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ETV5 transcription factor is overexpressed in ovarian cancer and regulates cell adhesion in ovarian cancer cells

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Epithelial ovarian cancer is the most lethal gynecological malignancy and the fifth leading cause of cancer deaths in women in the Western world. ETS transcription factors are known to act as positive or negative regulators of the expression of genes that are involved in various biological processes, including those that control cellular proliferation, differentiation, apoptosis, tissue remodeling, angiogenesis and transformation. ETV5 belongs to the PEA3 subfamily. PEA3 subfamily members are able to activate the transcription of proteases, matrix metalloproteinases and tissue inhibitor of metalloproteases, which is central to both tumor invasion and angiogenesis. Here, we examined the role of the ETV5 transcription factor in epithelial ovarian cancer and we found ETV5 was upregulated in ovarian tumor samples compared to ovarian tissue controls. The *in vitro* inhibition of ETV5 decreased cell proliferation in serum-deprived conditions, induced EMT and cell migration and decreased cell adhesion to extracellular matrix components. ETV5 inhibition also decreased cell–cell adhesion and induced apoptosis in anchorage-independent conditions. Accordingly, upregulation of ETV5 induced the expression of cell adhesion molecules and enhanced cell survival in a spheroid model. Our findings suggest that the overexpression of ETV5 detected in ovarian cancer cells may contribute to ovarian tumor progression through the ability of ETV5 to enhance proliferation of ovarian cancer cells. In addition, upregulation of ETV5 would play a role in ovarian cancer cell dissemination and metastasis into the peritoneal cavity by protecting ovarian cancer cells from apoptosis and by increasing the adhesion of ovarian cancer cells to the peritoneal wall through the regulation of cell adhesion molecules.

Epithelial ovarian cancer is the most lethal gynecological malignancy and the fifth leading cause of cancer deaths in women in the Western world.[1] Largely asymptomatic, more than 70% of the patients are already at an advanced stage at initial diagnosis. The 5-year survival rate for women with advanced stage disease is less than 20%. In contrast, the cure rate is almost 90% when women are diagnosed at an early stage.[2]

The ETS family of transcription factors play a role in several physiological and pathological processes such as embryogenesis, wound healing and tumor progression.[3] ETS transcription factors have been implicated in the regulation of gene expression during a variety of biological processes including cell growth and differentiation.[4] In particular, ETS transcription factors are able to activate the transcription of

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proteases, matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteases (TIMPs).[5,6] The activation of proteolytic enzymes is central to both tumor invasion and angiogenesis.[7]

The ETS family of transcription factors is divided into subfamilies (ETS-1 and -2, ERG, GABP, PEA3, ELK, ELF and PU1) based mainly on the sequence and location of the ETS domain, consisting of an 84 amino acid sequence, which is present in all members of the family. ETS proteins bind to DNA sequences with the core motif A/GGAA/T in cooperation with other transcriptional factors and cofactors that mediate transcriptional activation or repression.[8,9] Family members of the PEA3 subfamily, which includes PEA3, ER81 and ERM/ETV5 have been associated with the progression and invasion of tumors. PEA3 has been shown to induce an invasive phenotype in human oral, breast and cervical carcinomas and in mouse fibrosarcoma through the activation of MMPs.[10–12] In lung cancer cells, PEA3 transfection results in enhanced motility and invasion.[13] We have previously characterized the upregulation of the ETV5 gene in endometrial cancer with a specific and significant increase in those tumor stages associated with myometrial invasion.[14,15] In addition, we have demonstrated that the overexpression of ETV5 promotes cell migration and invasion through activation of MMP2 in an endometrial cancer cell line.[16]

An association between PEA3 mRNA expression and poor survival in ovarian carcinoma patients has been previously described by Davidson *et al.*[17] More recently, Cowden Dahl *et al.*[18] found that PEA3 overexpression is sufficient to induce expression of MMP9 and MMP14, cell migration and invasion in ovarian tumor cells. ETS-1 mRNA expression has also been reported as a biological marker of poor survival in both solid lesions and effusions from two cohorts of patients diagnosed with ovarian carcinoma.[19,20]

Epithelial ovarian cancer comprises 90% of ovarian cancer cases. In our study, we investigated the role of ETV5 in epithelial ovarian cancer. We analyzed expression of ETV5 in ovarian tumor samples by quantitative real-time PCR (RT-PCR) and immunohistochemistry and found that ETV5 was upregulated in tumor samples compared to tissue controls. We also examined the biological effects of modulating ETV5 expression in ovarian cancer cells.

Material and Methods

Cell lines and tumor samples

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 in Barcelona, Spain. Informed consent was obtained from all patients included in our study. Tumor staging was performed according to the International Federation of Gynecology and Obstetrics classification. Supporting Information Table 1 summarizes the clinicopathological characteristics of the tumor samples.

Ovarian cancer cell lines were cultured in a mixture (1:1) of MCDB 105 and M-199 mediums (Biological Industries, Israel) with 2 mM L-glutamine and supplemented with 15% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad) and penicillin–streptomycin (1:100; Gibco) at 37°C in a humidified atmosphere of 5% CO_2.

Samples and tissue microarrays for immunohistochemistry

Ten individual paraffin-embedded sections of 5 μm were obtained from normal and ovarian tumor samples. In addition, tissue microarrays (TMAs) were constructed at the Pathology Department of the Vall d'Hebron University Hospital. Representative areas from 88 paraffin-embedded ovarian carcinomas (12 clear cell, 14 endometrioid, six undifferentiated, 15 mucinous, 38 serous and three metastasis) were carefully selected and marked on individual paraffin blocks. Two tissue cores of 1-mm in diameter were obtained from each paraffin block and were precisely arrayed in a new paraffin block. Sections of 5 μm were obtained from all TMA paraffin blocks. ETV5 was detected by the indirect immunoperoxidase assay with ethylenediaminetetraacetic acid (EDTA) pH 8 buffer for antigen retrieval. Sections were incubated with a primary antibody against ETV5 (sc-22807, Santa Cruz Biotechnologies, Santa Cruz, CA) for 2 hr at a dilution of 1:100 and thereafter incubated with peroxidase-conjugated rabbit anti-goat immunoglobulin (EnVision, Dako, Glostrup Denmark) for 30 min and detected using the Envision Plus Detection System (Dako). Quantitative and qualitative ETV5 immunostaining was evaluated by two independent investigators. ETV5 nuclear staining was scored using a weighted histoscore method. Histoscores were calculated from the sum of (1 × % cells staining weakly positive) + (2 × % cell staining moderately positive) + (3 × % cells staining strongly positive) with a maximum of 300. The mean of the two scores of observers was used for the analysis.

