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Research Article

Expression Profiling Identifies Altered Expression of Genes That Contribute to the Inhibition of Transforming Growth Factor-β Signaling in Ovarian Cancer

Jan S. Sunde,[1] Howard Donninger,[2] Kongming Wu,[4] Michael E. Johnson,[5] Richard G. Pestell,[4] G. Scott Rose,[1] Samuel C. Mok,[5] John Brady,[3] Tomas Bonome,[2] and Michael J. Birrer[2]

[1]Walter Reed Army Medical Center, Washington, District of Columbia; [2]Department of Cell and Cancer Biology and [3]Laboratory of Cellular Oncology Virus Tumor Biology Section, National Cancer Institute, Bethesda, Maryland; [4]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania; and [5]Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

Abstract

Ovarian cancer is resistant to the antiproliferative effects of transforming growth factor-β (TGF-β); however, the mechanism of this resistance remains unclear. We used oligonucleotide arrays to profile 37 undissected, 68 microdissected advanced-stage, and 14 microdissected early-stage papillary serous cancers to identify signaling pathways involved in ovarian cancer. A total of seven genes involved in TGF-β signaling were identified that had altered expression >1.5-fold ($P < 0.001$) in the ovarian cancer specimens compared with normal ovarian surface epithelium. The expression of these genes was coordinately altered: genes that inhibit TGF-β signaling (*DACH1, BMP7*, and *EVI1*) were up-regulated in advanced-stage ovarian cancers and, conversely, genes that enhance TGF-β signaling (*PCAF, TFE3, TGFBRII*, and *SMAD4*) were down-regulated compared with the normal samples. The microarray data for *DACH1* and *EVI1* were validated using quantitative real-time PCR on 22 microdissected ovarian cancer specimens. The *EVI1* gene locus was amplified in 43% of the tumors, and there was a significant correlation ($P = 0.029$) between gene copy number and *EVI1* gene expression. No amplification at the *DACH1* locus was found in any of the samples. DACH1 and EVI1 inhibited TGF-β signaling in immortalized normal ovarian epithelial cells, and a dominant-negative DACH1, DACH1-ΔDS, partially restored signaling in an ovarian cancer cell line resistant to TGF-β. These results suggest that altered expression of these genes is responsible for disrupted TGF-β signaling in ovarian cancer and they may be useful as new and novel therapeutic targets for ovarian cancer. (Cancer Res 2006; 66(17): 8404-12)

Introduction

Ovarian cancer remains the most lethal type of gynecologic cancer in the United States, with modest improvements in survival over the last 40 years. It is anticipated that there will be 20,180 new cases of ovarian cancer and 15,310 ovarian cancer–related deaths diagnosed in the United States in 2006 (1). Typically, ovarian cancer has few symptoms early in its course, and therefore, the majority of patients are diagnosed with advanced-stage disease, which has a 5-year survival rate of only 30% (2). In contrast, early-stage ovarian cancer is often curable with a 5-year survival rate of 95%. The advent of new molecular techniques, such as gene expression profiling, for evaluating cellular processes now allows the identification of alterations in gene expression that may elucidate the mechanisms of cancer development and progression.

One of the hallmarks of cancer cells is the loss of response to inhibitory growth signals, such as transforming growth factor-β (TGF-β). Cellular processes that are affected by the TGF-β pathway include regulation of differentiation, inhibition of epithelial cell proliferation, and apoptosis (3). TGF-β ligands exert their activity by binding to a family of transmembrane serine/threonine kinase receptors (4). TGF-β binding to the receptors initiates a signal cascade, with the Smad proteins being the primary signal transducers (4). Activated TGFBRI phosphorylates and activates the downstream signaling components Smad2 and Smad3 (4). Phosphorylated Smad2 and Smad3 form a complex with Smad4 followed by translocation from the cytoplasm to the nucleus where the complex binds to and activates or represses specific TGF-β-responsive genes involved in proliferation (5). In addition, TGF-β is also capable of using alternate pathways, such as the direct binding of Smad3 to 12-*O*-tetradecanoylphorbol-13-acetate-responsive gene promoter elements in the absence or presence of c-Jun and c-Fos (6).

More recent work has identified genes with regulatory activity in the TGF-β signaling pathway. Two genes that inhibit TGF-β signaling have been described, *DACH1* (7) and *EVI1* (8). DACH1, a protein with homology to SKI and SKIL, has recently been shown to be a repressor of the TGF-β pathway in breast cancer cell lines by binding to the Smad3/Smad4 complex and preventing transcription (7). *EVI1* is a gene that plays a role in progression of hematopoietic malignancies, such as acute myelogenous leukemia (AML; ref. 9), by forming a chimeric protein with AML1. It inhibits TGF-β signaling by physically interacting with Smad3 to suppress its transcriptional activity (8) and recruiting the corepressor COOH-terminal binding protein CTBP1 into the nucleus (10).

Resistance to the antiproliferative effects of TGF-β is important in the development of many epithelial cancers (3), including ovarian carcinoma (11, 12). Although many molecular events have been shown to contribute to this resistance in other epithelial cancers, the mechanism(s) involved in ovarian cancer remains unclear. The role of mutational inactivation of TGF-β/Smad pathway genes in ovarian cancer is still unclear. There have been conflicting reports about the presence of receptor mutations in ovarian cancer (13–16). It has also been reported that enhanced expression of the TGF-β1 and TGF-β3 ligands and loss of

Note: J.S. Sunde and H. Donninger contributed equally to this work.

Requests for reprints: Michael J. Birrer, Department of Cell and Cancer Biology, National Cancer Institute, 37 Convent Drive, Room 1130, Bethesda, MD 20892. Phone: 301-402-9586; Fax: 301-480-4756; E-mail: birrerm@bprb.nci.nih.gov.

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expression of TGFBRI and TGFBRIII may contribute to ovarian carcinogenesis (17). A more recent extensive study has shown that primary ovarian epithelial cultures were resistant to the anti-proliferative effects of TGF-β, although there was no difference in the expression of the TGF-β receptors or the downstream Smad signaling components compared with normal human ovarian surface epithelium (12). This report suggested that TGF-β signaling is blocked downstream of the Smad complex formation.

In an attempt to better define the mechanism(s) behind the loss of TGF-β responsiveness in ovarian cancer, we analyzed microarray data from 119 ovarian cancers and 10 ovarian surface epithelium samples for altered expression of genes involved in the TGF-β signaling pathway. We identified seven genes (*DACH1, PCAF, TFE3, BMP7, EVI1, SMAD4*, and *TGFBRII*) with altered expression from normal ovarian surface epithelium. Those genes that enhanced TGF-β signaling (*PCAF, TFE3, SMAD4*, and *TGFBRII*) were down-regulated, and those that inhibited TGF-β signaling (*DACH1, EVI1*, and *BMP7*) were up-regulated; thus, the net effect of the altered expression of all these genes was the inhibition of the TGF-β signaling pathway in these tumor samples. Further analysis of two of these genes (*DACH1* and *EVI1*) showed that both were able to inhibit TGF-β signaling in immortalized ovarian epithelial cells. Expression of a dominant-negative DACH1 into an ovarian cancer cell line that expresses high levels of endogenous DACH1 and is nonresponsive to TGF-β signaling partially restored TGF-β signaling.

