Analysis of Gene Expression Regulated by the ETV5 Transcription Factor in OV90 Ovarian Cancer Cells Identifies FOXM1 Overexpression in Ovarian Cancer

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Abstract

Epithelial ovarian cancer is the most lethal gynecologic malignancy and the fifth leading cause of cancer death in women in the Western world. ETS transcription factors have been implicated in the regulation of gene expression during a variety of biologic processes including cell growth and differentiation. We recently examined the role of the ETS transcription factor ETV5 in epithelial ovarian cancer and described ETV5 as being upregulated in ovarian tumor samples as compared with ovarian tissue controls. In ovarian cancer cells, we showed that ETV5 regulated the expression of cell adhesion molecules, enhancing ovarian cancer cell survival in anchorage-independent conditions and suggesting that it plays a role in ovarian cancer cell dissemination and metastasis into the peritoneal cavity. To understand the role of ETV5 transcription factor during ovarian cancer cell dissemination, we analyzed by gene expression microarray technology those genes whose expression was altered in an ovarian cancer cell line with a stable downregulation of ETV5. The analysis of the genes and signaling pathways under the control of ETV5 in OV90 cells has unraveled new signaling pathways that interact with ETV5, among them the cell-cycle progression and the TGFβ signaling pathway. In addition, we found that the downregulation of ETV5 reduced the expression of the oncogenic transcription factor FOXM1. Consistently, FOXM1 was overexpressed in ovarian tumor samples, and its transcriptional levels increased with ETV5 transcription in ovarian tumor samples. Moreover, FOXM1 expression levels increased with tumor grade, suggesting a role in the progression of ovarian cancer. Mol Cancer Res; 1–11. ©2012 AACR.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy and the fifth leading cause of cancer death in women in the Western world (1). Reasons for this high lethality include the late stage at which women are diagnosed and the inherent aggressive biology of this cancer. Largely asymptomatic, more than 70% of patients are already at an advanced stage at initial diagnosis. The 5-year survival rate for women with advanced stage disease (stages II, III, and IV) is less than 20%. In contrast, the cure rate is almost 90% when women are diagnosed at an early stage (stage I). Although up to 80% of patients will initially respond to chemotherapy, only 10% to 15% of those patients will achieve and maintain a total response to treatment (2).

Ninety percent of the cases of ovarian carcinoma arise from the ovarian surface epithelium (OSE), which is composed of a single layer of epithelial cells that overlie the surface of the ovary. It possesses an uncommitted phenotype that expresses common mesenchymal markers, as well as epithelial markers. During neoplastic progression, the OSE loses its stromal characteristics and undergoes aberrant epithelial differentiation. Preneclastic inclusion cysts and epithelial ovarian carcinoma express E-cadherin and are nonmigratory. Therefore, unlike most epithelial tumors, ovarian tumors do not follow the classic model of epithelial-to-mesenchymal transition (EMT); rather, they show an increase in E-cadherin expression during tumor progression (3, 4). In addition, and unlike other solid tumors, which must infiltrate the surrounding tissue to progress, ovarian cancer spreads as tumor cells are shed from the primary tumor into the peritoneal cavity (5). In the peritoneal cavity,
tumor cells aggregate as spheroids, to maintain cell-to-cell contact and survive under anchorage-independent conditions. These spheroids can then attach to the extravascular mesothelial wall and, subsequently, invade, establishing tumors at secondary sites. Both cell-to-cell adhesion and cell–extracellular matrix interacting molecules are believed to play a role in the process of spheroid formation and tumor invasion (6–8).

We recently examined the role of the ETS transcription factor ETV5 in epithelial ovarian cancer and described ETV5 as being upregulated in ovarian tumor samples as compared with ovarian tissue controls (9). ETS transcription factors have been implicated in the regulation of gene expression during a variety of biologic processes including cell growth and differentiation (10). Moreover, ETS family members have been correlated to tumor progression in various cancer types (11–13). In ovarian cancer cells, we showed that ETV5 regulated the expression of cell adhesion molecules, enhancing ovarian cancer cell survival in anchorage-independent conditions and suggesting that it plays a role in ovarian cancer cell dissemination and metastasis into the peritoneal cavity (9).

