

Endothelin-1 Promotes Epithelial-to-Mesenchymal Transition in Human Ovarian Cancer Cells

Laura Rosanò,¹ Francesca Spinella,¹ Valeriana Di Castro,¹ Maria Rita Nicotra,² Shoukat Dedhar,³ Antonio Garcia de Herreros,⁴ Pier Giorgio Natali,¹ and Anna Bagnato¹

¹Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute; ²Molecular Biology and Pathology Institute, National Research Council, Rome, Italy; ³British Columbia Cancer Research Centre, Vancouver, British Columbia, Canada; and ⁴Unitat de Biologia Cel·lular i Molecular, Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Barcelona, Spain

Abstract

Despite considerable efforts to improve early detection and advances in chemotherapy, metastatic relapses remain a major challenge in the management of ovarian cancer. The endothelin A receptor (ET_AR)/endothelin-1 (ET-1) axis has been shown to have a significant role in ovarian carcinoma by promoting tumorigenesis. Here we show that the ET-1/ET_AR autocrine pathway drives epithelial-to-mesenchymal transition (EMT) in ovarian tumor cells by inducing a fibroblastoid and invasive phenotype, down-regulation of E-cadherin, increased levels of β -catenin, *Snail*, and other mesenchymal markers, and suppression of E-cadherin promoter activity. Activation of ET_AR by ET-1 triggers an integrin-linked kinase (ILK)-mediated signaling pathway leading to glycogen synthase kinase-3 β (GSK-3 β) inhibition, *Snail* and β -catenin stabilization, and regulation of transcriptional programs that control EMT. Transfection of dominant negative ILK or exposure to an ILK inhibitor suppresses the ET-1-induced phosphorylation of GSK-3 β as well as *Snail* and β -catenin protein stability, activity, and invasiveness, indicating that ET-1/ET_AR-induced EMT-promoting effects depend on ILK. ET_AR blockade by specific antagonists or reduction by ET_AR RNA interference reverses EMT and cell invasion by inhibiting autocrine signaling pathways. In ovarian carcinoma xenografts, ABT-627, a specific ET_AR antagonist, suppresses EMT determinants and tumor growth. In human ovarian cancers, ET_AR expression is associated with E-cadherin down-regulation, N-cadherin expression, and tumor grade. Collectively, these findings provide evidence of a critical role for the ET-1/ET_AR axis during distinct steps of ovarian carcinoma progression and identify novel targets of therapeutic intervention. (Cancer Res 2005; 65(24): 11649-57)

Introduction

Ovarian cancer, the leading cause of death from gynecologic malignancies, is a highly metastatic disease characterized by widespread peritoneal dissemination and ascites (1). Because treatment of patients in advanced stages is still penalized by low survival rates, the development of new treatment protocols depends on improved knowledge of the molecular mechanisms controlling metastasis. The endothelin (ET) family is composed of

three isopeptides, ET-1, ET-2, and ET-3, which act through two distinct subtypes of G-protein coupled receptors (i.e., ET_A and ET_B). The ET_A receptor (ET_AR) is highly specific for ET-1 whereas ET_BR binds ET-1, ET-2, and ET-3 with the same affinity (2). ET-1 has been implicated in the pathophysiology of a wide range of human tumors (3), including ovarian carcinoma (4, 5). ET-1 and the ET_AR are overexpressed in primary and metastatic ovarian carcinomas and ET-1 is present at high levels in ovarian tumor ascites (5, 6). In ovarian tumor cells, ET-1 acts as an autocrine growth, survival, and angiogenic factor selectively through the ET_AR (4, 6–9), activating diverse signaling pathways (10); these include mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase-dependent Akt activation, src-mediated epidermal growth factor receptor transactivation (9), which is partly responsible for MAPK phosphorylation (11), and p125 focal adhesion kinase and paxillin activation, which are thought to transduce signals involved in tumor cell invasion (10). Thus, ET-1, acting through ET_AR, consistently induces the activity of multiple metastasis-related proteinases, such as matrix metalloproteinases (MMP) and the urokinase-type plasminogen activator system (12). Moreover, ET-1 inhibits gap junction intercellular communication by inducing phosphorylation of connexin 43, allowing tumor cells to escape growth control and invasion (13).

In epithelial cancer, acquisition of invasiveness is often accompanied by loss of the epithelial features and gain of a mesenchymal phenotype, a process known as epithelial-to-mesenchymal transition (EMT).

A primary event that governs EMT is the disruption of the E-cadherin-mediated stable interactions between the cells (14–18). Normal cells of ovarian surface epithelium express little or no E-cadherin. Although many primary ovarian carcinomas express E-cadherin, its expression is reduced in many advanced carcinomas, confirming the paradigm of EMT as an integral component of the acquisition of the invasive phenotype (19, 20). Interestingly, a recent study shows that E-cadherin expression was significantly increased in the metastatic lesions compared with the respective primary ovarian tumors (21), indicating that E-cadherin down-regulation is a dynamic event that is required during the initial invasion stage whereas the regrowth of the secondary tumor as metastasis requires the reexpression of E-cadherin.