Materials and Methods

Tissue samples. Tissue from 37 stage III or IV grade 3 undissected fresh-frozen papillary serous ovarian cancers was obtained from the Cooperative Human Tissue Network and the Gynecologic Oncology Group tissue bank. Sixty-eight microdissected fresh-frozen advanced-stage grade 3 papillary serous ovarian cancers, 14 early-stage grade 3 papillary serous ovarian cancers, 8 low-grade papillary serous ovarian cancers, 20 low-malignant potential (LMP) ovarian cancers, and 10 normal ovarian surface epithelium cytobrushing specimens were obtained from the Brigham and Women's Hospital (Boston, MA). All samples were obtained under Institutional Review Board–approved protocols. All tissue samples were stored at −140°C until processed.

Cell lines and culture conditions. IOSE80 immortalized ovarian surface epithelial cells were maintained in a 1:1 mixture of 199 (Invitrogen Life Technologies, Inc., Carlsbad, CA) and 105 (Sigma, St. Louis, MO) medium supplemented with 3% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA), 1% L-glutamine (Invitrogen Life Technologies), and 1% penicillin/streptomycin (Invitrogen Life Technologies). All ovarian cancer cell lines, with the exception of OVCAR429, were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. OVCAR429 cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin.

Reporter constructs and expression plasmids. The pKW10 vector expressing hemagglutinin (HA)-tagged DACH1 as well as pKW10 expressing a dominant-negative DACH1 (DACH1-ΔDS) have previously been described (7). The MSCV vector expressing HA-EVI1 was a kind gift from Dr. G. Nucifora (University of Illinois, Chicago, IL) and has been described previously (18). The $CAGA_{12}$-Luc reporter plasmid (19) was kindly provided by Drs. S. Jakowlew and L. Ozbun [National Cancer Institute (NCI), Rockville, MD].

Microarray analysis. cRNA from the tissue samples and cytobrushings was prepared according to the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA) and hybridized to the arrays as described previously (20). Global normalization at a target value of 500 was applied to all of the arrays under consideration using GeneChip Operating Software (Affymetrix), and the normalized data were uploaded into the NCI's Microarray Analysis Database.[6] Biometrics Research Branch (BRB) ArrayTools version 3.2.2[7] was used for statistical analysis of the array data. Probe sets scored as absent (A) at $\alpha_1 = 0.05$ or marginal (M) at $\alpha_2 = 0.065$ were excluded from the analysis. In addition, only those transcripts present in >50% of the arrays and displaying a variance in the top 50th percentile were evaluated. This analysis yielded a filtered data set containing 14,119 informative probe sets. Differentially expressed genes were identified for tumor and ovarian surface epithelium specimens using a multivariate permutation test in BRB-ArrayTools. A total of 2,000 permutations was completed to identify the list of probe sets containing <10 false positives at a confidence of 95%. Differential expression was considered significant at $P < 0.001$. The statistic applied to each probe set was a random variance t test. A list of ~3,000 genes with altered expression ($P < 0.001$) was generated using these analyses criteria.

Identification of signaling pathways. The gene list generated by microarray analysis was analyzed using PathwayAssist software version 2.5 (Iobion Informatics, LLC, La Jolla, CA) to identify genes in specific cellular pathways as reported previously (20).

Comparative genomic hybridization and quantitative PCR. Frozen sections (7 μm) were cut, and microdissection was done using a Leica LMD system (Leica, Germany). DNA was extracted and purified using the QIAamp DNA Micro kit according to the manufacturer's instruction (Qiagen, Inc., Valencia, CA). Whole-genome amplification was done on the purified DNA by the GenomiPhi DNA Amplification System (Amersham Biosciences, Piscataway, NJ) with the addition of 1.5 μL 50 mmol/L aminoallyl-dUTP (Ambion, Austin, TX). The mixture was incubated for 17 hours at 30°C followed by 10 minutes at 65°C and cooling to 4°C. Amplified DNA was then digested with *Hae*III (New England Biolabs, Boston, MA) for 2 hours, precipitated, and resuspended in 9 μL coupling buffer [0.1 mol/L sodium bicarbonate (pH 9.0)]. Reconstituted 4.76 mmol/L Cy3 and Cy5 monoreactive dye (2 μL; Amersham Biosciences) was then added to the aminoallyl-dUTP–labeled tumor DNAs and normal female control DNAs, respectively. After 1 hour of incubation at room temperature in the dark, the coupling reaction was quenched by adding 4.5 μL of 4 mol/L hydroxylamine into the reaction mix. After 15 minutes, the volume of each sample was adjusted to 50 μL with nuclease-free water and purified with the NucAway spin column (Ambion). A total of 50 μg human Cot-1 DNA and 100 μg yeast tRNA was added to the pooled normal and tumor DNA sample, dried under vacuum, and resuspended in 100 μL hybridization buffer (Hybridization buffer 1, Ambion). Labeled DNAs were hybridized to a 54,675-element oligo array produced by the NCI Microarray Facility. After hybridization, the microarray was scanned using the ScanArray 4000 XL fluorescent scanner (Perkin-Elmer, Boston, MA), and the resulting images were analyzed using the QuantArray Express software (Perkin-Elmer, Downers Grove, IL). The relative fluorescent level or fluorescent ratio representing the relative amount of target sequences in the probe mix was analyzed by comparing the fluorescent intensity of corresponding individual spots after local background subtraction and normalization. The average local background and SD over all the array spots were also calculated.

DNA copy number changes of both *DACH1* and *EVI1* in tumor tissue samples were validated using Taqman real-time PCR (RT-PCR) amplification with Taqman Universal PCR Master Mix with SYBR Green and an ABI Prism 7300 Sequence Detection System (PE Applied Biosystems, Foster City, CA). DNA content was normalized to that of Line-1 DNA. Sequences for the primers used were as follows: *EVI1*, 5′-AAAGCCGCTCAACTACATGG-3′ (forward) and 5′-TGCTTTGAATGCGTCCCAGAG-3′ (reverse); *DACH1*, 5′-CTTGTCAGAGGGAAGGGTGG-3′ (forward) and 5′-GCACTGTTTGCCGCTTTACTT-3′ (reverse); and Line-1, 5′-AAAGCCGCTCAACTACATGG-3′ (forward) and 5′-TGCTTTGAATGCGTCCCAGAG-3′ (reverse). DNA amplification was done using 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. DNA quantification was assessed by the fluorescence intensity emitted during

[6] http://nciarray.nci.nih.gov/index.shtml.
[7] http://linus.nci.nih.gov/BRB-ArrayTools.html.