To understand the role of the transcription factor ETV5 during ovarian cancer cell dissemination, we analyzed by gene expression microarray technology those genes whose expression was altered in ovarian cancer cell lines with a stable downregulation of ETV5. An analysis of the deregulated genes indicated changes in cell-cycle progression and TGFβ signaling. In particular, we found that the down-regulation of ETV5 reduced the expression of the oncogenic transcription factor FOXM1. Consistently, FOXM1 was overexpressed in ovarian tumor samples, and its transcriptional levels increased with ETV5 transcription in ovarian tumor samples. In addition, the FOXM1 expression levels increased with tumor grade, suggesting a possible role in the progression of ovarian cancer.

Material and Methods

Cell lines and ovarian tumor samples for microarray analysis

OV90 ovarian cancer cells were transduced with stable (sh)RNA EGFP lentiviral producing vector (FSV), to knockdown ETV5 expression and to generate 2 stable OV90-modified cell lines. Two short hairpin (sh) ETV5 sequences were used. ETV5 i3, 5′-CTCTACAACATTTGTGCC-TAT-3′ and ETV5 i4, 5′-CGGCAAATGTCAGAACC-TATT-3′ to generate 2 stable OV90-modified cell lines (OV90i3 and OV90i4, respectively). OV90 ovarian cancer cell lines were cultured, as described by Llauradó and colleagues (9). Forty-eight ovarian tumor samples and 6 ovarian tissue controls were collected from patients who underwent surgery in the Department of Gynecological Oncology at the Hospital Vall d’Hebron in Barcelona, Spain (Supplementary Table S1). Informed consent was obtained from all patients included in this study. Tumor staging was conducted according to the International Federation of Gynecology and Obstetrics (FIGO) classification.

Gene expression analysis of ovarian cancer cell lines

cDNA microarrays were achieved using the Affymetrix microarray platform and the GeneChip Human Gene 1.0 ST Array (Affymetrix). Triplicates of OV90 and OV90i4 cells were used to conduct this study. Total RNA was extracted with the RNeasy Mini Kit (Qiagen), following the instructions provided by the manufacturer. Starting material consisted of 100 ng of total RNA from each sample. The quality of the isolated RNA was measured first by the Bioanalyzer Assay (Agilent). Briefly, sense single-strand (DNA) suitable for labeling was generated from the total RNA with the Ambion WT Expression Kit (Ambion), according to the manufacturer’s instructions. Sense ssDNA was fragmented, labeled, and hybridized to the arrays using the GeneChip WT Terminal Labeling and Hybridization Kit from Affymetrix. The chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000. Images were processed using the Microarray Analysis Suite 5.0 (Affymetrix). The data were submitted to non-specific filtering, to remove low-signal genes and low variability genes. Following the Smyth methodology, the selection of differentially expressed genes between conditions was based on a linear model analysis with empirical Bayes moderation of the variance estimates (14). This type of analysis yields information similar to standard test statistics, such as fold changes (FC) or P values. This information can then be used to determine differentially expressed genes. All of our statistical analyses were carried out using the free statistical language R and the Bioconductor Project’s libraries developed for microarray data analysis.

Gene expression analysis of ovarian tumor samples

Total RNA was extracted using the RNeasy Mini Kit (Qiagen), following the instructions provided by the manufacturer. The microarrays for gene expression were designed by the Tethys algorithm using the ENSEMBL database. Cy3- and Cy5-labeled aRNA was produced using the MessageAmplification Kit by Ambion. Microarray hybridization was conducted at 60°C with 17 hours of hybridization time, according to Agilent indications. Initial raw data were obtained using an Agilent DNA Microarray Scanner (G2505B) and Agilent acquisition software (Feature Extraction Software). The expression levels for particular genes in the normal samples (controls) were compared with the same reference pool, and final expression FCs between tumor and normal ovarian tissue were generated in silico, eliminating the reference pool.