Constructs and stable cell line generation

OV90 ovarian cancer cells were transduced with stable (sh)RNA Enhanced Green Fluorescent Protein (EGFP) lentiviral producing vector (FSV) to knockdown expression of ETV5. Two short hairpin ETV5 sequences were used: ETV5 i3, 5′ CTCTACAACTATTGTGCCTAT 3′ and ETV5 i4, 5′ CGGCAAATGTCAGAACCTATT 3′ to generate two stable OV90-modified cell lines (OV90i3 and OV90i4, respectively). Controls were uninfected OV90 cells (OV90C). Transfection of the LinXE retrovirus packaging cell line was carried out using Lipofectamine (Invitrogen) according to the conditions recommended by the manufacturer. Retroviral supernatants were harvested 48 hr after transfection and were concentrated by centrifugation for 1 hr at 16,000*g*. Concentrated lentivirus was used to infect cells in the presence of 8 μg/ml polybrene. The infection efficiency was assessed by EGFP expression using flow cytometry and was greater than 80%. The TOV-112 ovarian cancer cells were stably transfected with pEGFP-ETV5 or pEGFP without insert as a control[16] using Lipofectamine Plus reagent (Invitrogen), according to the manufacturer's instructions. Transfected cells were then selected with G418 (Gibco) at a concentration of 800 μg/ml.

Quantitative real-time PCR

Samples were examined by a pathologist. None of them presented tumor necrosis and the percentage of tumor *versus* nontumor cells was more than 90%. Total RNA was collected and purified using the RNeasy kit (Qiagen, Valencia, CA). The RNA integrity number was always higher than 6.5. One microgram of total purified RNA was subjected to a reverse transcriptase reaction using Superscript III (Invitrogen) according to the manufacturer's conditions. cDNA, corresponding to approximately 1 μg of starting RNA, was used in three replicates for quantitative PCR (Taqman, Applied Biosystems, Foster City, CA). For the analysis of ovarian tumor samples and controls, we used the ETV5-Hs00231790_m1 probe and the POLR2A-Hs00172187_m1 probe for normalization (Applied Biosystems). For the analysis of ovarian cancer cell lines and primary ovarian epithelial cultures, we used the ETV5-Hs00231790_m1 probe and the 18s ribosomal RNA C6_4308329 probe for normalization (Applied Biosystems). The analysis of the expression of E-cadherin repressors was performed on the OV90 control and the modified cells. We used the SNAIL (Hs00195591_m1) probe, ZEB1 (Hs00232783_m1) probe and 18S RNA (4319413E-0406012) probe for normalization (Applied Biosystems).

Immunofluorescence

Cells were plated on glass coverslips and incubated for 48 hr. Cells were fixed for 10 min in 4% paraformaldehyde/phosphate-buffered saline (PBS), treated with 50 mM NH_4Cl for 30 min to prevent the autofluorescence and permeabilized in 4% Triton X-100–PBS for 10 min. Cells were incubated with primary antibody followed by a Texas red-conjugated secondary antibody. Cells were counterstained with combined Vectashield Mounting Medium (Vector, Burlingame, CA) and 4,6-diamidino-2-phenylindole (Sigma, St Louis, MO). Primary antibodies used for immunofluorescence were anti-β-catenin (610153), anti-E-cadherin (610181, BD Transduction Laboratories, Palo Alto, CA), anti ZO-1 (33-9100, Zymed Laboratories, San Francisco, CA); Phallodin-TRITC (77418, Sigma) was used for detecting polymeric F-actin.

Western blot analysis

Whole cell extracts were prepared using Laemmli Buffer. Samples were run on a 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% milk solution (Tris buffered saline (TBS)–0.1% Tween) for 1 hr at room temperature and incubated with indicated primary antibody in 5% milk solution overnight at 4°C. The membranes were washed three times for 10 min in TBS–0.1% Tween at room temperature and incubated for 1 hr with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected by the enhanced chemiluminiscence system (Amersham Pharmacia Biotech) as described by the manufacturer's instructions.

The primary antibodies used for Western blot were anti-E-cadherin (610181), anti-integrin β1 (610467), anti N-cadherin (610920, BD Transduction Laboratories), Biosciences Pharmigen, anti-ERM/ETV5 (sc-22807, Santa Cruz Biotechnologies), anti-integrin alpha 5 (AB1949, Chemicon, Millipore, MA), anti α-tubulin (2125, Cell signaling) and anti ZO-1 (61-7300, Zymed Laboratories).

Cell proliferation assay

2.4×10^5 OV90C, OV90i3 and OV90i4 cells were plated in triplicate on p60 plates in complete medium (15% FBS). The following day, the complete medium was replaced by medium with 10% FBS or by serum-deprived medium (2% FBS) and allowed to grow for a 10-day period. Cells were counted at days 3, 7 and 10 using a Newbauer chamber.

Wound healing assay

The OV90 cells were plated at confluence on 24-well plates and incubated overnight. A straight line was then gently performed at the bottom of the dish. Cells were washed, incubated in medium with 2% FBS and kept in a computer-controlled mini-incubator, which provided stabilized temperature of 37°C with 95% humidity, 5% CO_2 and optical transparency for microscopic observations. The incubator was fastened to an inverted microscope (Live Cell Imaging CellR, Olympus, Japan) to monitor cell migration. Images were taken with the $4\times + 1.6\times$ objectives every 15 min and were analyzed using the ImageJ software (Wright Cell Imaging Facility, UHNR, CA). Initial wound area (mm^2) and time needed to close the wound (hr) were the variables used to calculate the migration speed of the cells.

Transwell migraton assay

Cell migration assays were performed using the CytoSelect™ 24-Well Cell Migration Assay kit (CBA-101-C, Cell Biolabs, San Diego, CA). 0.5×10^6 OV90 control cells and OV90i3 and i4 transduced cells were plated on 2% serum media in triplicate and incubated for 3 days. Migration assays were performed for a minimum of three times.

Transwell invasion assay

Cell invasion assays were performed using the CytoSelect™ 24-Well Cell Invasion Assay kit, Fluorimetric Format (CBA-101-C, Cell Biolabs). 0.5×10^6 OV90 control cells and OV90i3 and i4 transduced cells were plated on 2% serum media in triplicate and incubated for 3 days. Invasion assays were performed for a minimum of three times.