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Table 1. Expression of selected TGF-β pathway genes in 37 undissected advanced-stage papillary serous ovarian cancers compared with normal surface ovarian epithelium

Gene	Fold change
DACH1	4.5*
BMP7	2.0
PCAF	−2.2
TFE3	−2.7

*Fold change compared with normal ovarian surface epithelium.

each PCR cycle. Copy number changes were calculated by using the formula $2^{(Nt - N\text{line}) - (Tt - T\text{line})}$, where N_t is the threshold cycle observed for either the *DACH1* or *EVI1* primer set in the normal female DNA sample, N_{line} is the threshold cycle number observed for the Line-1 primer set in the normal DNA sample, T_t is the average threshold cycle observed for either the *DACH1* or *EVI1* primer set in a tumor DNA sample, and N_t is the average threshold cycle number observed for the Line-1 primer set in the tumor DNA sample.

RNA isolation. Total RNA from selected samples was extracted using Trizol (Invitrogen Life Technologies) as per the manufacturer's instructions followed by purification using RNeasy Mini columns (Qiagen).

Western blot analysis. Cell lysates from the panel of ovarian cancer cell lines and two immortalized normal ovarian surface epithelial cell lines were prepared by lysing the cells in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mmol/L Tris (pH 7.4)] supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 100 μg/mL leupeptin, and 100 μg/mL aprotinin. The cell lysates were sonicated and centrifuged to remove debris, and equal amounts of protein were separated on polyacrylamide gels as described (7) and incubated with antibodies to DACH1 (7) and β-actin. The signal was detected by enhanced chemiluminescence.

Quantitative RT-PCR. Quantitative RT-PCR was used to validate the differential expression of *DACH1* and *EVI1*. RNA from 22 microdissected tumor specimens used for microarray analysis and 10 ovarian surface epithelium samples was used. In addition, RNA from a panel of 14 ovarian cancer lines and 2 immortalized ovarian surface epithelial cell lines was also evaluated for expression of *EVI1*. Quantitative RT-PCR was done using an iCycler Real-time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with the QuantiTect SYBR Green RT-PCR kit (Qiagen) as per the manufacturer's instructions with the following primers: *DACH1,* 5′-GGAATGGATTGTGGCTGAAC-3′ (forward) and 5′-GGTATTGGACTGGTACATCAAG-3′ (reverse) and *EVI1,* 5′-TGCTATGATGCTGTCACTGTC-3′ (forward) and 5′-CGTGGCTTATGGACTGGATAG-3′ (reverse). The fold change for each target gene was calculated using the $2^{-\Delta\Delta CT}$ method as described previously (20) with β-glucuronidase (*GUSB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and cyclophilin A as the reference genes for the tumor samples and *β-actin* as the reference gene for the cell line samples. Sequences of the β-actin primers have been described previously (20).

Transient transfections and luciferase reporter assays. Exponentially growing cells were transfected with 200 ng of the $CAGA_{12}$-Luc promoter reporter construct alone or in combination with DACH1, DACH1-ΔDS, or EVI1 expression constructs using Fugene 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN). *Renilla* luciferase (20 ng; Promega, Madison, WI) was cotransfected to control for transfection efficiency. After overnight incubation, the medium was removed and replaced with serum-free medium and the cells were allowed to grow for 36 hours. Recombinant TGF-β1 (Calbiochem, San Diego, CA) at a final concentration of 1 ng/mL was then added to the cells for 12 hours, after which luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Knockdown of DACH1 in A547 cells by short hairpin RNA. Three retroviral DACH1 short hairpin RNA (shRNA) constructs (Open Biosystems, Huntsville, AL) were individually transfected into Phoenix A cells using Fugene 6 transfection reagent. Forty-eight hours after transfection, the supernatants were harvested and 1 mL was added to 2 mL of RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin and overlayed onto 1×10^6 A547 cells in 100-mm dishes. Twenty-four hours after infection, cells were trypsinized and serially diluted and stable clones were selected using puromycin. Clones were screened by Western blot analysis to determine DACH1 expression.

Immunohistochemistry. A total of 31 optimally debulked stage III grade 3 serous adenocarcinoma cases was used for EVI1 immunostaining. Immunolocalization of EVI1 was done using the EnVision System/AP (DakoCytomation, Carpinteria, CA). In brief, 7-μm-thick ovarian tissue sections were mounted on SuperFrost/Plus microscopic slides (Fisher Scientific, Pittsburgh, PA). EVI1 protein antigens were retrieved by microwave in 0.01 mol/L citric acid buffer (pH 6.0) for 10 minutes. A polyclonal anti-EVI1 antibody (ProSci, Inc., Poway, CA) at a dilution of 0.5 μg/mL in 1% bovine serum albumin (BSA)-PBS was used. Negative controls were done by incubating the tissue sections with 1% BSA-PBS instead of the primary antibody. An alkaline phosphatase–conjugated polymer followed by fast red was subsequently applied to the sections. The intensity of the immunopositive signal was scored with a four-point scale: 0, negative; 1, weak; 2, medium; 3, strong. Five to seven regions were randomly taken from each section for scoring. The mean score for all the cases was calculated. The results obtained were correlated with *EVI1* mRNA expression levels using the Kruskal-Wallis test. The level of critical significance was considered to be $P < 0.05$. The results showed that samples

Table 2. Expression of selected TGF-β pathway genes in advanced-stage, early-stage, and low-grade microdissected papillary serous ovarian carcinomas and microdissected LMP tumors ($P < 0.001$) as determined by microarray analysis

Gene	Advanced (n = 68)	Early (n = 14)	LMP (n = 20)	Low grade (n = 8)
DACH1	4.0* (72)	4.2 (93)	4.6 (85)	4.4 (87)
EVI1	11.9 (100)	18.2 (93)	9.9 (100)	8.6 (100)
BMP7	3.2 (47)	4.2 (57)	NS (N/A)	NS (N/A)
PCAF	−3.1 (88)	−3.5 (93)	−3.3 (100)	−3.6 (100)
TFE3	−1.7 (65)	NS (N/A)	−1.5 (50)	NS (N/A)
TGFBR2	−3.2 (96)	−4.6 (93)	−3.3 (95)	−4.3 (87)
SMAD4	−2.2 (76)	−2.0 (86)	−2.5 (100)	−2.8 (100)

NOTE: Numbers in parentheses indicate percentage of tumors overexpressing the indicated gene.
Abbreviations: NS, not significantly differentially expressed; N/A, not applicable.
*Fold change compared with normal ovarian surface epithelium.