Signaling pathway analysis

Functional pathway and network analyses were conducted through the use of Ingenuity pathway Analysis (IPA: version 9.0, Ingenuity Systems). IPA identified those canonical pathways, biologic processes, and gene interaction networks that were most significant to the genes selected from the microarray expression analysis (differentially expressed genes with log-FC <-1; >1 and an adjusted P < 0.01). Each gene designation was mapped in the Ingenuity Pathways Knowledge Base.
TGFB cancer cell line treatment
A total of 2.4 × 10^5 OV90C and OV90i4 cells were plated in triplicate on p60 plates. Cells were either treated with 100 pmol/L TGFB or with 2 μmol/L of the SB431542 inhibitor for 7 days. Then, whole protein extracts were prepared using Laemmli buffer.

Ovarian tumor samples for real-time quantitative PCR
A panel of 34 ovarian tumor samples and 11 ovarian tissue controls were obtained from patients who underwent surgery in the Department of Gynecological Oncology at the Hospital Vall d’Hebron (Supplementary Table S2; ref. 9).

Real-time quantitative PCR
Total RNA was collected and purified using the RNeasy Kit (Qiagen). One microgram of total purified RNA was subjected to a reverse transcriptase reaction using Superscript III (Invitrogen Life Technologies) according to the manufacturer’s conditions. cDNA, corresponding to approximately 1 μg of starting RNA, was used in 3 replicates for quantitative PCR (TaqMan; Applied Biosystems). For the analysis of ovarian tumor samples and controls, we used the *ETV5*-Hs00231790_m1 and the *FOXM1*-Hs00153543_m1 probes and the POLR2A-Hs00172187_m1 probe for normalization (Applied Biosystems). The gene expression profiles of OV90C and OV90i4 cells were cultured on coverslips and incubated for 20 minutes at 37°C in serum-free MCDB-105:M199-E (1:1) medium containing 10 μmol/L DHE (Invitrogen). Upon removal of the medium and wash with PBS, fluorescence was directly monitored on an Olympus fluorescence microscope (excitation, 510 nm; emission, 590 nm). Induced oxidative stress was determined in OV90 controls and OV90i4 cells treated with 0.5 mmol/L of H2O2 from 30 minutes to 2 hours. Then, cells were incubated with the DHE probe, and the fluorescence production was monitored as described above.

Western blot analysis
Whole-cell extracts were prepared using Laemmli buffer. Samples were run on an 8% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked in 5% milk solution (TBS-0.1% Tween) for 1 hour at room temperature and incubated with indicated primary antibody in 5% milk solution overnight at 4°C. The membranes were washed 3 times for 10 minutes in TBS-0.1% Tween at room temperature and incubated for 1 hour with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected by the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech) as described by the manufacturer’s instructions.

The primary antibodies used for Western blotting were anti-ERM/ETV5 (sc-22807), anti-FOXM1 (C20, sc-502), anti-ERK1/2 (sc-06-182), and anti-CYCLIN B1 (sc-245; Santa Cruz Biotechnologies); anti-pSMAD2 (3101; Cell Signaling); and anti-α-tubulin (2125; Cell Signaling). Proteins were detected by the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech) as described by the manufacturer’s instructions.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) on OV90 cells was conducted using aAb ERM/ETV5 and the Magna ChIP Kit (Millipore), according to the manufacturer’s instructions. Rabbit acetyl-histone H3 and normal rabbit IgGs and no antibody were used as positive and negative controls, respectively. For PCR analysis, 1 μL of input DNA extraction and 2 μL of immunoprecipitated DNA were used for 40 cycles of amplification (annealing temperature, 60°C) with the following primers for the *FOXM1* promoter: *FOXM1* reverse, 5′-TTC AGT TTT TTC CGC TGT TGT-3′ and *FOXM1* forward, 5′-GGA AGA AAG CGG AGA TAA TAC-3′. In addition, we have used a Custom TaqMan Assay (Applied Biosystems, ref 4331348) that includes the 3 ETS-binding sites located in the *FOXM1* promoter to conduct an real-time quantitative PCR (RT-Q-PCR) on the samples obtained from the ChIP. The probe and the primers used for the RT-Q-PCR are the following: 5′-CAGACGATCGTT-CACTG-3′, 5′-GATAATACCGACCCCTCAAAGG-3′, and 5′-CTGGGCCCCTTCTTCT-3′.