E-cadherin down-regulation can be accompanied by increased expression of mesenchymal N-cadherin which promotes inappropriate survival signals and the malignant phenotype through interactions with the stromal cells (22–25). Loss of E-cadherin gene expression is mainly due to up-regulation of the transcription factor *Snail*, a zinc finger protein that represses E-cadherin by binding the E-boxes present in its promoter (15). Increased expression of *Snail* has been correlated with loss of E-cadherin expression *in vitro* (26, 27) and *in vivo* (28–31).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Anna Bagnato, Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute, Via delle Messi D'Oro 156, 00158 Rome, Italy. Phone: 39-06-5266-2565; Fax: 39-06-5266-2600; E-mail: bagnato@ifo.it.

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The intracellular domain of E-cadherin interacts with catenin proteins, called α -, β -, γ -, and p120-catenin, to form the cytoplasmic adhesion complex linked to actin filaments (22). Another characteristic cellular event of EMT is an increase in the nuclear amount of β -catenin. In addition to its pivotal role in cadherin-based cell adhesion, β -catenin can act as a transcriptional activator through its interaction with T-cell-specific transcription factor/lymphoid enhancer factors (TCF/LEF). Activity of β -catenin/TCF complex is essential for the transcription of genes that direct cell fate, polarity, and proliferation of tumor cells (14). Cytosolic β -catenin is normally phosphorylated by glycogen synthase kinase-3 β (GSK-3 β) at serine and threonine residues in its NH₂-terminal domain. This region is then recognized and ubiquitinated by a multiprotein complex containing the F-box protein β -TrCP with resultant degradation of the polyubiquitinated β -catenin by the proteasome (17). Alternatively, the canonical Wnt signaling pathway can inhibit the ability of GSK-3 β to phosphorylate target substrates, with resultant increases in β -catenin levels. The stabilization of β -catenin consequently leads to enhanced nuclear accumulation and its transcriptional activity through binding to TCF/LEF complex (32). Different signaling pathways could stabilize the pool of cytosolic β -catenin that is released from E-cadherin-bound sites as a consequence of *Snail*-mediated E-cadherin repression.

Recent findings support a model wherein GSK-3 β activity also controls *Snail* phosphorylation, β -TrCP-directed ubiquitination, and proteasomal degradation. Consistent with this, GSK-3 β inhibition induces stability and increased nuclear levels of *Snail* protein. Moreover, a block of GSK-3 β has been shown to increase the transcription of *Snail* gene (33–37).

The integrin-linked kinase (ILK), a component of focal adhesion plaques which interacts directly with the cytoplasmic domains of β 1-integrin subunits, has an essential role in EMT by connecting the cell-adhesion molecules, integrins, and growth factors to the actin cytoskeleton and to a range of signaling pathways. Overexpression of ILK induces down-regulation of E-cadherin expression, nuclear translocation of β -catenin, and activation of β -catenin and *Snail* transcriptional activity (38–40). Expression of ILK inhibits GSK-3 β activity, indicating that GSK-3 β is a substrate of ILK (41). Consistent with the premise that GSK-3 β regulates *Snail* and β -catenin levels and transcriptional programs in a cooperative fashion, we showed that activation of the ET-1/ET_AR pathway results in inhibition of GSK-3 β through ILK. This stabilizes *Snail* and β -catenin proteins which concordantly engage the transcriptional activities that converge on the EMT process in human ovarian cancer cells. A small-molecule ET_AR antagonist, ABT-627, suppresses EMT determinants and tumor growth in an ovarian xenograft tumor model. These findings indicate that the ET_AR signaling is essential during distinct steps of tumor progression by interfering with EMT process.

Materials and Methods

Cell culture. Human ovarian carcinoma cell lines, HEY and OVCA 433, were cultured as previously described (12). All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). Cells were cultured in serum-free medium for 24 hours before ET-1 (100 nmol/L; Peninsula Laboratories, Belmont, CA) stimulation. ET_AR antagonists, ABT-627 (1 μ mol/L; kindly provided by Abbott Laboratories, Abbott Park, IL) and BQ 123 (1 μ mol/L; Peninsula Laboratories), were added 15 minutes before the agonist. Pretreatment with KP-392 (10 μ mol/L; Quadra Logic

Technologies QLT, Vancouver, BC), MG132 (10 μ mol/L), cycloheximide (20 μ mol/L; all from Calbiochem, San Diego, CA), or LiCl (40 mmol/L; Sigma, St. Louis, MO) was done for 30 to 60 minutes before the addition of ET-1.

ELISA. Subconfluent HEY cells were serum-starved for 24 hours and incubated for the indicated times. ET-1 release was measured in triplicate on microtiter plates by an ELISA kit (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. ET-1 may be measured in the range 0 to 120 pg/mL. The sensitivity is <1.0 pg/mL.