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with higher levels of EVI1 immunostaining scores had significantly higher levels of *EVI1* mRNA levels.

Results

Whole-genome expression profiling identifies alterations in the expression of genes involved in the TGF-β pathway. We recently reported 1,191 genes that were differentially regulated in 37 undissected papillary serous ovarian cancer specimens compared with normal ovarian surface epithelium (20). Using PathwayAssist, we identified four genes that mediate TGF-β signaling that were differentially expressed ($P < 0.01$) in the papillary serous ovarian cancer specimens compared with the normal ovarian surface epithelium by >1.5-fold (Table 1). Those genes that inhibit TGF-β signaling (*DACH1* and *BMP7*) were up-regulated in the cancer specimens compared with the normal ovarian epithelium, and those genes that enhance TGF-β signaling (*PCAF* and *TFE3*) were down-regulated, suggesting that TGF-β signaling in the cancer specimens was inhibited. To confirm that these genes were differentially regulated in the epithelial cells rather than the stroma, we analyzed microarray data from an independent set of microdissected papillary serous ovarian tumors consisting of 68 advanced-stage and 14 early-stage specimens using PathwayAssist. The differential regulation of the genes identified in the undissected samples (Table 1) was confirmed in the microdissected samples ($P < 0.001$; Table 2), and furthermore, an additional three genes (*EVI1, TGFBRII*, and *SMAD4*) involved in TGF-β signaling were identified as being differentially regulated by ≥1.5-fold in the microdissected samples compared with normal ovarian surface epithelium (Table 2). All the genes showed altered expression in a high percentage (47-100%) of advanced tumors. To attempt to address at what stage during the development of ovarian cancer the expression of these genes becomes altered, we determined their expression in microdissected early-stage ovarian cancer. All the genes, with the exception of *TFE3*, were significantly altered in early-stage papillary serous ovarian cancer specimens compared with the normal ovarian surface epithelium (Table 2). Of further note, the pattern of expression, with respect to fold change, for the genes described in Table 2 was similar in both the advanced-stage and the early-stage cancers. *DACH1* mRNA expression, as determined by microarray analysis, was elevated ≥1.5-fold compared with the normal ovarian surface epithelium in 72% (49 of 68) of the advanced-stage cancers and 93% (13 of 14) of the early-stage cancers (Table 2). *EVI1* mRNA expression was increased in all of the advanced-stage cancers and in the majority of the early-stage cancers (93%; Table 2). *BMP7* was only increased in 47% of the advanced-stage cancers and 57% of the early-stage cancers. *PCAF, TGFBRII,* and *SMAD4* were all similarly decreased in both the advanced-stage and the early-stage cancers (Table 2), whereas *TFE3* was only decreased in the advanced-stage cancers (65%). It was not statistically significantly altered in the early-stage cancers (Table 2). The differences in the percentages of advanced-stage and early-stage tumors differentially expressing each gene were not statistically significant ($P > 0.1$ for each gene). None of these differentially regulated genes showed any correlation with survival (hazard ratio, 0.726-1.151; $P > 0.1$), and furthermore, for the early-stage tumors, the relative expression of these genes was not associated with recurrence (0.9- to 1.7-fold change; $P > 0.2$). These results suggest that alteration in the expression of genes involved in TGF-β signaling is common to both early-stage and advanced-stage ovarian cancers and further

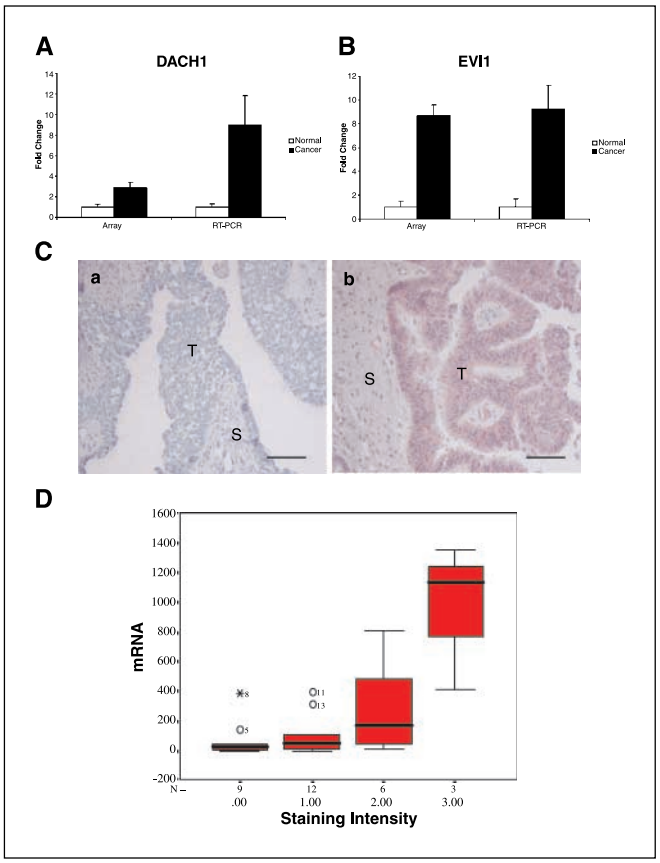

Figure 1. Comparison of microarray and quantitative RT-PCR analysis for *DACH1* (*A*) and *EVI1* (*B*) in 22 dissected papillary serous ovarian cancer specimens compared with 10 normal ovarian epithelium samples. The fold change for the quantitative RT-PCR analysis was calculated by determining the expression of *DACH1* and *EVI1* in each sample relative to the expression of three housekeeping genes, *GUSB, GAPDH*, and *cyclophilin A*, and then comparing this relative expression with that of the normal samples as described in Materials and Methods. *C,* immunohistochemical staining for EVI1 showing representative sections of advanced-stage ovarian cancer samples negative (*a*) and positive (*b*) for EVI1. EVI1 is expressed predominantly in the tumor (*T*) with minimal staining in the stroma (*S*). Bar, 10 μm. *D,* Kruskal-Wallis analysis showed a significant correlation ($P < 0.05$) between EVI1 protein and mRNA expression in the advanced-stage tumors.

suggest that TGF-β signaling is inhibited early in the development of ovarian cancer. In addition, we found that expression of these genes was also altered in microdissected low-grade and LMP tumors (Table 2), suggesting that deregulation of these TGF-β pathway genes is an important early event in the pathogenesis of all ovarian cancers.