Oxidative stress assay
Basal oxidative stress levels were determined by measuring dihydroethidium (DHE) fluorescence. OV90C and OV90i4 cells were cultured on coverslips and incubated for 20 minutes at 37°C in serum-free MCDB-105:M199-E (1:1) medium containing 10 μmol/L DHE (Invitrogen). Upon removal of the medium and wash with PBS, fluorescence was directly monitored on an Olympus fluorescence microscope (excitation, 510 nm; emission, 590 nm).

Statistical analysis
Statistical analyses were conducted using the Statistical Package for Social Science version 15.0 (SPSS, IBM). The Student t test was conducted to compare the means of the mRNA expression levels. The Pearson correlation test was applied to analyze the correlation between *ETV5* and *FOXM1* expression in the human tumor samples. *P* < 0.05 was considered statistically significant.

Results
Characterization of ETV5-regulated genes in OV90 ovarian cancer cells
The gene expression profile of OV90 ovarian cancer cells with a stable downregulation of *ETV5* (OV90i4) was compared with OV90 control cells using microarray technology. Approximately, 8,500 genes were detected after nonspecific filtering to remove low-signal and low variability genes. Of those, we selected 798 (9.4%), which showed log-FCs >1 in differential expression, for further analysis.

The differentially expressed genes (log-FC < -1; >1 and adjusted *P* < 0.01) were subjected to IPA, to identify the biologic mechanisms, pathways, and functions most relevant to the genes of interest. Of the 25 networks generated, the "cell cycle, cellular assembly and organization, DNA replication, recombination, and repair" network was identified as the top scorer (Table 1). Fourteen networks had scores greater than 2. The score indicates the likelihood that the assembly of a set of genes in a network could be explained by random chance alone. Networks with scores of 2 or higher...
have at least a 99% confidence of not being generated by random chance alone. In addition, the differentially expressed transcripts were subjected to canonical pathways and biologic functions analysis using the IPA software. The biologic functions were grouped by the IPA software into disease and disorders, molecular and cellular functions and physiological system development and functions. The biologic functions most represented in each group were cancer, cell-cycle, and tissue development, respectively. The most significant canonical pathway represented was the “role of BRCA1 in DNA damage response.”

A more detailed analysis of the molecules and connections included in the top 2 networks pointed to TGFβ signaling (network 1) and cell-cycle regulation (network 2) as being 2 signaling pathways altered under the control of ETV5 in the OV90 ovarian cancer cell line (Fig. 1A). These results support our previous work on ETV5 and ovarian cancer, where we showed that ETV5 can regulate the proliferation rate of OV90 cells and the induction of the EMT through upregulation of the Zeb1 transcription factor and, subsequently, the repression of E-cadherin in OV90 ovarian cancer cells (9). To confirm the significance of the TGFβ pathway in relation to ETV5, we treated OV90C and OV90i4 cells with TGFβ and the TGFβ receptor kinase–specific inhibitor SB431542 that blocks the TGFβ signaling pathway. We observed that the OV90 controls and the i4 cells possessed different basal levels of pSmad2 protein, indicating a difference in the levels of TGFβ pathway activation, which, in turn, suggests that ETV5 can modulate the activity of the TGFβ signaling pathway. The addition of TGFβ increased the level of Smad2 phosphorylation, whereas the addition of SB431542 blocked signal transduction through the receptor, thus indicating that the activation of the signaling pathway through the receptor was unaffected in OV90 cells (Fig. 1A). Our data confirmed the interaction of the TGFβ pathway and the ETV5 transcription factor, although exhaustive research must still be conducted, to unravel the significance of this interaction.

Network 2 includes the genes involved in cell-cycle regulation and DNA repair, most of which are already implicated in ovarian carcinogenesis (Fig. 1B). In particular, cyclin B1 was one of the core proteins found in this network. We have already confirmed the downregulation of cyclin B1 protein levels in OV90i4 cells (Fig. 1B).