Anchorage-independent cell cultures and apoptosis assays

Ovarian cell aggregates were generated using a liquid overlay technique as previously described.[21] Briefly, 12-well plates were coated with 0.5% agarose (SeaKem®LE agarose, Lonza, Basel, Switzerland) in serum-free medium. OV90C, OV90 i3 and OV90 i4 cells were released from the monolayer cultures and resuspended in complete medium. 2×10^5 cells were deposited in each well and maintained at 37°C for 72 hr. Cell death was measured using Annexin V-phycoerythrin (PE)

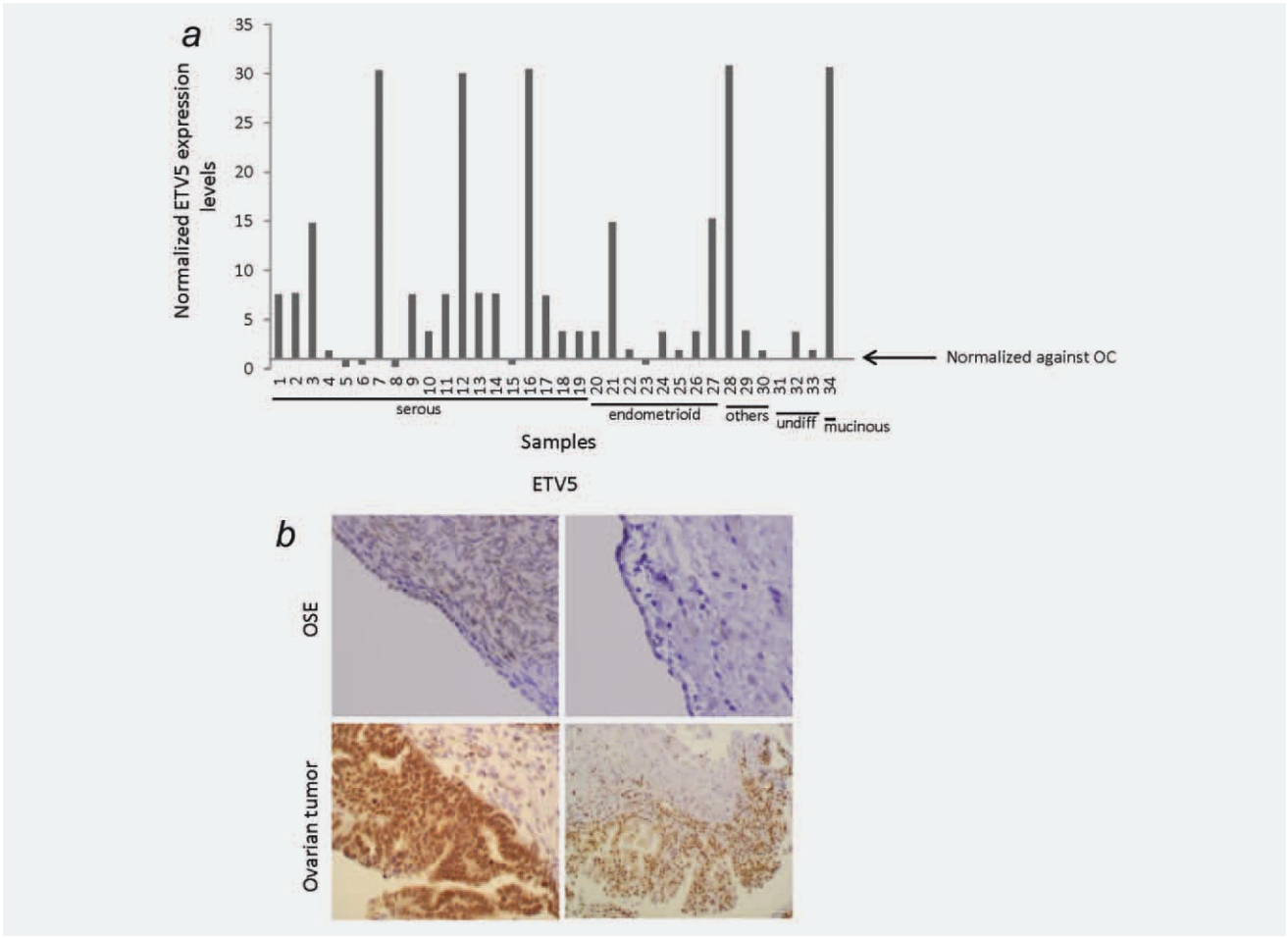

Figure 1. ETV5 expression in ovarian tumor samples. (*a*) ETV5 RNA expression levels from a panel of 34 ovarian tumor samples normalized against 11 ovarian tissue controls (OC). ETV5 expression levels above one mean that ETV5 expression is upregulated compared to OC. (*b*) ETV5 immunoreactivity in normal ovarian surface epithelium (OSE) and ovarian tumor tissues (×10). ETV5 immunoreactivity is undetectable in OSE and shows high intensity in ovarian tumor samples. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and 7-Amino-actinomycin D (7-AAD) staining (559763, BD Biosciences). Analysis was carried out using FACSCalibur (Becton Dickinson, San Jose, CA). Cell death was also monitored by Western blot using an anti-poly (ADP-ribose) polymerase (PARP) antibody.

Cell adhesion assay

Adhesion assays were performed using the CytoSelect 48-Well Cell Adhesion Assay, extracellular matrix (ECM) array (CBA-070, Cell Biolabs). OV90C, OV90i3 and OV90i4 cells were maintained in 2% FBS media overnight. Then, 1×10^5 cells were plated in triplicate on the p48-coated plate. After 30 min of incubation at 37°C, adherent cells were fixed, stained and photographed. The stain was extracted and quantified colorimetrically in a plate reader at 560 nm.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS, IBM) version 15.0. The Student's *t*-test was performed to compare means in the migration (wound healing and transwell), adhesion and spheroid apoptosis assays. The nonparametric Sperman's rho test was applied to analyze the correlation between expression of ETV5 and E-cadherin in the human tumor samples. The probability of $p < 0.05$ was considered statistically significant in both statistical analyses. The Student's *t*-test was performed to compare means in the ETV5 histoscores between tumor subtypes, stages and grade.

Results

Expression of ETV5 is upregulated in ovarian tumor samples

ETV5 mRNA was analyzed by quantitative RT-PCR in 34 ovarian tumor samples corresponding to different histological subtypes and clinical stages (Supporting Information Table 1). ETV5 expression in the ovarian tumor samples was normalized against 11 ovarian tissue controls (Fig. 1*a*). The relative quantification revealed an upregulation of ETV5 in the