Immunoblotting. Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce Biotechnology, Rockford, IL) was used for the enrichment of membrane protein whereas NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology) were used to separate cytoplasmic and nuclear fractions. Total cell lysates, membrane, cytoplasmic, or nuclear fractions, or homogenized HEY tumor specimens were subjected to SDS-PAGE and processed by immunoblotting using antibodies to E-cadherin, N-cadherin, β -catenin (BD Transduction Laboratories, Heidelberg, Germany), phosphorylated GSK-3 β (pGSK-3 β), GSK-3 β (Cell Signaling, Beverly, MA), ILK (Upstate, Charlottesville, VA), β -actin (Oncogene, Darmstadt, Germany), ET_AR (Abbott Laboratories), vimentin (DAKO, Denmark), and *Snail* (12). The proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified using NIH image (Scion, Frederick, MD).

Reverse transcription-PCR. Total RNA from HEY cells was extracted using TRIzol (Invitrogen). Reverse transcription-PCR (RT-PCR) was done using AccessQuick RT-PCR System (Promega, Madison, WI) according to the instructions of the manufacturer. The primers sets were as follows: *ET_AR*, 5'-CACTGGTTGGATGTGTAATC-3' and 5'-GGAGATCAATGACCACATAG-3'; *GAPDH*, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Thirty-five cycles of amplification were done.

Northern blotting. Total RNA was transferred to a nylon membrane, which was hybridized in the QuikHyb hybridization Solution (Stratagene, La Jolla, CA). The cDNA probe was prepared using RT-PCR products by SUPERScript One-Step RT-PCR System (Invitrogen). Briefly, 1 μ g of RNA was reverse transcribed using the following primers: *E-cadherin*, 5'-AACAGGATGCTGAAGGTGA-3' and 5'-AAAATCCAAGCCCTTTGCTG-3'; *Snail*, 5'-TTCACGACGCCCTACGACCAG-3' and 5'-GCCTTCCCAC-TAGTCTCATC-3'. Probes were labeled with [α -³²P]dCTP using a random primer oligolabeling kit (Amersham). The relative intensity of signals was quantified using NIH image (Scion).

Luciferase reporter gene assay. To measure the transcriptional activity of *Snail*, E-cadherin promoter, and β -catenin, 3×10^5 cells per well were transiently transfected using LipofectAMINE reagent (Invitrogen) with 0.5 μ g of pGL3-SNA (-869/+59) vector containing luciferase (*Luc*) gene under the control of the human *Snail* promoter (40), with 0.5 μ g pGL2 Ecad3/luc construct (kindly provided by Dr. E.R. Fearon, University of Michigan, Ann Arbor, MI), or with a plasmid containing three copies of the TCF-4 binding site upstream a firefly luciferase reporter gene (pTOP-Flash, Cell Signaling), respectively, or with empty control vectors (Promega). Where indicated, HEY cells were transfected overnight with 1 μ g of ILK cDNA (kinase dead) in pUSEamp (E359K mutant) or with empty vector (Upstate). All constructs were cotransfected with pCMV- β -galactosidase plasmid (Promega). After 24 hours of transfection, serum-starved cells were treated with ET-1 and/or inhibitors for additional 24 hours. Luciferase activity was determined in the cell lysates using the Luciferase assay system (Promega) and normalized to β -galactosidase activity. The mean of three independent experiments done in sextuplicate was reported.

Small-interfering RNA treatment. HEY cells were transfected with 100 nmol/L small-interfering RNA (siRNA) duplexes against ET_AR mRNA (SMART pool) or scrambled mock siRNA obtained commercially (Dharmacon, Lafayette, CO). siRNA transfection using LipofectAMINE reagent (Invitrogen) was done according to the protocol of the manufacturer. Cells were harvested 48 hours later and ET_AR mRNA and protein levels were determined. In the luciferase assay, cells were transfected with 0.5 μ g of pGL3-SNA vector or 0.5 μ g of pGL2 Ecad3/luc construct 24 hours after being transfected with 100 nmol/L ET_AR siRNA. Cells were then treated with ET-1 (100 nmol/L) and harvested after 24 hours.

Immunohistochemistry. Indirect immunoperoxidase staining of tumor xenografts was done on acetone-fixed 4- μ m tissue sections as previously described (43). E-cadherin, N-cadherin, and β -catenin expressions were detected using antibodies described above with the Vector MOM immunodetection kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole as chromogenic substrate and Mayer's hematoxylin as nuclear counterstain. Sections incubated with isotype-matched immunoglobulins served as negative control.