**ETV5-dependent regulation of FOXM1 transcription factor**

To understand the mechanisms of cell-cycle regulation by ETV5, we focused our attention on those genes in network 2 that are involved in cell-cycle regulation. Among the deregulated proteins, FOXM1 expression was found to be downregulated 2-fold (log-FC = 1.048) in ETV5-downregulated OV90 cells compared with controls. FOXM1 is a transcription factor that has been associated with carcinogenesis, as it is overexpressed in various human malignancies (15–19). FOXM1 has been shown to be an important cell-cycle regulator of both the transition from G1 to S-phase and the progression to mitosis. Loss of FOXM1 expression generates mitotic spindle defects, delays cells during mitosis, and induces mitotic catastrophe. Moreover, FOXM1 has been shown to regulate the transcription of cell-cycle genes essential for G1–S and G2–M progression, including Cdc25A, Cdc25B, cyclin B, cyclin D1, p21, and p27 (20). We found that most of the genes that have been shown to be targets of FOXM1 were also downregulated in OV90i4 cells (21, 22). This suggests that FOXM1 could be the mediator of the cell proliferation delay found in OV90i4 cells (Fig. 2A). The downregulation of FOXM1 was further validated by RT-q-PCR and Western blotting. These analyses confirmed that the downregulation of FOXM1 was concomitant to the downregulation of ETV5 (Fig. 2B). The downregulation of FOXM1 was validated by Western blotting in a second OV90-modified cell line with ETV5 downregulation (OV90i3), confirming that the downregulation was not due to off-target effects (Supplementary Fig. S1). To check whether ETV5 directly regulates FOXM1 transcription, we analyzed whether or not ETV5 binds to the FOXM1 promoter using ChIP. Three putative ETS-binding sites were identified in the FOXM1 promoter region by bioinformatic prediction. ChIP analysis showed that ETV5 did bind to the proximal promoter region of FOXM1, suggesting that FOXM1 could be regulated by the direct binding of ETV5 to its promoter region (Fig. 2C; Supplementary Fig. S2).

**FOXM1 is a regulator of oxidative stress in OV90 ovarian cancer cells with ETV5 downregulation**

FOXM1 has also been described as a critical regulator of oxidative stress during oncogenesis. Oxidative stress has been found in many tumors. It is caused by an imbalance between the generation of reactive oxygen species (ROS) and a cell’s

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**Table 1. List of the 5 top networks ranked by the IPA software**

<table>
<thead>
<tr>
<th>Top networks</th>
<th>Associated network functions</th>
<th>Score</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cell cycle, cellular assembly and organization, DNA replication, recombination, and repair</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>2.</td>
<td>Cell cycle, cancer, and genetic disorder</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>3.</td>
<td>DNA replication, recombination and repair, cell cycle, and connective tissue development and function</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>4.</td>
<td>Lipid metabolism, small-molecule biochemistry, and vitamin and mineral metabolism</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>5.</td>
<td>Inflammatory response, cell death, and cellular growth and proliferation</td>
<td>15</td>
<td>19</td>
</tr>
</tbody>
</table>

NOTE: Networks with scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone.
ability to rid itself of oxidants. Moreover, elevated levels of ROS have recently been proposed to be involved in tumor metastasis (23, 24) as an important messenger for EMT (25), cell migration, invasion (26), and angiogenesis (27).

A negative feedback loop involving FOXM1 regulating ROS has been identified (28). In our research, elevated FOXM1 was found to significantly upregulate the expression of ROS scavenger genes, such as MnSOD and catalase. Previous work in our laboratory has also described ETV5 as a regulator of the enzymes involved in antioxidant defense (29). We decided to investigate the possible link between ETV5, FOXM1, and oxidative stress in OV90 cells. We analyzed the production of ROS in OV90i4 and OV90 control cell lines. Cells were incubated with the DHE probe. DHE oxidation to ethidium stained the nucleus with a bright red fluorescence. As can be observed in Fig. 3A, the OV90 cells with a downregulation of ETV5 displayed more ROS, linking the downregulation of ETV5 with a decrease in protection against oxidative stress. To further explore a putative link between an increase in oxidative stress, ETV5, and FOXM1 in OV90 cell lines, we exposed OV90 cells to 0.5 mmol/L concentrations of H2O2 for different periods of time. This resulted in an increase in ROS production and an increase in ETV5 and FOXM1 protein levels. This was most apparent in OV90i4 cells with low ETV5 endogenous levels, suggesting that the downregulation of ETV5 sensitizes OV90 cells to oxidative stress, possibly through the downregulation of FOXM1 (Fig. 3B and C).
Expression of **FOXM1** in ovarian tumor samples