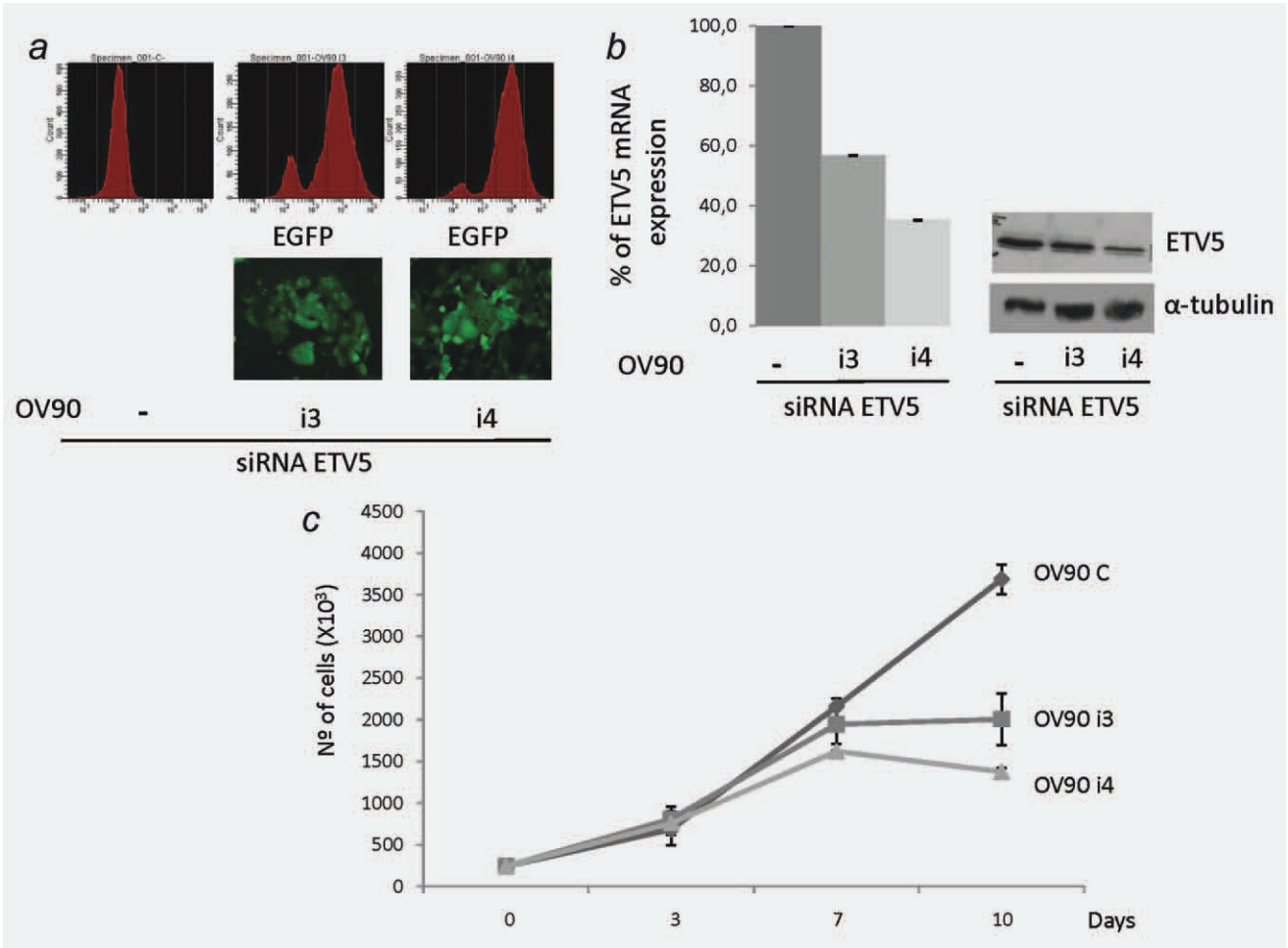

Figure 2. ETV5 knockdown in OV90 ovarian cancer cells inhibits cell growth under serum-deprived condition. (*a*) OV90 ovarian cancer cells were transduced with stable short hairpin (sh)RNA lentiviral constructs. The infection efficiency was assessed by EGFP expression using flow cytometry. (*b*) Inhibition of ETV5 expression was validated at the mRNA level by quantitative RT-PCR and at the protein level by Western blot in OV90 transduced cell lines (OV90i3 and OV90i4). Controls were uninfected OV90 cells. (*c*) 2.4×10^5 OV90 sh-modified and control cells were seeded in triplicate in serum-deprived conditions and allowed to grow for a 10-day period. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

majority of samples. We could not find a correlation between the levels of ETV5 expression and tumor subtype, tumor stage or tumor grade. Immunohistochemical analysis of ETV5 expression in ovarian surface epithelium and epithelial ovarian tumors showed an increased ETV5 staining in both the nuclei and cytoplasm of ovarian tumoral cells as compared to normal ovarian surface epithelial cells (Fig. 1*b*). These results suggested a possible role for ETV5 in ovarian cancer progression.

To further analyze the expression of ETV5 protein expression throughout ovarian tumourigenesis, we used TMA immunohistochemistry. TMAs consisted of 88 tumor samples corresponding to different histological subtypes and clinical stages (Supporting Information Table 2). Although there was no statistically significant association between the levels of ETV5 expression and tumor subtype, tumor stage or tumor grade (Supporting Information Fig 1), our analysis showed a decrease in the ETV5 protein levels associated with an increase in the tumor grade and tumor stage, suggesting that increased levels of ETV5 are associated with a more differentiated phenotype and initial stages where the ovarian tumor cells express high E-cadherin levels

Knockdown of ETV5 by shRNA in OV90 ovarian cancer cells inhibits cell growth under serum-deprived conditions

To examine the role of ETV5 in ovarian cancer, we knocked down ETV5 in the OV90 ovarian cancer cell line. OV90 ovarian cancer cells were transduced with two different stable short hairpin (sh)RNA lentiviral constructs to reduce ETV5 expression (Fig. 2). Two stable modified cell lines OV90i3 and OV90i4 were established. The infection efficiency was assessed by EGFP expression using flow cytometry and was greater than 80% (Fig. 2*a*). To check the effectiveness of shRNA at knocking down ETV5 expression, ETV5 expression levels were validated both at the