Chemoinvasion assay. Chemoinvasion assay was done with a 48-well modified Boyden chamber (NeuroProbe) and 8- μ m pore size polyvinyl pyrrolidone-free polycarbonate Nucleopore filters (Costar, New York, NY) as previously described (12). The filters were coated with an even layer of 0.5 mg/mL Matrigel (BD Transduction Laboratories). The lower compartment of the chamber was filled with ET-1 (100 nmol/L) and/or ABT-627 (1 μ mol/L). Serum-starved HEY cells (5×10^5 /mL) were placed in the upper compartment (55 μ L/well). BQ 123 and ABT-627 were previously added to the cells and preincubated for 15 minutes at 37°C. In the chemoinvasion assay using ET_AR siRNA, HEY cells were transfected for 48 hours and then incubated in the upper compartment. After 24 hours of incubation at 37°C, the filters were removed, stained with Diff-Quick (Merz-Dade, Dudinggen, Switzerland), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

Xenografts in nude mice. Female athymic (*nu^{-/-}/nu^{-/-}*) mice, 4 to 6 weeks of age, were used (Charles River Laboratories, Milan, Italy). The treatment protocol followed the guidelines for animal experimentation of the Italian Ministry of Health. Mice were injected s.c. into one flank with 1.5×10^6 viable HEY cells. After 7 days, when tumor reached ~0.2 to 0.3 cm in diameter, mice were randomized in two groups ($n = 10$) to receive different treatments. One group was treated i.p. for 21 days with 2 mg/kg/d of ABT-627. Control mice were injected with drug vehicle. On day 40 after tumor injection, tumors were removed from control and treated mice and snap frozen for immunohistochemical and immunoblot analysis. Tumor size was

measured with calipers and was calculated using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Tissue samples. Immunohistochemical analysis of ovarian cancers was done on archival from 50 tumors collected with informed consent as indicated by our Institutional Review Board, which were classified according to WHO criteria. Primary tumors include 36 serous and 14 endometrioid carcinomas. Avidin-biotin indirect immunoperoxidase staining was done as previously described (5) by using antibodies described above and monoclonal antibody to ET-1 (clone TR.ET.48.5, Affinity Bioreagents, Golden, CO). The avidin-biotin assays were done using the Vectastain Elite kit (Vector Laboratories). 3-Amino-9-ethylcarbazole was used as chromogenic substrate and Mayer's hematoxylin as nuclear counterstain.

Statistical analysis. Statistical analysis was done using χ^2 test, Student's test, or Fisher's exact test as appropriate. The time course of tumor growth was compared across the groups with the use of two-way ANOVA with group and time as variables (SPSS, Chicago, IL). All statistical tests were two sided. $P < 0.05$ was considered statistically significant.

Results

Endothelin-1/endothelin A receptor autocrine pathway is required for epithelial-to-mesenchymal transition. ET_AR over-expression is often accompanied in ovarian tumor cells by production of ET-1, and autocrine stimulation via ET-1 has been implicated in tumor progression (4, 5). Because of the unknown invasive responses elicited by ET-1, such as EMT, we first analyzed cell plasticity (42) of the two human ovarian cancer cell lines, HEY and OVCA 433, which release ET-1 and express functional ET_AR (4). The ET-1 levels released from HEY and OVCA 433 cells were within the physiological range needed for activation of ET_AR in an