Oligonucleotide microarrays comparing the gene expression profiles of ovarian tumor samples and normal ovarian tissue had been previously used in our laboratory to identify diagnostic biomarkers for ovarian cancer (unpublished results). Analysis of the microarray data showed that **FOXM1** and many **FOXM1** target genes (21, 22) were overexpressed in ovarian tumors compared with controls (Table 2). To validate the overexpression of **FOXM1** in ovarian tumors, we analyzed the expression levels of **FOXM1** in a new panel of ovarian tumor samples using quantitative RT-Q-PCR. A panel of 34 ovarian tumor samples and 11 ovarian tissue controls was used to measure the expression levels of **FOXM1** and 5 of the most upregulated **FOXM1** target genes (Table 3). Gene expression in the ovarian tumor samples was normalized against ovarian tissue controls. **FOXM1** relative quantification revealed an upregulation of **FOXM1** in the majority of samples (Fig. 4A). Statistical analysis comparing the levels of expression between tumor subtypes showed that there were significantly higher **FOXM1** mRNA expression levels in grade III tumors than in grade I tumors (Student t test; \( P < 0.05; \) Fig. 4B). These data indicated that increased levels of **FOXM1** expression were associated with a more aggressive phenotype. Furthermore, as grade III tumors are associated with poor prognosis, this also suggests a role for **FOXM1** in the progression of ovarian cancer.

Finally, we tested whether an increase in **ETV5** mRNA expression levels was associated with an increase in **FOXM1** levels in the same set of human tumor samples. We found that the mRNA levels of both molecules were significantly correlated (Pearson correlation coefficient = 0.496, \( P = 0.003; \) Fig. 4C).

**Discussion**

We have recently shown that **ETV5** transcription factor is overexpressed in ovarian cancer and regulates cell adhesion molecules in ovarian cancer cells (9). To understand the molecular pathways associated with **ETV5** that are also involved in ovarian cancer cell progression and
dissemination, we analyzed the expression profile of ETV5 downregulation in ovarian cancer cells by gene expression microarray technology.

On the basis of gene interactions and molecular relationships, an analysis of the deregulated genes in OV90i4 cells pointed to the TGFβ signaling and cell-cycle pathways as the main networks altered in OV90 cells with ETV5 downregulation. It is well known that TGFβ plays a dual role as tumor suppressor and pro-oncogenic factor (30–33). In advanced cancer stages, it promotes tumor invasion,

**Table 2. Microarray data for FoxM1 and FoxM1 target genes upregulated in ovarian tumor samples compared with controls**

<table>
<thead>
<tr>
<th>ID</th>
<th>Entrez gene name</th>
<th>FC (tumor vs. normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXM1</td>
<td>Forkhead box M1</td>
<td>1.79</td>
</tr>
<tr>
<td>CCNB2</td>
<td>Cyclin B2</td>
<td>1.67</td>
</tr>
<tr>
<td>CDKN3</td>
<td>Cyclin-dependent kinase inhibitor 3</td>
<td>1.47</td>
</tr>
<tr>
<td>CEP55</td>
<td>Centrosomal protein 55 KDa</td>
<td>2.09</td>
</tr>
<tr>
<td>BIRC5</td>
<td>Baculoviral IAP repeat containing 5; apoptosis inhibitor survivin</td>
<td>1.73</td>
</tr>
<tr>
<td>AURKB</td>
<td>Aurora kinase B</td>
<td>1.39</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer 2, early onset</td>
<td>1.12</td>
</tr>
<tr>
<td>CCNA2</td>
<td>Cyclin A2</td>
<td>1.25</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin B1</td>
<td>1.05</td>
</tr>
<tr>
<td>CDC20</td>
<td>Cell division cycle 20 homolog (S. cerevisiae)</td>
<td>1.44</td>
</tr>
<tr>
<td>NEK2</td>
<td>NIMA (never in mitosis gene a)-related kinase 2</td>
<td>1.42</td>
</tr>
<tr>
<td>PLK1</td>
<td>Polo-like kinase 1</td>
<td>1.09</td>
</tr>
<tr>
<td>SKP2</td>
<td>S-phase kinase-associated protein 2 (p45)</td>
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</tr>
<tr>
<td>KIF20A</td>
<td>Kinesin family member 20A</td>
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</tr>
<tr>
<td>STMN1</td>
<td>Stathmin 1</td>
<td>1.27</td>
</tr>
<tr>
<td>MMD</td>
<td>Monocyte to macrophage differentiation-associated</td>
<td>1.87</td>
</tr>
</tbody>
</table>