Validation of microarray data. To further analyze the role of these genes in TGF-β resistance, we focused on the two genes with the highest level of overexpression, *DACH1* and *EVI1*, and validated the expression of each by quantitative RT-PCR on 22 microdissected advanced-stage ovarian cancer specimens and 10 normal ovarian surface epithelial samples (Fig. 1). Expression of both *DACH1* (Fig. 1*A*) and *EVI1* (Fig. 1*B*) was enhanced ($P = 0.01$ and 0.0001, respectively) in these samples compared with the normal ovarian epithelium samples as determined by quantitative RT-PCR analysis, and these results agreed with the microarray data (Fig. 1). Immunohistochemistry using an anti-EVI1 antibody showed that EVI1 protein was also increased in the advanced-stage ovarian cancer specimens (Fig. 1*C*) and there was a significant correlation ($P = 0.018$) between mRNA and protein

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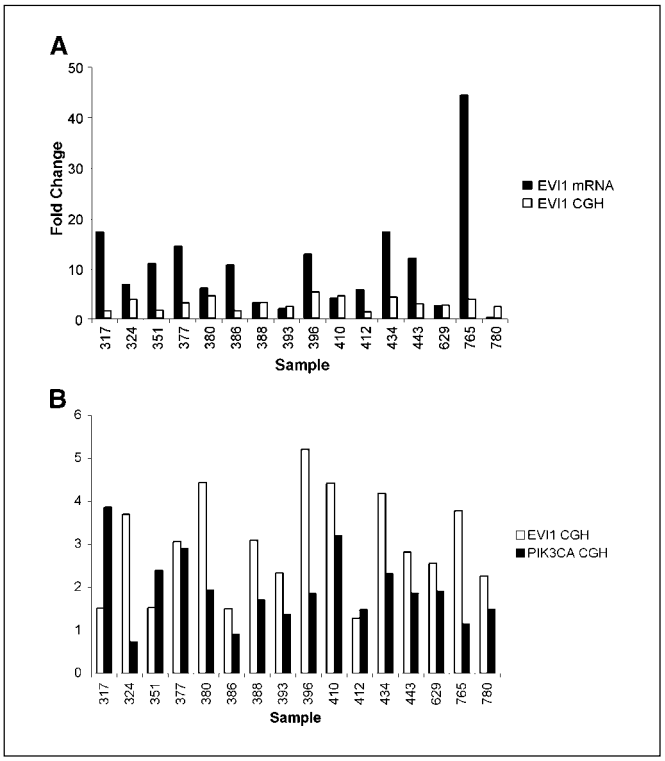

Figure 2. *A,* comparison of the fold changes obtained by CGH as determined by quantitative PCR and quantitative RT-PCR for *EVI1* in 16 microdissected ovarian cancer samples. *B,* comparison of gene amplification for *EVI1* and *PIK3CA* as determined by CGH.

levels as determined by quantitative RT-PCR and immunohistochemistry, respectively (Fig. 1*D*).

Comparative genomic hybridization and quantitative PCR. One of the possible mechanisms of enhanced expression of *DACH1* and *EVI1* in the ovarian cancer specimens is gene amplification. We randomly selected 23 microdissected advanced-stage ovarian cancer specimens (with variable expression) to analyze gene copy number by comparative genomic hybridization (CGH) and subsequent quantitative PCR analysis. Our analysis did not show any increase in gene copy number for *DACH1* (located at 13q22) in 23 cases of the advanced-stage cancers, of which 8 samples had increased mRNA levels as determined by microarray analysis. On the other hand, 43% (10 of 23) of the microdissected ovarian cancer specimens showed increased gene copy number (>1.2-fold) for *EVI1* (at 3q24-28) by CGH and the fold changes correlated significantly with those identified by quantitative PCR (r = 0.613; P = 0.002). Furthermore, in 16 cases from which both quantitative PCR and quantitative RT-PCR data were available, there was a significant correlation between *EVI1* DNA copy numbers and levels of *EVI1* mRNA expression (r = 0.529; P = 0.029; Fig. 2*A*). These results suggest that, at least for a portion of the microdissected ovarian cancer specimens, the enhanced expression of *EVI1* is the result of increased gene copy number. Because the *phosphatidylinositol 3-kinase* (*PI3K*) locus has been reported to be amplified in ovarian cancer (21, 22) and it is located close to the *EVI1* locus, we analyzed *PI3K* gene copy number by CGH and quantitative PCR in the same subset of tumor samples. Interestingly, there was not a direct correlation between *EVI1* and *PI3K* gene amplification (Fig. 2*B*). The majority of the samples analyzed showed a higher level of amplification at the *EVI1* locus than the *PI3K* locus, with only few samples showing a higher level of amplification at the *PI3K* locus than the *EVI1* locus (Fig. 2*B*). Only one sample showed similar gene amplification at both gene loci. These results suggest that both loci are not amplified to the same extent in ovarian cancer and, because the *EVI1* locus seems to be the predominantly amplified one, imply that amplification of this locus may be more important in ovarian cancer than amplification of *PI3K*.

***DACH1* and *EVI1* expression in ovarian cancer cell lines.** *DACH1* and *EVI1* expression in a panel of 14 ovarian cancer cell lines and 2 immortalized ovarian cell lines was analyzed by Western blotting and quantitative RT-PCR, respectively. Analysis of DACH1 protein levels in the panel of ovarian cancer cell lines showed that A224, OVCAR3, CP70, A2780, AD10, 222, CAOV3, and A547 cells expressed DACH1 protein at levels at least 2-fold higher than the two immortalized ovarian cell lines, IOSE80 and IOSE120 (Fig. 3*A* and *B*), with A547 cells showing the highest expression. *EVI1* mRNA expression was increased by ≥1.5-fold in only 43% of cell lines compared with the normal ovarian cell lines (Fig. 3*C*), with A364 cells having the greatest fold increase in *EVI1* mRNA. EVI1 expression was not analyzed by Western blotting, as a suitable antibody that gave consistent results could not be obtained. It is important to note that overexpression of these genes in cell lines seems to occur at a lower rate than that found in primary tumors. This discrepancy may result from (*a*) underestimation of the