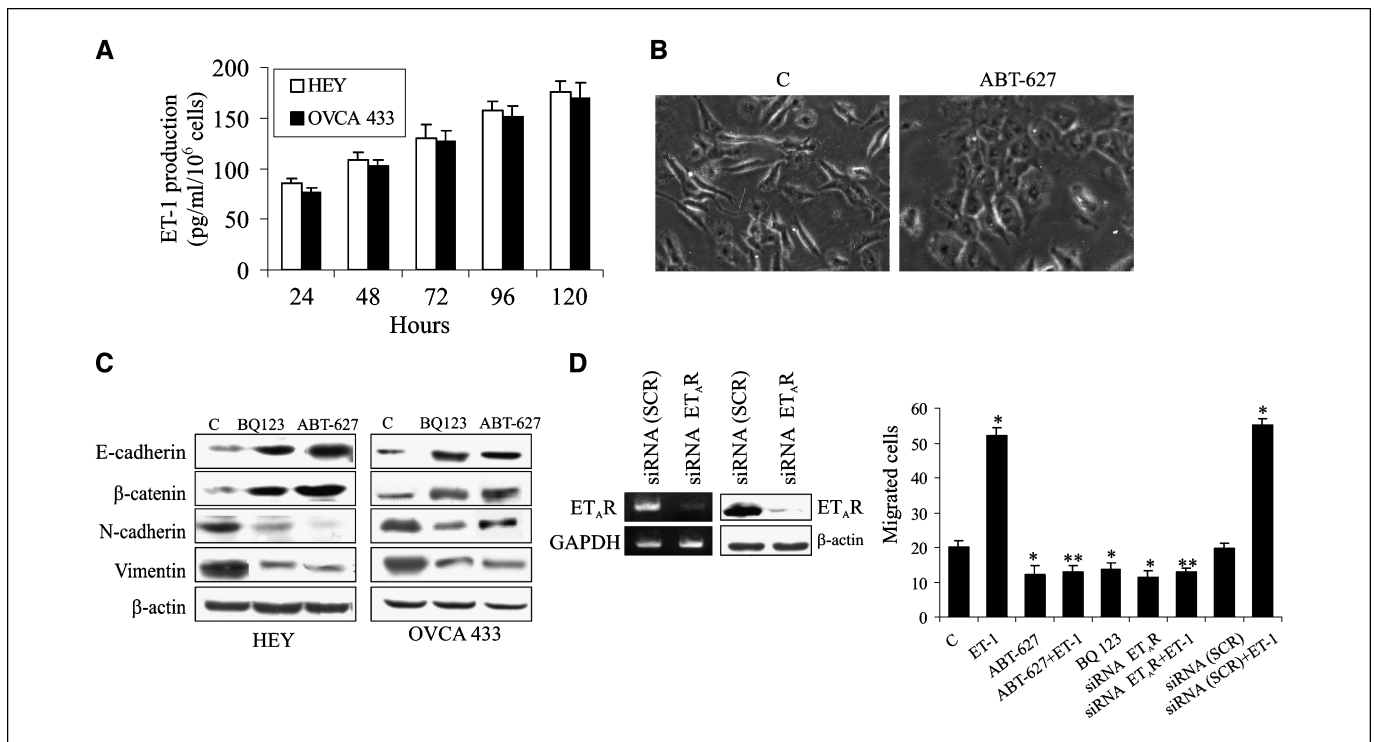


Figure 1. ET-1/ET_AR autocrine pathway is required for EMT. **A**, ET-1 secretion was measured in conditioned medium of serum-starved HEY and OVCA 433 cells using ELISA kit. *Columns*, mean of results from three experiments each done in triplicate; *bars*, SD. **B**, effect of ET_AR antagonist, ABT-627, on serum-starved HEY cell morphology evaluated by phase-contrast microscopy after 48 hours of incubation. **C**, effect of BQ 123 or ABT-627 on the expression of E-cadherin, β -catenin, N-cadherin, and vimentin evaluated by immunoblotting of HEY and OVCA 433 cells. **D**, HEY cells were transfected for 48 hours with ET_AR siRNA or scrambled siRNA (SCR) and mRNA and protein levels were determined. Effect of ET_AR antagonists and ET_AR siRNA on basal and ET-1-induced cell invasion. *Columns*, mean of three independent experiments, each done in triplicate; *bars*, SD. *, $P \leq 0.001$, compared with control; **, $P \leq 0.001$, compared with ET-1.

