of FILIP1L expression. A transcription activator, CREB, binds to the CREB/ATF site in the CpG island of the FILIP1L promoter. Overall, these findings suggest that downregulation of FILIP1L associated with DNA methylation is related with the invasive phenotype in ovarian cancer and indicates that FILIP1L has the potential to be a biomarker for invasive potential and a target for novel ovarian cancer therapy.

To identify common intracellular mediators of proliferation, migration, and apoptosis, we previously analyzed gene expression profiles of endothelial cells after treatment with angiogenesis inhibitors such as endostatin, fumagillin, and EMAP-II (3, 4). FILIP1L was upregulated in endothelial cells in response to these inhibitors. We subsequently showed that overexpression of FILIP1L resulted in inhibition of cell proliferation and migration and increased apoptosis (5). In addition, targeted expression of FILIP1L in tumors inhibited tumor growth in vivo (5). These findings suggested that FILIP1L may be an important inhibitor of cell proliferation and migration as well as an inducer of apoptosis. Our previous work suggested that agents, which increase the expression of FILIP1L in cells, have the effect of inhibiting the proliferation and migration of those cells. The findings from the present study suggest that downregulation of FILIP1L is associated with the invasive phenotype in ovarian cancer and that modulation of FILIP1L expression has the potential to be a target for ovarian cancer therapy.

Ovarian borderline tumors are epithelial ovarian cancers with histologic and biologic features intermediate between benign and malignant neoplasms. They display evidence of epithelial proliferation with frequent high-grade nuclear features but rare to no invasive implants. Approximately 15% of ovarian cancers are borderline tumors (ovarian tumors of low malignant potential; ref. 17). We have shown that FILIP1L expression is significantly lower in invasive serous carcinoma than in noninvasive serous borderline tumors (Fig. 3). The methylation of the CpG island in the FILIP1L promoter was significantly higher in invasive serous carcinomas than that in noninvasive serous borderline tumors (Fig. 6A and B). Others have shown that borderline tumors show a lower degree of methylation than malignant ovarian cancers. Genes such as IGFBP-3, ER-α, p16, BRCA1, hMLH1, and TGFBI are less methylated in borderline tumors than in invasive ovarian cancers (27–31). Serous is the most frequent subtype of epithelial ovarian cancer (17). Although we have shown that FILIP1L downregulation is implicated in serous ovarian cancers, it will be important to expand our study to examine if the same mechanism is also involved in other epithelial ovarian cancer subtypes.

We have shown that a DNA demethylating agent DAC, but not the histone deacetylase inhibitor TSA, resulted in increased mRNA (Fig. 5A) and protein expression (Fig. 5B) of FILIP1L in FILIP1L-low expressing ovarian cancer cells, suggesting that DNA methylation, but not histone modification, is associated with the downregulation of FILIP1L in these cells. However, although FILIP1L mRNA was downregulated, percent DNA methylation was low in ovarian cancer cell lines such as SKOV3 and OVCA429 (Fig. 6D) as well as in ovarian serous carcinomas (Fig. 6A). Whether low/moderate level of DNA methylation can downregulate FILIP1L in these specimens needs to be further investigated. It is also possible that DNA methylation is not the only mechanism by which FILIP1L is downregulated.

Standard therapy for ovarian cancer is surgical cytoreduction followed by combination chemotherapy using a taxane (paclitaxel) and platinum (carboplatin; ref. 32). The majority of ovarian cancers will unfortunately recur despite optimal front line therapy (17). Recurrent cancer is often resistant to conventional agents. Platinum resistance has been shown to be associated with DNA methylation, specifically with methylation of the CpG units of the MLH1 mismatch repair gene (33, 34). Studies have shown a clinical effect of a combination of carboplatin and DNA demethylating agents (35, 36). Epigenetic-based therapies that lead to the reexpression of tumor suppressor genes may have a role in desensitizing tumors to cytotoxic chemotherapies. In patients whose ovarian cancers have low expression of FILIP1L, there may be utility to the addition of a demethylating agent to increase FILIP1L expression. This therapy may revive the tumor suppressor-like function of FILIP1L. In conjunction with cytotoxic therapies, agents targeted to increase the expression of FILIP1L may have the potential to improve progression-free survival and overall survival in a disease with a dismal outcome. Determining the level of expression of FILIP1L in ovarian cancer may also be useful in predicting the invasive potential of those tumors and assist in staging, grading, and management.

Differential expression of the FILIP1L gene has been shown in other histologies. cDNA microarray analysis was used to identify FILIP1L as one of the genes whose transcription is induced in senescent human prostate epithelial cells but significantly repressed in immortalized prostate epithelial cells (37, 38). FILIP1L mRNA expression was downregulated in microvascular endothelial cells infected with Kaposi's sarcoma-associated herpes virus but not in uninfected microvascular endothelial cells (39). Furthermore, mRNA expression was downregulated in B cells transformed with the oncogene TaxBLV (bovine leukemia virus Tax) but not in untransformed B cells (40). Therefore, future studies to test whether DNA methylation in the
FILIP1L promoter plays a role in mediating downregulation of FILIP1L in other tumor histologies will be of interest. In summary, we have shown that downregulation of FILIP1L is associated with the invasive phenotype in ovarian cancer. Downregulation of FILIP1L is mediated by promoter methylation. Further characterization of the mechanism of FILIP1L downregulation may improve the understanding of the role played by FILIP1L in ovarian carcinogenesis and lead to the development of more effective anticancer agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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