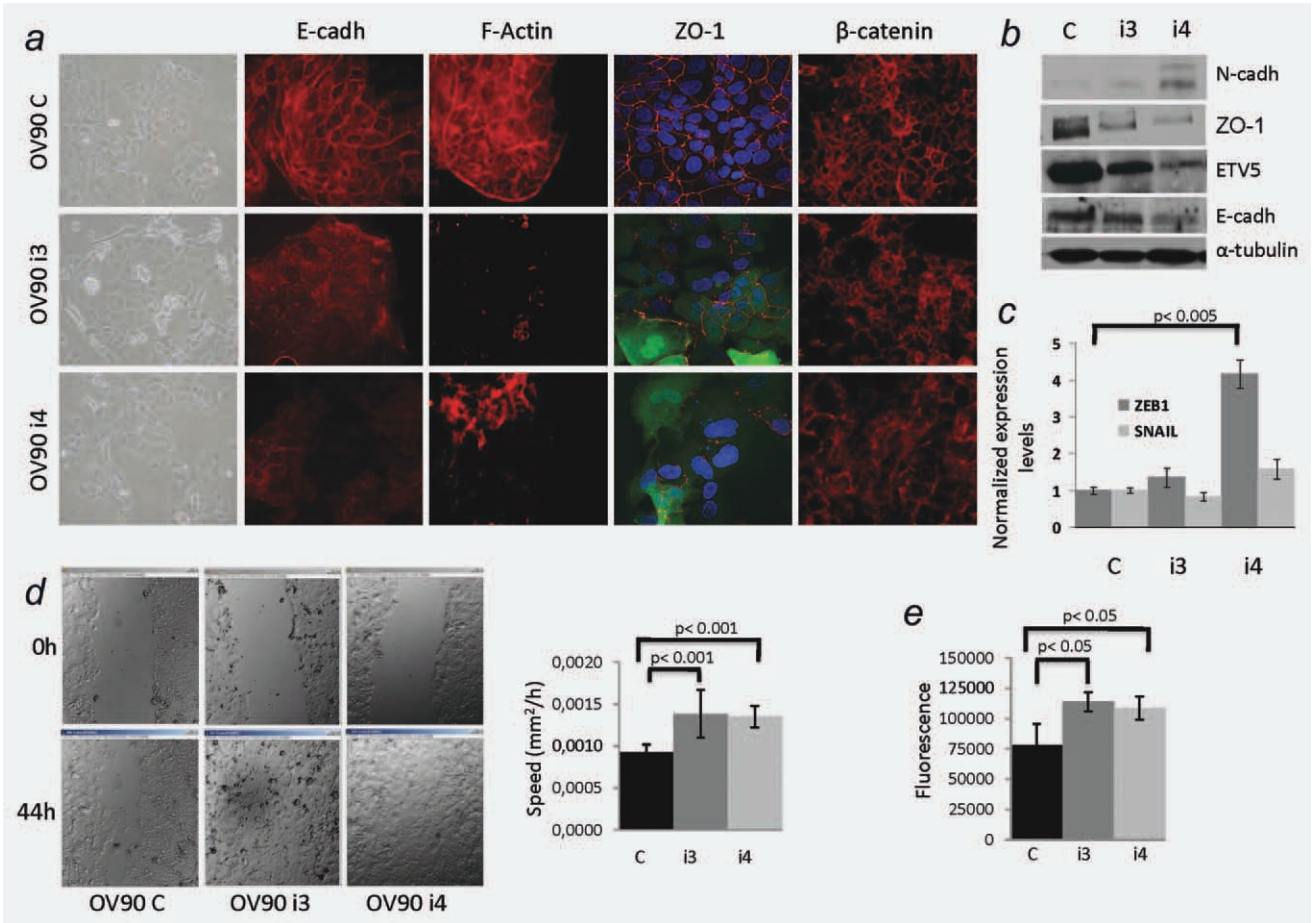

Figure 3. Inhibition of ETV5 expression in OV90 ovarian cancer cells induces downregulation of E-cadherin expression. (*a*) Phase-contrast images and immunofluorescence analysis of E-cadherin, F-actin, ZO-1 and β-catenin of OV90 controls and OV90i3 and OV90i4 transduced cells (×40). (*b*) Western blot analysis of EMT markers in OV90 control and transduced cells. (*c*) Analysis by semiquantitative RT-PCR of the E-cadherin repressors Zeb1 and Snail in OV90 control cells and OV90i3 and i4 transduced cells. (*d*) Cell migration was monitored in OV90 control and transduced cells using the wound healing assay. Statistical analyses were performed using Student's *t*-test. $p < 0.001$ was considered statistically significant. (*e*) A transwell migration assay was performed in triplicate using the Cytoselect 24-well cell migration assay (Cell Biolabs) in OV90 control and transduced cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mRNA level by quantitative RT-PCR and at the protein level by Western blot in OV90-transduced cell lines. The reduction in ETV5 expression was approximately 50% and 70% for the OV90i3 and OV90i4 cells, respectively, compared to the OV90 control cells (Fig. 2*b*). As ETS transcription factors have been found to be involved in the regulation of cell proliferation, among other processes,[22] we first examined the effect of reducing ETV5 expression on cell proliferation in the presence and absence of serum over a 10-day period. We found that in the presence of serum there were no significant differences (data not shown) in cell growth between the OV90 control and the OV90-modified cells. However, the OV90-modified cells that were grown in serum-deprived conditions (2% FBS) did show a reduction in the number of cells over time (Fig. 2*c*). This indicated that the inhibition of ETV5 expression can reduce cell proliferation and sensitize cells to serum deprivation.

Inhibition of ETV5 expression in OV90 ovarian cancer cells induces downregulation of E-cadherin expression

A comparison of the morphology between the OV90 control and the shRNA-modified OV90 cell lines (OV90i3 and OV90i4) revealed that inhibition of ETV5 induced loss of cell to cell contacts. Immunofluorescence analysis of the junctional adherens protein E-cadherin showed a significant reduction in the staining of the protein leading to a more disrupt pattern at the membrane (Fig. 3*a*). We found that the downregulation of ETV5 in the OV90 cells inhibited the expression of E-cadherin at the mRNA (Fig. 4*b*) and protein levels (Fig. 3*b*). It is well known that the loss of E-cadherin is a hallmark of the epithelial–mesenchymal transition (EMT). EMT involves the loss of intercellular cohesion and the modification of the cytoskeleton, leading to increased motility and invasion.[23,24] We evaluated whether the downregulation of ETV5 induced an EMT in the OV90 cells. In addition to

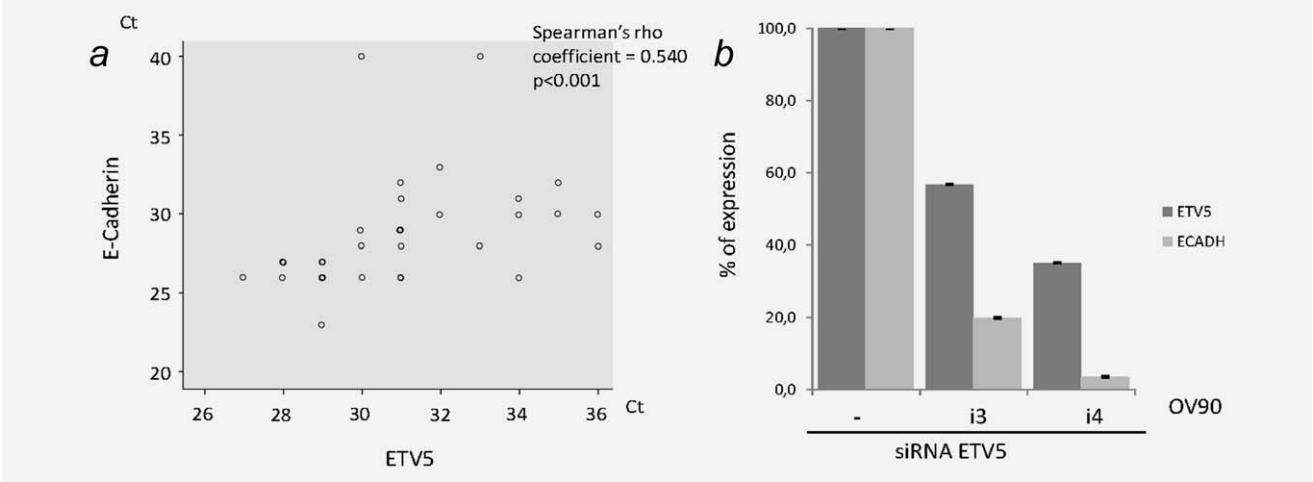

Figure 4. ETV5 and E-cadherin mRNA levels are correlated in human tumor samples and in OV90 ovarian cancer cells. (*a*) A total of 34 ovarian tumor samples were analyzed. The expression of E-cadherin showed a significantly positive correlation with ETV5 ($p < 0.001$, two-tailed Spearman's rho coefficient = 0.540). (*b*) Inhibition of ETV5 expression in OV90 ovarian cancer cells induces E-cadherin downregulation at the mRNA level.