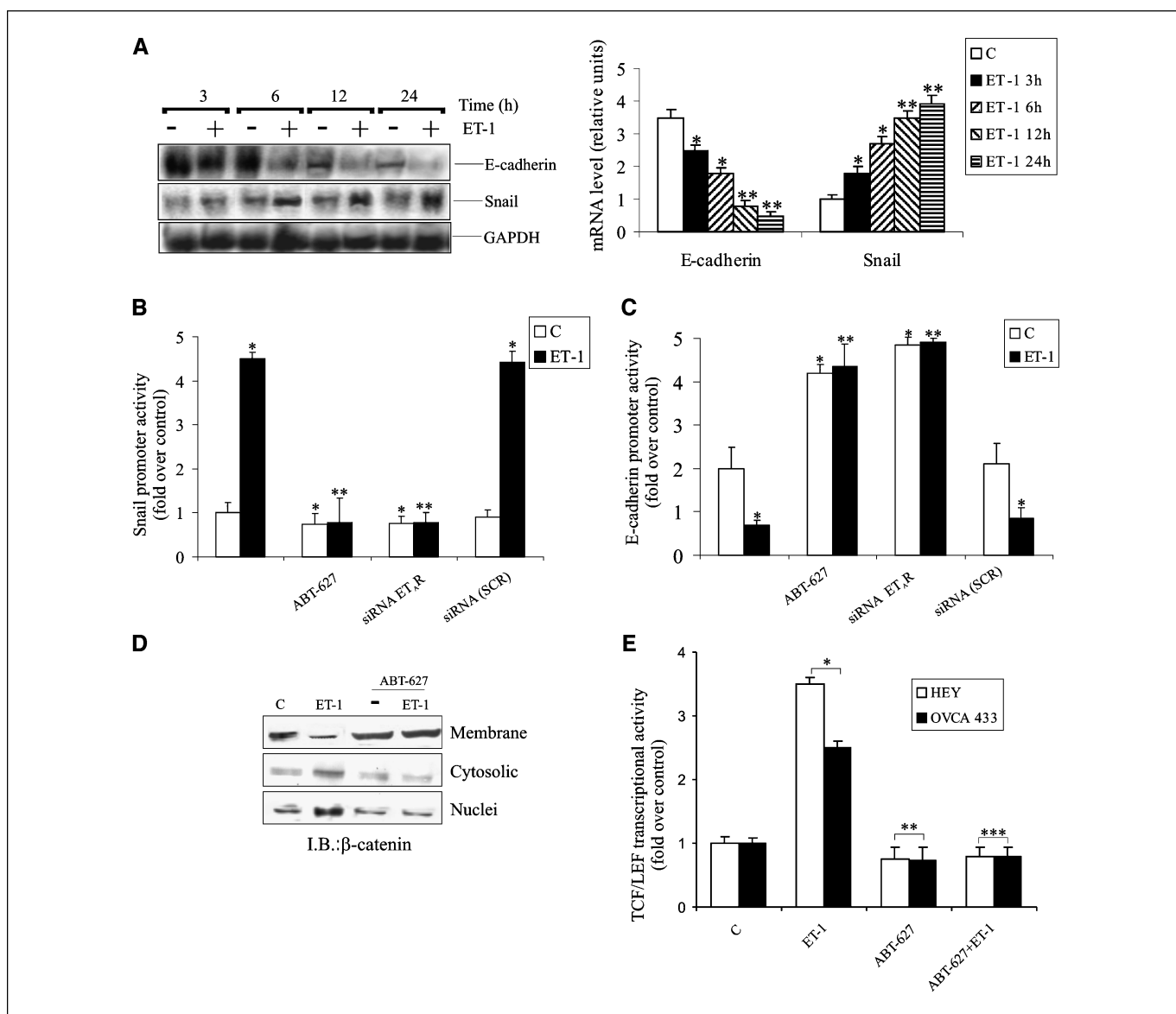


Figure 2. ET-1 down-regulates E-cadherin through *Snail* and β -catenin. **A**, time-dependent effect of ET-1 on *Snail* and E-cadherin mRNA in HEY cells as shown by Northern blotting analysis. The relative density of mRNA content was analyzed statistically; columns, average value of three independent Northern blots; bars, SD; *, $P < 0.01$, compared with control (C); **, $P < 0.001$, compared with control. **B** and **C**, effect of ABT-627 or ET_AR siRNA and/or ET-1 on either *Snail* promoter or E-cadherin promoter activity. **D**, immunoblotting for β -catenin expression in membrane, cytoplasmic, and nuclear fractions of HEY cells treated with ET-1 and/or ABT-627. **E**, transcriptional activity of β -catenin-TCF/LEF-1 in HEY and OVCA 433 cells. Relative transcriptional activity as a ratio to β -galactosidase activity; columns, mean of three independent experiments each done in sextuplicate; bars, SD. *, $P < 0.001$, compared with control; **, $P < 0.01$, compared with control; ***, $P < 0.001$, compared with ET-1.

autocrine fashion (Fig. 1A). To test whether the interference with ET-1/ET_AR autocrine loop would affect EMT, we used two pharmacologic antagonists of ET_AR: BQ 123, a selective peptide antagonist, and ABT-627, a small molecule that potently ($K_i = 34$ pmol/L) and selectively inhibits ET-1 signaling at the level of interaction with ET_AR (43). In ovarian cancer primary cultures and cell lines, ABT-627 and BQ 123 cause inhibition of spontaneous growth rate. No inhibitory effect is observed in a cell line (C33A) not expressing ET_AR, further strengthening the specificity of these ET_AR antagonists. Moreover, addition of the ET_BR antagonist BQ 788 does not affect the basal cell growth rate, showing that endogenous ET-1 acts as an autocrine modulator of ovarian cancer cells selectively through the ET_AR (4, 5, 43). Sustained ET_AR

signaling caused by an autocrine ET-1/ET_AR loop can be required for the maintenance of EMT in HEY cells as shown by a spindle-shaped and motile fibroblastoid phenotype (Fig. 1B). Interestingly, a large percentage of HEY cells reverted to an epithelial phenotype in which the cells formed compact structures in the presence of 1 μ mol/L ABT-627 for 48 hours (Fig. 1B). To determine whether EMT-associated molecular alterations have occurred in these cells, we examined the expression of epithelial and mesenchymal markers by immunoblotting. Both ET_AR antagonists reversed ET-1-induced EMT, in association with repression of N-cadherin and vimentin, and regained expression of endogenous E-cadherin and β -catenin (Fig. 1C). To further verify the role of ET_AR in regulating EMT, we employed RNA interference (siRNA) technology, which