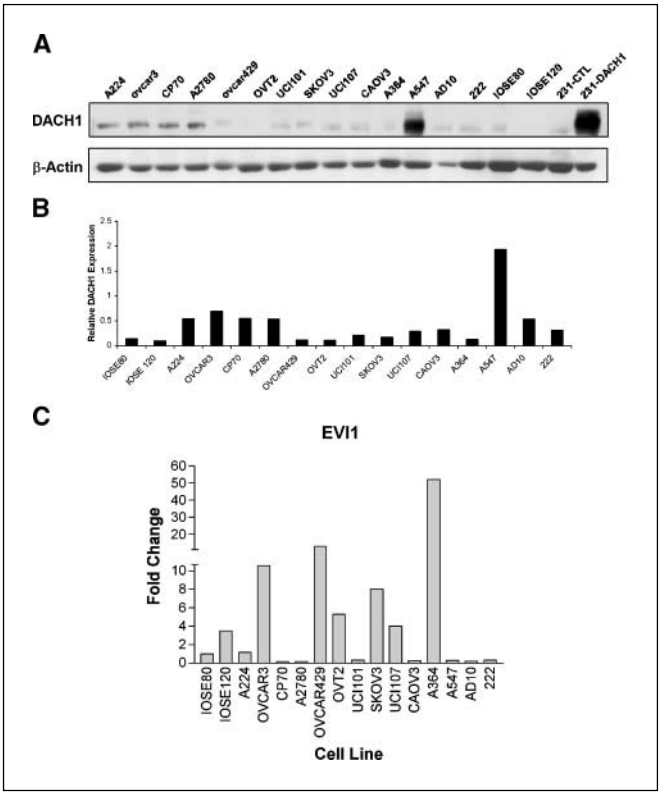

Figure 3. Analysis of DACH1 and EVI1 expression in a panel of ovarian cancer cell lines compared with two immortalized ovarian surface epithelial cell lines (IOSE80 and IOSE120). *A,* DACH1 expression was analyzed by Western blotting. 231-CTL and 231-DACH1 are negative and positive control cell lines, respectively, for DACH1 expression. β-Actin was used as a control for protein loading. *B,* densitometric quantification of the Western blot in (*A*) showing expression of DACH1 relative to β-actin expression. *C, EVI1* expression in the ovarian cancer cell lines as determined by quantitative RT-PCR. Fold changes were calculated by determining the relative expression of EVI1 to *β-actin* for each cell line and then comparing this relative expression with that of IOSE80.

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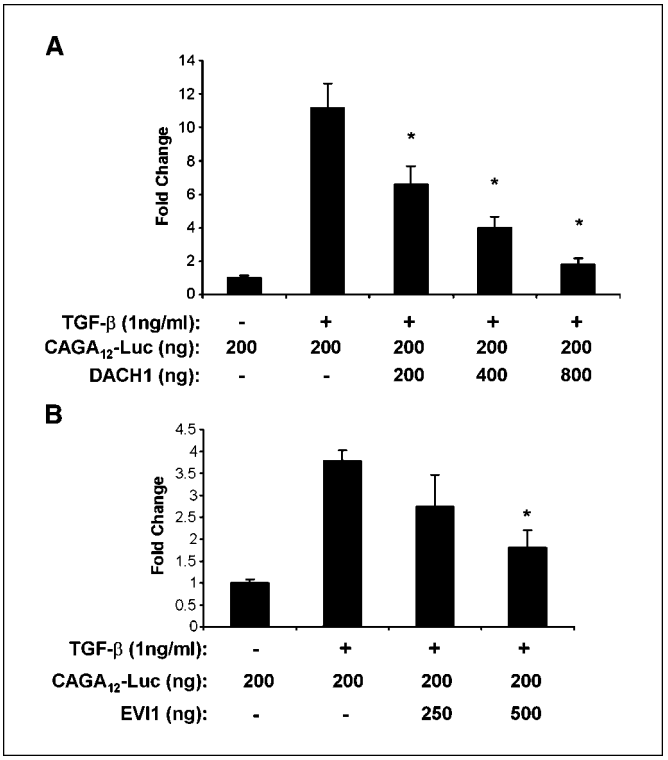

Figure 4. DACH1 and EVI1 inhibit TGF-β signaling in normal ovarian cells. IOSE80 cells were transiently transfected with the TGF-β-responsive promoter luciferase construct $CAGA_{12}$-Luc alone or with a DACH1 (*A*) or EVI1 (*B*) expression plasmid, serum starved for 36 hours, and then stimulated with TGF-β (1 ng/mL) for 12 hours. Data are the fold change relative to unstimulated cells after normalizing to *Renilla* luciferase activity as a control for transfection efficiency. *Columns,* mean of triplicate experiments; *bars,* SD. *, $P < 0.05$, compared with TGF-β-stimulated cells alone.

expression levels of these genes because expression was compared with "immortalized" ovarian epithelial cell lines that may already have elevated expression of these genes and (*b*) changes occurring within tumor cells subjected to cell culture conditions during which maintenance of high-level expression of these genes is no longer required.

DACH1 and EVI1 inhibit TGF-β signaling in ovarian cells. To determine whether DACH1 and EVI1 affect the TGF-β signaling pathway in ovarian cancer, we examined this pathway in the IOSE80 immortalized ovarian epithelial cell line and a panel of ovarian cancer cell lines using a TGF-β-responsive promoter luciferase construct, $CAGA_{12}$-Luc. TGF-β stimulation of IOSE80 cells resulted in a 4- to 12-fold increase ($P = 0.002$) in promoter activity from this luciferase construct (Fig. 4*A*), and cotransfection of DACH1 (Fig. 4*A*) or EVI1 (Fig. 4*B*) resulted in an 85% ($P = 0.004$) and a 50% ($P = 0.007$) reduction of this TGF-β-stimulated promoter activity. A547 cells that express high levels of DACH1 (Fig. 3) also did not respond to TGF-β (data not shown). To determine whether this lack of response to TGF-β in the A547 cells was, in part, due to the high level of DACH1 expression (Fig. 3), we expressed a dominant-negative DACH1 (DACH1-ΔDS) in these cells together with the $CAGA_{12}$-Luc reporter. This resulted in a dose-dependent increase in promoter activity ($P = 0.007$; Fig. 5) under treatment with TGF-β, suggesting that the high level of DACH1 is responsible, in part, for the lack of response of A547 cells to TGF-β stimulation.

To confirm this finding, we used shRNA constructs to stably knock down DACH1 expression in A547 cells. Of three different shRNA constructs used, only one resulted in knockdown of DACH1, and we isolated three independent clones that exhibited lower DACH1 than parental A547 cells (Fig. 6*A* and *B*). We used the other shRNA constructs that did not result in knockdown of DACH1 as controls (Fig. 6*A* and *C*) as well as three clones transfected with the vector alone (Fig. 6*A* and *B*). We tested these clones for their ability to respond to TGF-β using luciferase assays with the $CAGA_{12}$-Luc reporter. Transfection of the knockdown clones with the $CAGA_{12}$-Luc reporter resulted in a small (1.5- to 1.7-fold) but significant ($P < 0.05$) increase in response to TGF-β (Fig. 6*D*) compared with the lack of response to TGF-β seen in the parental A547 cells. No response to TGF-β was seen in any of the vector or control shRNA clones (Fig. 6*D*). The small increase in promoter activity in response to TGF-β is possibly due to the fact that DACH1 expression in these cells was not completely knocked down (Fig. 6*A* and *B*). To determine whether the growth-inhibitory effects of TGF-β could be partially restored in the DACH1 knockdown cells, we did cell growth assays in the presence of TGF-β and there was no statistically significant difference in the growth of the DACH1 knockdown clones compared with the parental or vector clones (data not shown). It is likely that restoration of TGF-β growth inhibition requires more substantial knockdown of DACH1 levels. These results, together with the luciferase assays using the dominant-negative DACH1, strongly suggest that DACH1 is at least partly responsible for the lack of response to TGF-β in A547 ovarian cancer cells.