Figure 3. FOXM1 as a protector against oxidative stress in OV90 ovarian cancer cells with ETV5 downregulation. A, basal oxidative stress levels determined by measuring DHE fluorescence. As can be observed, OV90 i4 nuclear staining was bright red compared with OV90 control cells. Pictures (i) and (ii) were taken at ×20 magnification, whereas (iii) and (iv) were taken at ×40 magnification. B, induced oxidative stress. OV90 controls and OV90i4 cells were treated with 0.5 mmol/L of H₂O₂ from 30 minutes to 2 hours. Pictures (i) and (ii) represent cells not treated; (iii) and (iv) represent cells treated with 0.5 mmol/L of H₂O₂ for 1 hour; and (v) and (vi) represent cells treated with 0.5 mmol/L of H₂O₂ for 2 hours. C, OV90 controls and OV90i4 cells were treated with 0.5 mmol/L of H₂O₂ from 30 minutes to 2 hours. The protein extracts were analyzed by Western blotting against ETV5 and FOXM1. Total ERK1/2 was used as a loading control.
angiogenesis, and metastasis (34). In ovarian cancer, TGFβ enhances ovarian cancer metastatic potential through the induction of an EMT (35, 36). In addition, ETS transcription factors have already been described to mediate TGFβ-induced EMT (37–39). Also, ETS-1 oncogenic activity has been shown to be mediated by the TGFα mitogen (37). Our previous work has described that the downregulation of ETV5 induces EMT through the induction of Zeb1, which delays the rate of cell proliferation (9). Analysis of the TGFβ pathway in OV90 controls and in i4 cells showed an interaction between the TGFβ pathway and the ETV5 transcription factor. This confirms that the analysis of gene interactions using the IPA software is a useful approach for identifying key pathways. In addition, in our analysis of network 2, cell-cycle regulation was found to be altered in OV90i4 cells with cyclin B1 indicated as the core molecule. ETS transcription factors have been shown to activate the expression of cyclins D1 and D3 (40, 41). We confirmed the downregulation of cyclin B1 in OV90i4 cells,

**Table 3.** Validation of FoxM1 and FoxM1 target genes by quantitative RT-Q-PCR in ovarian tumor samples compared with controls

<table>
<thead>
<tr>
<th>Detector ID</th>
<th>Entrez gene name</th>
<th>FC (tumor vs. normal)</th>
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<tr>
<td>FOXM1-Hs00153543_m1</td>
<td>FOXM1</td>
<td>3.68</td>
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<td>CCNB2-Hs00270424_m1</td>
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<td>CEP55-Hs00216688_m1</td>
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<tr>
<td>BIRC5-Hs00153353_m1</td>
<td>BIRC5</td>
<td>7.49</td>
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</table>

Figure 4. FOXM1 expression in ovarian tumor samples. A, FOXM1 mRNA expression levels from a panel of 34 ovarian tumor samples normalized against 11 ovarian tissue controls. B, box plot of FoxM1 expression levels according to tumor grade. A statistically significant association between the levels of FoxM1 mRNA expression and tumor grade (Student t test; P < 0.05) was found. C, the expression of FOXM1 showed a significantly positive correlation with ETV5 (P < 0.003; Pearson correlation coefficient = 0.496).
suggesting that the observed decrease in cell proliferation was because of an inhibition of cell-cycle progression.