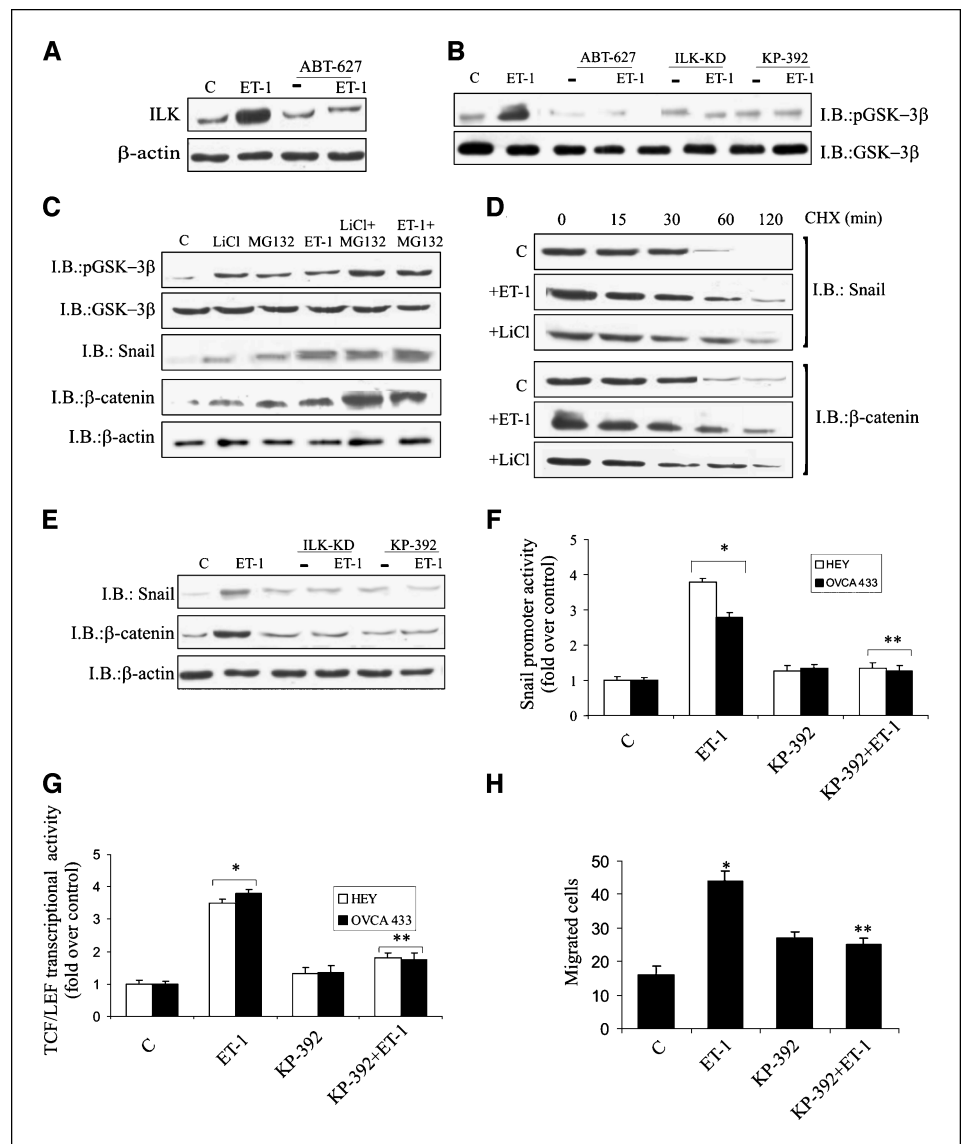
targets ET_AR. Transfection with ET_AR siRNA, but not with the control scrambled siRNA, markedly reduced both ET_AR mRNA and protein levels (Fig. 1D). ET_AR antagonists, as well as ET_AR siRNA, resulted in a significant decrease in the basal activity of HEY cells and ET-1-induced cell invasion whereas the control siRNA was ineffective (Fig. 1D). Taken together, these data indicate that an ET-1/ET_AR autocrine loop in ovarian carcinoma cells has a critical role in inducing EMT morphologic and molecular changes and cell invasion.

Endothelin-1 induces transcriptional up-regulation of *Snail*. Because the transcription factor *Snail* functions as a potent repressor of E-cadherin, we examined whether ET-1 can regulate *Snail* at multiple levels. In HEY cells, ET-1 up-regulates *Snail* mRNA in a time-dependent manner. This effect was paralleled by the down-regulation of E-cadherin mRNA levels (Fig. 2A). In addition, ET-1 induced a significant increase in the promoter activity of *Snail*. The ability of the ET_AR antagonist ABT-627 to block ET-1-induced *Snail* promoter activity indicated that this effect is mediated by the ET_AR (Fig. 2B). Because *Snail* has been implicated in repression of E-cadherin transcription, we analyzed the effect of

ET-1 on E-cadherin promoter activity. ET-1 treatment suppressed the transcriptional activity of E-cadherin through ET_AR as indicated by the inhibitory effect of ABT-627 (Fig. 2C). Additionally, HEY cells were transiently cotransfected with ET_AR siRNA and either *Snail* promoter or E-cadherin promoter. As shown in Fig. 2B and C, transfection with ET_AR siRNA, but not with control siRNA, reverted the ET-1-induced suppression of E-cadherin promoter and remarkably prevented that induced by endogenous ET-1. By contrast, transfection with ET_AR siRNA caused a decrease in both basal and ET-1-induced *Snail* promoter activity, resulting in E-cadherin down-regulation at the transcriptional level.