Discussion

Gene expression profiling is a powerful tool for identifying genes that are aberrantly expressed in cancer specimens compared with their normal counterparts. To better understand the role of genes involved in the resistance to the antiproliferative effects of TGF-β associated with ovarian cancer, we used expression profiling to identify genes in the TGF-β pathway that are aberrantly expressed in ovarian cancers compared with normal surface ovarian

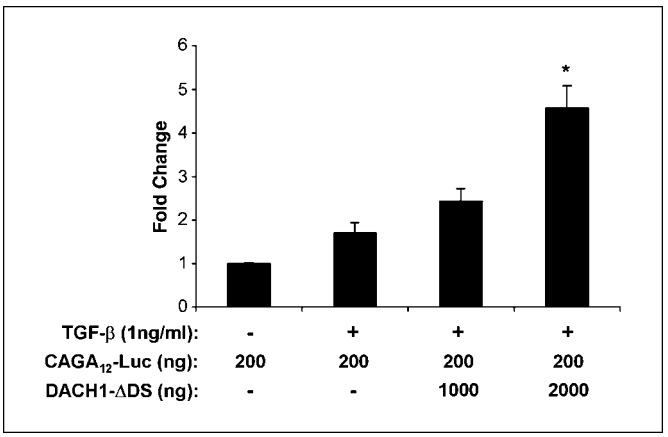

Figure 5. Dominant-negative DACH1 restores TGF-β signaling in ovarian cancer cells. A547 cells were transiently cotransfected with the $CAGA_{12}$-Luc promoter luciferase construct and increasing amounts of a DACH1 dominant-negative (DACH1-ΔDS) expression plasmid, serum starved for 36 hours, and then stimulated with TGF-β (1 ng/mL) for 12 hours. Promoter activity was determined by luciferase assays. Results are fold change relative to unstimulated cells after normalizing to *Renilla* luciferase to control for transfection efficiency. *Columns,* mean of triplicate experiments; *bars,* SD. *, $P < 0.05$, compared with unstimulated cells.

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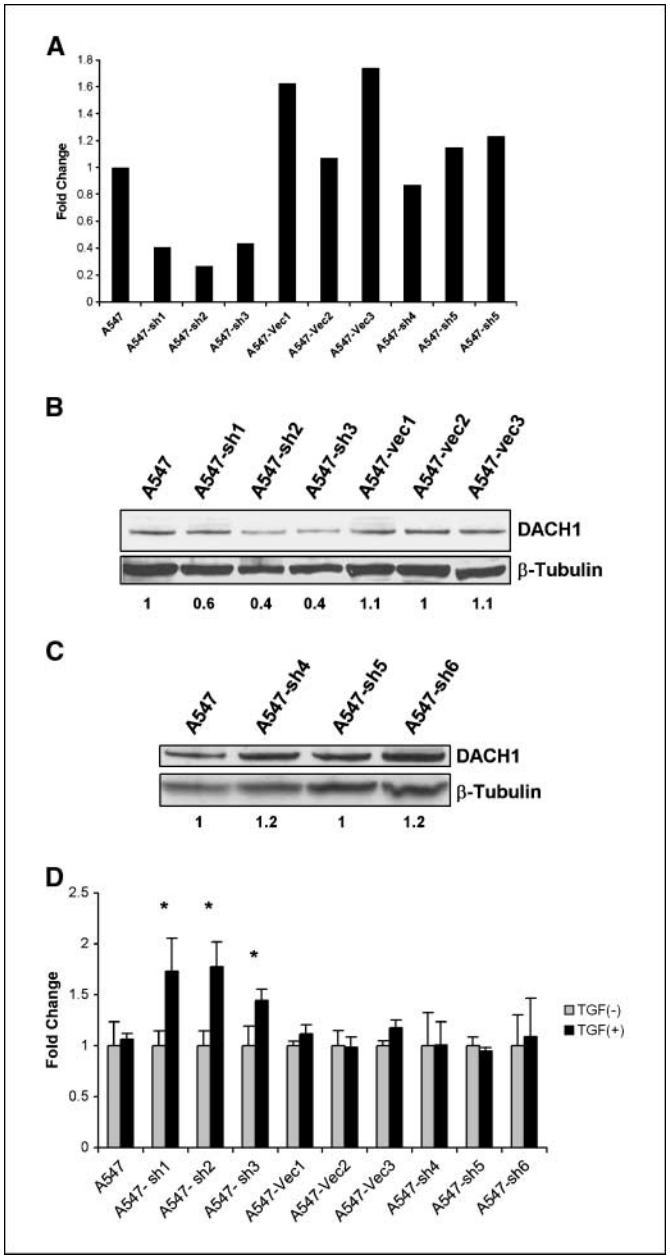

Figure 6. Knockdown of DACH1 expression partially restores TGF-β signaling in A547 cells. *A,* quantitative RT-PCR analysis of *DACH1* expression in three clones stably transfected with a DACH1 shRNA construct (A547-sh1, A547-sh2, and A547-sh3) compared with parental A547 cells, three clones transfected with a vector control (A547-vec1, A547-vec2, and A547-vec3), and three clones transfected with a DACH1 shRNA construct that did not cause a decrease in DACH1 expression (A547-sh4, A547-sh5, and A547-sh6). *B,* Western blot analysis of DACH1 expression in A547 cells, the three clones stably transfected with a DACH1 shRNA construct, and three clones transfected with a vector control. β-Tubulin was used as a control for protein loading. *Numbers,* fold change relative to A547 cells after normalizing to β-tubulin. *C,* Western blot analysis of DACH1 expression in the three clones stably transfected with a DACH1 shRNA construct that did not cause a decrease in DACH1 expression. *Numbers,* fold change in DACH1 expression relative to the parental A547 cells after normalization to β-tubulin expression. *D,* parental A547, the three vector control clones, three DACH1 knockdown clones, and the three control shRNA clones (A5467-sh4, A547-sh5, and A547-sh6) were transfected with the $CAGA_{12}$-Luc promoter luciferase reporter, serum starved for 36 hours, and then stimulated with 1 ng/mL TGF-β for 12 hours. Promoter activity was measured by luciferase assay, and results represent the fold change over unstimulated cells after normalization to *Renilla* luciferase to control for transfection efficiency. Results of triplicate experiments. *, $P < 0.05$, compared with unstimulated cells.

epithelium. We identified seven genes that were differentially regulated in ovarian cancer specimens compared with normal ovarian surface epithelium. Those genes that inhibit TGF-β signaling (*DACH1, EVI1*, and *BMP7*) were up-regulated in the cancer specimens, and those genes that enhanced TGF-β signaling (*TGFBRII, SMAD4, TFE3*, and *PCAF*) were down-regulated, resulting in a net inhibition of TGF-β signaling in these ovarian cancer specimens.