Several studies have described the overexpression of FOXM1 in various human malignancies, including prostate, breast, lung, ovary, colon, pancreas, stomach, bladder, liver, and kidney (42) cancers, suggesting that it may play a role in the development and progression of human cancers. Analysis of data obtained from a microarray study previously conducted in our laboratory comparing ovarian tumor samples with ovarian controls showed FOXM1 among the genes upregulated in ovarian tumors and downregulated in ETV5-modified cells. We found that FOXM1 is regulated by ETV5 in ovarian cancer cells through the direct binding of ETV5 to its promoter region. Our group has previously described the role of ETV5 in the protection against oxidative stress (29), and we suggest that this effect may be due, in part, to the regulation of FOXM1. Increased levels of ROS are observed in OV90 cells with ETV5 downregulation, which is indicative of an association between these 2 factors. We showed that in OV90 cells, exogenous H2O2 was able to induce an increase in FOXM1 protein levels concomitant to an increase in ETV5 protein levels. Interestingly, in tumor cells, ROS is an important factor that directly regulates the expression of ETS-1, the founder member of the ETS family of transcription factors (43, 44). Likewise, H2O2 is able to transcriptionally upregulate ETS-1 in ovarian cancer cells (45). There is also evidence that ETS-2, a closely related family member of ETS-1, is transcriptionally upregulated by H2O2 treatment in NIH3T3 fibroblasts and that cells with defects to their antioxidant defense systems display endogenously increased levels of ETS-2 (46). The concomitant regulation of FOXM1 through the direct binding of ETV5 to its promoter suggests that ETV5 may, in part, protect cells from oxidative stress through the upregulation of FOXM1. FOXM1 has been previously shown to protect cells from oxidative stress, regulating the intracellular levels of ROS through the upregulation of antioxidant genes (28).

To better understand the putative role of FOXM1 in ovarian cancer, we analyzed the expression of FOXM1 in a gene expression microarray experiment previously conducted in our laboratory, ETV5, FOXM1, and many of its target genes (21, 22) were upregulated in ovarian tumors. FOXM1 overexpression was further confirmed in new set of ovarian tumor samples by RT-Q-PCR. We found a statistically significant association between the levels of FOXM1 mRNA expression and tumor grade (Student t test; \( P < 0.05 \)). In patients with breast cancer, it has been shown that a higher expression of FOXM1 is associated with poor prognosis (47). Moreover, it has been shown that FOXM1 expression could serve as an independent predictor of poor survival in gastric cancer (48). Our results indicated that in ovarian cancer, increased levels of FOXM1 expression were also associated with a more aggressive phenotype, suggesting a role in the progression of ovarian cancer.

In addition, we found that there was a clinical correlation between the expression of ETV5 and FOXM1 in human ovarian tumor samples. This suggests that ETV5 may contribute to the upregulation of FOXM1 during ovarian tumor development.

Even though FOXM1 has already been described to be overexpressed in various malignancies, to our knowledge, this is the first report that has shown FOXM1 to be overexpressed in ovarian cancer (reviewed in ref. 49). FOXM1 plays a key role in cell-cycle progression at the S- and G2–M phases (mitotic division; refs. 21, 50), and it has been shown to regulate the maintenance of chromosomal segregation and genomic stability (21). FOXM1 has been shown to be induced by increased oncogenic stress (28) and to counteract oxidative stress–induced premature senescence (51). A recent report has also hypothesized that FOXM1 may induce cancer initiation through stem/progenitor cell expansion (52). FOXM1 can also promote drug resistance to herceptin, paclitaxel (53), and cisplatin (54) in breast cancer cells.

In conclusion, the analysis of the genes and signaling pathways under the control of ETV5 in OV90 cells has unraveled new signaling pathways that interact with ETV5. In addition, it has confirmed the role of this transcription factor as a protector against oxidative stress during oncogenesis, as well as having identified FOXM1 as a target gene that is overexpressed in ovarian cancer. Future research will identify the biologic role of FOXM1 in ovarian cancer tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Llauradó, J. Reventos, A. Ruiz

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