E-cadherin loss, we found that the OV90-modified cells exhibited a downregulation of other epithelial markers, such as ZO-1, and an upregulation of mesenchymal markers, such as N-cadherin. This indicated that the inhibition of ETV5 induced an EMT in the OV90 cells (Fig. 3*a*). Concomitant with E-cadherin loss, we also found a reduction in actin fibers and cytoskeleton disruption (F-actin and β-catenin, Fig. 3*a*). To further investigate the mechanism of E-cadherin transcriptional regulation by ETV5, we examined the expression of the E-cadherin transcriptional repressors Zeb1, Zeb2, Snail, Slug and Twist and found an induction of Zeb1 in the OV90-modified cells (Fig. 3*c*). No obvious upregulation of Snail (Fig. 3*c*), Zeb2, Slug or Twist mRNA was detected (data not shown). Our results showed that ETV5 can regulate E-cadherin expression through the induction of the Zeb1 transcriptional repressor, promoting a loss of cell to cell contact. Concomitant to the EMT phenotype, the OV90-transduced cells exhibited enhanced migration, which was measured both by wound healing and transwell assays (Fig. 3*d*). Additional analysis of cellular invasion using transwell invasion assays did not show a significant difference in invasion between OV90 control cells and OV90 cells with ETV5 downregulation (Supporting Information Fig. 2).

To check whether an increase in ETV5 expression was associated with an increase in E-cadherin levels in human tumor samples, we measured E-cadherin mRNA levels in the same panel of ovarian tumoral samples by quantitative RT-PCR (Supporting Information Table 1) and found that the mRNA levels of both molecules were significantly correlated (Fig. 4*a*). Accordingly, the downregulation of ETV5 in the OV90 cells inhibited E-cadherin expression at the mRNA level (Fig. 4*b*).

Modulation of ETV5 expression regulates apoptosis in ovarian cancer cells grown under anchorage-independent conditions

It has been proposed that cells shed from the ovarian primary tumor aggregate as spheroids within the abdominal cavity, to maintain cell–cell contact and survive under anchorage-independent growth conditions.[25,26] Cell membrane molecules such as cadherins and integrins mediate cell adhesion and cell survival in these conditions.[27,28]

We grew ovarian cancer cells as aggregates, to model ovarian tumor cell dissemination by spheroids. Because we found that ETV5 can modulate E-cadherin expression, we checked whether ETV5 could affect cell survival in cells grown under anchorage-independent conditions. As shown in Figure 5*a*, aggregates generated from the control OV90 cells had a compact morphology. However, aggregates generated from the OV90i3- and OV90i4-modified cell lines were visibly less compact. As ovarian cancer tumors show an upregulation of ETV5 compared to normal ovarian surface epithelium (OSE) (Fig. 1), we decided to check the effects of upregulating ETV5 in an ovarian cancer cell line. We overexpressed an EGFP-ETV5 fusion protein in the TOV112 ovarian cancer cell line and checked whether ETV5 could enhance cell survival in the TOV112-transfected cells grown as aggregates. We found that the TOV112 EGFP-ETV5 clones (GE11 and GE13) showed more aggregation than the TOV112 EGFP cells (G1 and G6) (Fig. 5*a*). Analysis by flow cytometry showed that in the OV90 cells, the inhibition of ETV5 significantly increased cell death (Fig. 5*b*). We compared levels of apoptosis between TOV112 EGFP control cells and the TOV112 EGFP-ETV5 overexpressing clones and found that TOV112 ETV5 overexpressing clones had a significantly reduced level of apoptosis (Fig. 5*b* and Supporting