Although none of the genes identified from our microarray analysis have been definitely associated with ovarian cancer, they have been linked to other epithelial cancers. Mutational inactivation of *TGFBRII* has been identified in pancreatic and biliary cancers (23) as well as in colon cancer (24), and altered expression of *SMAD4* is associated with endometrial cancer (25). TFE3 acts synergistically with the Smad3 and Smad4 complex to activate SERPINE1 and Smad7 transcription (26) is inactivated by gene fusions in renal carcinomas (27–29); BMP7 decreases nuclear accumulation of Smad3 and up-regulation of serine proteinase inhibitor (plasminogen activator inhibitor 1; ref. 30), thus inhibiting TGF-β signaling, is up-regulated in melanoma cell lines (31), and PCAF, a nuclear coactivator for EVI1 (32) and Smad3 (33), is not commonly associated with human epithelial cancers (34).

The mechanism(s) involved in the resistance to the antiproliferative effects of TGF-β in ovarian cancer has remained unclear. It is not due to functional mutations in the TGF-β receptors or Smads, as these occur infrequently (16, 35, 36). It has also been suggested that other factors, such as epidermal growth factor, abrogate the antiproliferative effect of TGF-β *in vivo* (37). Our data suggest that it is altered gene expression of TGF-β pathway genes that may account for the resistance of ovarian cancer to the antiproliferative effects of TGF-β. Several other studies have shown that it is not alterations in gene expression of TGF-β pathway genes that are responsible for the resistance of ovarian cancer to the antimitogenic effects of TGF-β but rather blockage of this pathway downstream of the Smad complex formation (12, 38). The discrepancy between these studies and our findings could be due to the type and number of specimens used in the various studies. In fact, it has been shown that primary ovarian cancer cells in culture are responsive to the growth-inhibitory effects of TGF-β (38, 39).

Two genes of interest that we identified from our microarray analysis were *DACH1* and *EVI1*, both of which inhibit TGF-β signaling. *DACH1* shares structural homology to the *Ski* and *Sno* proto-oncogenes (40), both of which repress activator protein-1 (AP-1) and Smad signaling associated with the TGF-β pathway (41, 42). DACH1 was recently shown to inhibit TGF-β-induced apoptosis in breast cancer cell lines by binding to Smad4 and NCoR (7), but it has not previously been identified to be associated with ovarian cancer. We showed that it is up-regulated in the majority of ovarian cancer specimens (72% of advanced-stage and 93% of early-stage ovarian cancers) profiled by microarray analysis. We believe that this is the first report implicating DACH1 in ovarian cancer, and our *in vitro* data showing that DACH1 inhibits TGF-β signaling in IOSE80 cells and a dominant-negative DACH1 (DACH1-ΔDS), as well as knockdown of DACH1 expression, restores TGF-β signaling in the A547 ovarian cancer cell line support a role for this protein in the inhibition of TGF-β signaling in ovarian cancer.

EVI1 is an oncogene that is frequently associated with acute and chronic myelogenous leukemia (43–45) and has also been

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implicated in lung cancer (46, 47) and is overexpressed in endometrial cancer cell lines (48). EVI1 affects cellular function via several pathways in hematopoietic cells, including activation of AP-1 and interaction with the Smad3/Smad4 complex at the nuclear level, to inhibit TGF-β-induced transcription (44). To date, only one study has reported altered *EVI1* expression in ovarian cancer (49). Our results indicate that it may play a significant role in ovarian cancer because the majority of all ovarian cancer specimens showing markedly increased expression of this gene by microarray analysis. The importance of EVI1 in ovarian cancer development is underscored by the fact that a subset of these tumors shows *EVI1* gene amplification. This is an early genetic event because 93% of patients with early-stage ovarian cancer have increased *EVI1* expression (see Table 2), suggesting that *EVI1* may be important early in the transformation process and for the development of ovarian cancer. Somewhat surprisingly, we found that amplification at the *EVI1* locus occurred at a higher level than at the *PI3K* locus, a locus frequently amplified in ovarian cancer (21, 22), further implicating amplification of this gene as an important event in the development of ovarian cancer. Increased *EVI1* gene copy number has been found in non–small cell lung cancer (46, 47) and esophageal cancer (50).

Our results support previous work, showing that elements of the TGF-β/Smad signaling pathway remain intact in primary ovarian cancer cell cultures (12). This study showed minimal differences in the expression of many genes involved in TGF-β signaling, including *Smad2, Smad3, Smad6, Smad7, SnoN*, and *Ski*. We also found no difference in expression of these genes in our study. Further, this group showed that Smad3 and Smad4 phosphorylation and complex formation were normal in ovarian cancer cells (12). This led to the suggestion that the TGF-β pathway is blocked downstream of Smad complex formation and other as yet unidentified components of the TGF-β pathway are responsible for this. The results from our study complement these findings and suggest that EVI1 and DACH1 may be two ideal candidates. EVI1 actively interferes with Smad3 transcriptional activity without interfering with Smad3/Smad4 complex formation (44), and DACH1 binds to Smad4 and inhibits TGF-β/Smad signaling (7). These actions would effectively inhibit the effects of TGF-β without altering the upstream signaling events. Of note, altered expression of these seven TGF-β pathway genes shows no prognostic value for patient survival. We hypothesize that this is the result of the redundant molecular lesions seen in many of these tumors where different genes within the TGF-β pathway are affected, resulting in the inhibition of the pathway. Further, we suspect that the inhibition of this pathway is an early event in the development of ovarian cancer. This is supported by the fact that altered expression of these seven TGF-β pathway genes from normal ovarian epithelium was found in early-stage tumors. Thus, essentially all ovarian cancers will have this critical pathway inhibited early on minimizing its potential to differentially affect patient survival.

In summary, we believe that global expression profiling applied to a large sample of carefully processed specimens coupled with the use of PathwayAssist has allowed us to identify the seven genes with altered expression in the TGF-β pathway, which may contribute to the observed TGF-β resistance of ovarian cancers. We show that two of these genes (*DACH1* and *EVI1*) inhibit TGF-β signaling in immortalized ovarian epithelial cells and they may be useful as novel therapeutic targets.

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