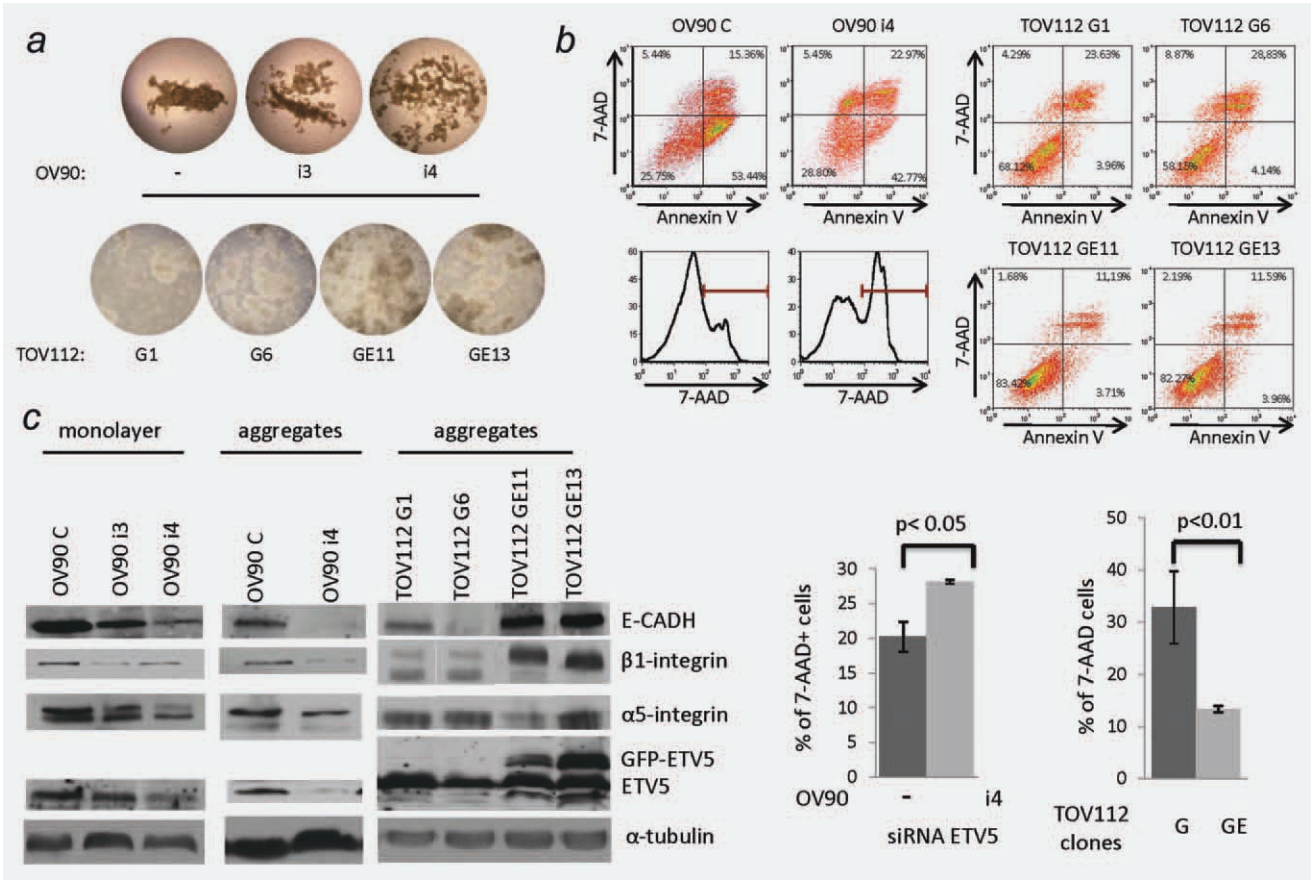

Figure 5. Modulation of ETV5 expression regulates apoptosis in ovarian cancer cells grown under anchorage-independent conditions. OV90 and TOV112 cells were grown as aggregates to model ovarian tumor cell dissemination. (*a*) Aggregate morphology of OV90 control and OV90-modified cell lines (OV90i3 and OV90i4) and TOV112 EGFP control (1 and 6) and TOV112 EGFP-ETV5 (11 and 13) overexpressing clones. Images were taken at 4× magnification. (*b*) Cell death was measured by flow cytometry using Annexin V-PE and 7AAD staining in OV90 control and transduced cells and in TOV112 EGFP control and TOV112 EGFP-ETV5 overexpressing clones grown as aggregates. Measurements were done in triplicate. The data are means ± standard deviation (SD) of the percentage of 7-AAD positive cells. (*c*) Western blot of adhesion molecules E-cadherin and α5 and β1 integrins in OV90 control and transduced cells and in TOV112 EGFP control clones and EGFP-ETV5 transfected clones. Effective overexpression of EGFP-ETV5 fusion protein in TOV112 GE clones is shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Information figure). The reduction in cell survival observed was associated with the downregulation of the cell adhesion proteins E-cadherin and also the α5 and β1 integrins in OV90 cells (Fig. 5*c*) while reduced levels of apoptosis in TOV112 EGFP-ETV5 overexpressing clones correlated to enhanced E-cadherin and β1 integrin expression (Fig. 5*c*).

Our results suggested that ETV5 may act as an antiapoptotic factor in ovarian cancer cells, which are shed from the ovarian primary tumor and remain as spheroids within the peritoneal cavity.

Inhibition of ETV5 in OV90 ovarian cancer cells reduces cell attachment to the extracellular matrix

Because we found α5 and β1 integrin levels decreased in the OV90 cells with ETV5 downregulation, we performed adhesion assays using different extracellular matrices. The OV90 cells with ETV5 downregulation exhibited reduced adhesion to collagen type I, fibronectin, laminin and collagen type IV (Fig. 6). The OV90 cells did not attach to fibrinogen (data not shown). These results suggested that ETV5 can also modulate specific cell–matrix interactions and may promote attachment to the mesothelial wall through the regulation of integrin cell matrix receptors.

Discussion

Ninety percentage of the cases of ovarian carcinomas arise from the OSE which is composed of a single layer of epithelial cells that overlies the surface of the ovary. It has an uncommitted phenotype expressing common mesenchymal markers (such as N-cadherin) as well as epithelial markers (such as keratins 7, 8, 18, 19 and MUC1 but not E-cadherin). It is well known that during neoplastic progression, the OSE loses its stromal characteristics and undergoes an aberrant

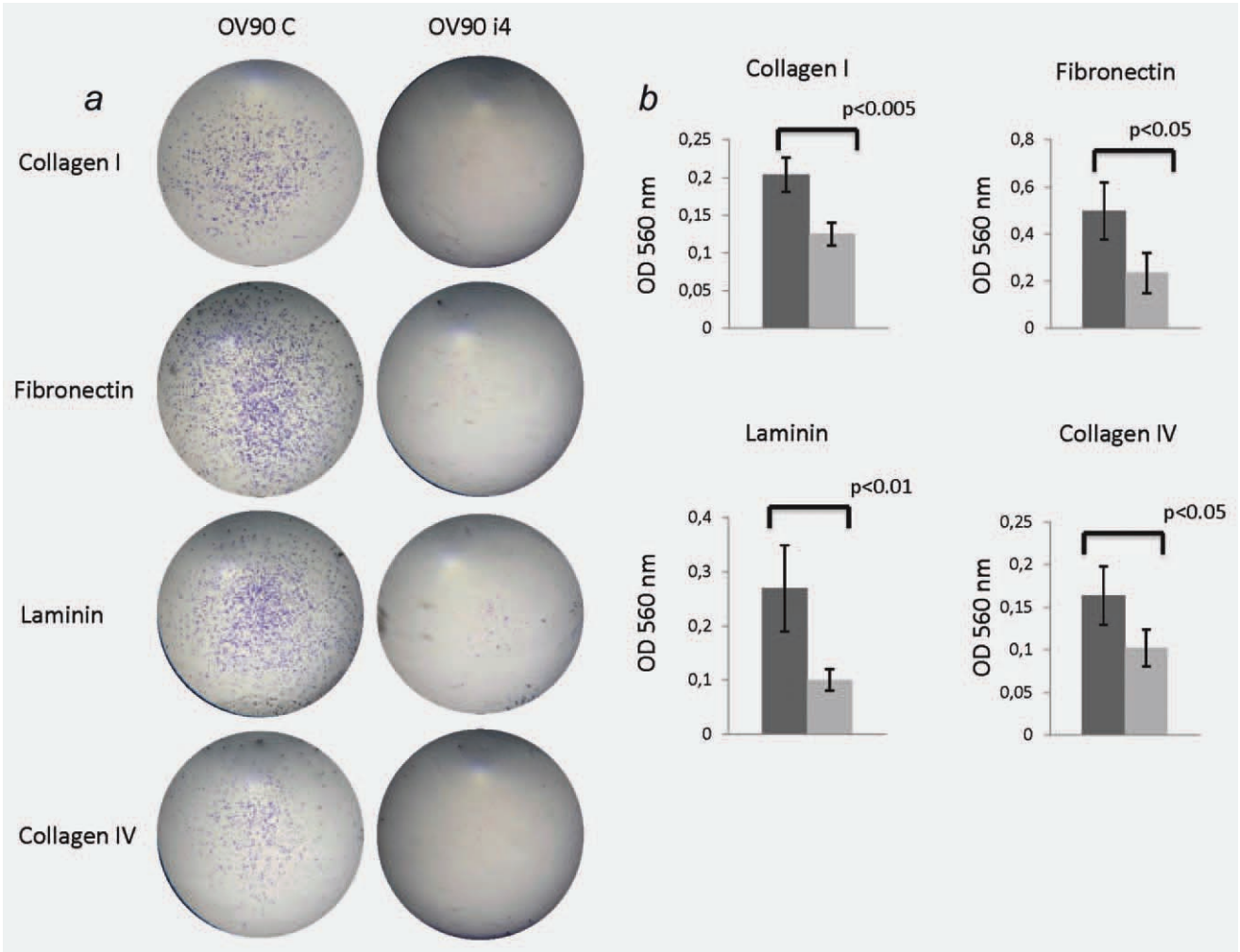

Figure 6. Inhibition of ETV5 in OV90 cancer cells reduces cell attachment to collagen type I, fibronectin, laminin and collagen type IV extracellular matrices. 1×10^5 cells were plated in triplicate on the p48 coated plate. ECM array included collagen type I, fibronectin, laminin, collagen type IV and fibrinogen-coated wells. (*a*) Pictures of stained adherent cells (×4). (*b*) Colorimetry was quantified at OD 560 nm after cell staining extraction. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

epithelial differentiation. Preneoplasic inclusion cysts and epithelial ovarian carcinoma express E-cadherin and are non-migratory. Therefore, unlike most epithelial tumors, ovarian tumors do not follow the classical model of EMT but show an increase in E-cadherin expression during tumor progression.[29,30]

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. 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 extraovarian mesothelial wall and, subsequently, invade establishing tumors at secondary sites. Both cell to cell adhesion and cell–ECM interacting molecules are believed to play a role in the process of spheroid formation and tumor invasion.[21,31–33]

In our study, we investigated the role of the ETS transcription factor ETV5 in epithelial ovarian cancer. We found an upregulation of ETV5 mRNA and protein expression in ovarian tumor samples compared to ovarian controls. The high heterogeneity found in the ETV5 expression levels between samples may be due to the percentage of disseminating tumor cells to nondisseminating tumor cells. As we show here, ETV5 is important in the dissemination and maintenance of ovarian tumor cells in the peritoneal cavity.

We could not find a statistically significant association between the levels of ETV5 protein expression and tumor subtype, tumor stage or tumor grade (Supporting Information Fig. 1). Even though, our analysis shows a decrease in the ETV5 protein levels associated with an increase in the tumor grade and tumor stage suggesting that increasing levels of ETV5 in primary tumors are associated with a more

differentiated phenotype and initial stages of carcinogenesis, when the ovarian tumor cells have been found to express high E-cadherin levels. We noticed that ETV5 expression was increased in both the nuclear and cytoplasmic compartments. Other ETS factors, including ETV5, have also been found in the cytoplasm.[14,34] As all ETS factors recognize a common DNA binding motif (GGAA/T), additional DNA sequence specificity regulation is likely to be achieved by other mechanisms of control. We propose that ETV5 location in the cytoplasm may be required for post-translational modifications to activate or repress target genes involved in the carcinogenic process. To further examine the role of ETV5 in ovarian cancer, we decided to both knockdown and upregulate the expression of ETV5 in two ovarian cancer cell lines (OV90 and TOV112) and examine its biological effects.

The inhibition of ETV5 clearly modulated cell proliferation in serum-deprived conditions, suggesting its role in ovarian tumor progression. Significantly, the *in vitro* modulation of ETV5 levels in two ovarian cancer cell lines was able to regulate expression of E-cadherin. The inhibition of ETV5 downregulated E-cadherin in the OV90 cells, while the overexpression of ETV5 upregulated E-cadherin expression in the TOV112 cells. Accordingly, we found a clinical correlation between expression of E-cadherin and ETV5 in human ovarian tumor samples, suggesting that ETV5 may contribute to the upregulation of expression of E-cadherin during ovarian tumor development.

We found that the regulation of E-cadherin levels through changes in ETV5 expression was associated with changes in the expression of integrins α5 and β1. The ectopic expression of ETV5 in the TOV112 ovarian cancer cell line induced the expression of E-cadherin and β1 integrin and enhanced cell survival, when cells were grown in a spheroid model. Supporting our results, Casey *et al.*[21] already reported that β1 integrins regulated the formation and adhesion of ovarian spheroids *in vitro*. We propose that the upregulation of cell adhesion molecules mediated by the overexpression of ETV5 would act as an antiapoptotic factor in ovarian spheroids, increasing cell aggregation, suppressing anchorage-dependent cell death and enhancing cell dissemination when the cells are shed from the primary tumor. Supporting our results, Kang *et al.*[35] have demonstrated an antiapoptotic role of E-cadherin in Ewing tumor cells grown as multicellular spheroids. They show that E-cadherin-mediated cell to cell contacts lead to activation of the ErbB4 tyrosine kinase and the Akt signaling pathway in Ewing tumor spheroids. E-cadherin has also been shown to mediate cell survival of squamous carcinoma tumor spheroids.[36] In addition, Elloul *et al.*[37] using an antibody against E-cadherin that prevents cell–cell adhesion show that E-cadherin is important for the maintenance of the spheroid morphology in ovarian cancer cells. In ovarian cancer effusions, Elloul *et al.* and others have also found a decrease in the expression of E-cadherin repressors together with an increase in expression of E-cadherin.[37–40]

Integrins are also known to be the main mediators of cell to matrix adhesion. As it is already known that β1integrin subunit inhibits spheroid invasion of mesothelial cells monolayers by 90%,[21] we decided to examine the adhesion of ovarian cells to the ECM components associated with mesothelial cells. We found that ETV5 could modulate adhesion to the extracellular matrices fibronectin and collagen type I, which are matrix components secreted by mesothelial cells lining the peritoneal wall. Moreover, fibronectin is also expressed in mesothelial cells in addition to being part of the ECM.[41] Our results suggest that the upregulation of ETV5 detected in ovarian tumors may enhance the cell attachment of ovarian cells to the mesothelial monolayer during ovarian cancer cell dissemination. Other studies based on ovarian cancer spheroids have demonstrated that integrins can also mediate the disaggregation of ovarian cancer spheroids on ECM through the activation of metalloproteases.[42]

Overall, our findings suggest that the overexpression of ETV5 detected in ovarian cancer cells may contribute to ovarian tumor progression through the ability of ETV5 to enhance ovarian cancer cell proliferation in a tumor microenviroment with a lack of cell nutrients. In addition, the upregulation of ETV5 would also play a role in ovarian cancer cell dissemination and metastasis into the peritoneal cavity by protecting ovarian cancer cells from apoptosis and by increasing the adhesion of ovarian cancer cells to the peritoneal wall through the regulation of cell adhesion molecules.

Our results on ETV5 and ovarian cancer cell progression and dissemination, along with our previous findings involving ETV5 in endometrial cancer migration and invasion,[16] highlights the role that ETV5 plays as a key regulator of the invasive phenotype in both tumor types. The biological behavior of ovarian cancer is unique, differing markedly from the classic and well-studied pattern of metastasis found in most other cancers, such as endometrial cancer. Ovarian cancer cells primarily disseminate within the peritoneal cavity and are only superficially invasive through the mesothelium rather than deeply invading the surrounding tissue.

Future research will identify how ETV5 regulates and alters the expression of ETV5 target genes promoting tumor cell migration and invasion through the myometrium in endometrial cancer and intraperitoneal dissemination in ovarian cancer.

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