Endothelin-1 promotes β-catenin nuclear translocation and β-catenin-dependent transcriptional activity. Upon Wnt-dependent or Wnt-independent stimulation, β-catenin accumulates in the cytosol and translocates to the nucleus where it may associate with the TCF/LEF, thereby activating target gene transcription and biological responses (14, 15). To analyze the translocation of β-catenin in response to activation of the ET-1/ET_AR pathway, we separated membrane, cytoplasmic, and nuclear fractions of HEY cells. ET-1 up-regulated the cytosolic and nuclear accumulation of

Figure 3. ET-1 induces *Snail* and β-catenin protein stability and transcriptional activity through ILK-dependent GSK-3β inhibition. Immunoblotting for expression of ILK in HEY cells treated with ET-1 and/or ABT-627 (A); of pGSK-3β and total GSK-3β in HEY cells or cells transfected with kinase-deficient ILK (*ILK-KD*) treated with ET-1 and/or ABT-627 and KP-392 (B); of pGSK-3β, total GSK-3β, *Snail*, and β-catenin in HEY cells stimulated with LiCl, MG132, or LiCl and MG132, or with ET-1 alone or with MG132 (C); of *Snail* and β-catenin in HEY cells treated with cycloheximide (CHX) for different times in the absence or presence of ET-1 or LiCl (D); of *Snail* and β-catenin in HEY cells or cells transfected with kinase-deficient ILK treated with ET-1 and/or indicated inhibitors (E). Transcriptional activity of *Snail* promoter (F) and β-catenin-TCF/LEF-1 (G) in HEY and OVCA 433 cells treated with ET-1 and/or KP-392. Columns, mean relative transcriptional activity of three independent experiments each done in sextuplicate; bars, SD. *, *P* < 0.001, compared with control; **, *P* < 0.001, compared with ET-1. H, invasion assay of HEY cells treated with KP-392 and/or ET-1. Columns, mean of three independent experiments each done in triplicate; bars, SD. *, *P* ≤ 0.001, compared with control; **, *P* ≤ 0.01, compared with ET-1.



β -catenin, and both were prevented by ABT-627 (Fig. 2D). In HEY and OVCA 433 cells, ET-1 treatment activated transcription of the TCF/LEF reporter. This response was completely blocked by ABT-627, pointing to ET_A as the relevant receptor in these events (Fig. 2E).

Endothelin-1 induces Snail and β -catenin protein stability and transcription through integrin-linked kinase-dependent glycogen synthase kinase-3 β inhibition. It has been shown that EMT induced by overexpression of ILK is accompanied by down-regulation of E-cadherin expression, activation of *Snail* gene expression, and increased nuclear activity of β -catenin TCF/LEF transcriptional complex (39, 44, 45). Moreover, ILK expression increases during ovarian cancer progression and soluble factors in ascitic fluid mediate sustained overexpression of ILK in ovarian tumors (46). ILK expression was up-regulated in ET-1-treated cells and this response was mediated by the ET_AR as shown by the blocking activity of ABT-627 (Fig. 3A).

ET-1 also promoted a significant increase in the Ser⁹-phosphorylated GSK-3 β (pGSK-3 β) through ET_AR as shown by abrogation of this effect by ABT-627 (Fig. 3B). To establish that GSK-3 β is a downstream substrate of ILK in the signaling activated by ET-1/ET_AR, we blocked ILK activity by transfecting HEY cells with a kinase-deficient, dominant negative form of ILK or with the small-molecule inhibitor of ILK activity, KP-392 (44). Under these conditions, ET-1 was unable to phosphorylate GSK-3 β (Fig. 3B), thus showing the critical role of ILK in GSK-3 β inactivation induced by the ET-1/ET_AR axis.

The demonstration that GSK-3 β inhibition promotes nuclear localization and transcription of *Snail*, as well as *Snail* and β -catenin protein stabilization, adds another level of complexity to

the regulation of EMT (34, 36). The proteasome inhibitor MG132, the GSK-3 β inhibitor LiCl, and ET-1 enhanced the expression of *Snail*, β -catenin, and p-GSK-3 β . These responses were increased in cells pretreated in association with lithium or ET-1, indicating that pGSK-3 β and the proteasome pathway are involved in the ET-1-induced regulation of *Snail* and β -catenin proteins (Fig. 3C).

Furthermore, in HEY cells treated with cycloheximide, the half-lives of *Snail* and β -catenin were prolonged in the presence of ET-1 and were similar to that observed following treatment of HEY cells with the GSK-3 β inhibitor LiCl (Fig. 3D and Supplementary Fig. S1A and B). This further underscores for the first time that ET-1 acts through an ET_AR-mediated pathway to stabilize *Snail* and β -catenin proteins in promoting an EMT program through GSK-3 β , probably by inhibiting the β -TrCP-mediated ubiquitination of both proteins. To establish the role of ILK in ET-1-induced *Snail* and β -catenin stability, we silenced ILK activity by dominant negative ILK and with KP-392. Under these conditions, ET-1 affected neither *Snail* nor β -catenin stability (Fig. 3E). Moreover, KP-392 significantly reduced ET-1-induced *Snail* and β -catenin/TCF/LEF transcriptional activity and cell invasiveness (Fig. 3F-H). These observations clearly place ILK at a crossroad of ET-1/ET_AR signaling to activate *Snail*- and β -catenin-driven transcriptional programs that cooperatively support an invasive EMT process.

Endothelin A receptor antagonist-induced inhibition of tumor growth *in vivo* is associated with a suppression of epithelial-to-mesenchymal transition molecular determinants. We next determined whether ET_AR blockade resulted in the inhibition of ET-1-induced EMT effectors and tumor growth by treating mice bearing established HEY tumors with ABT-627.

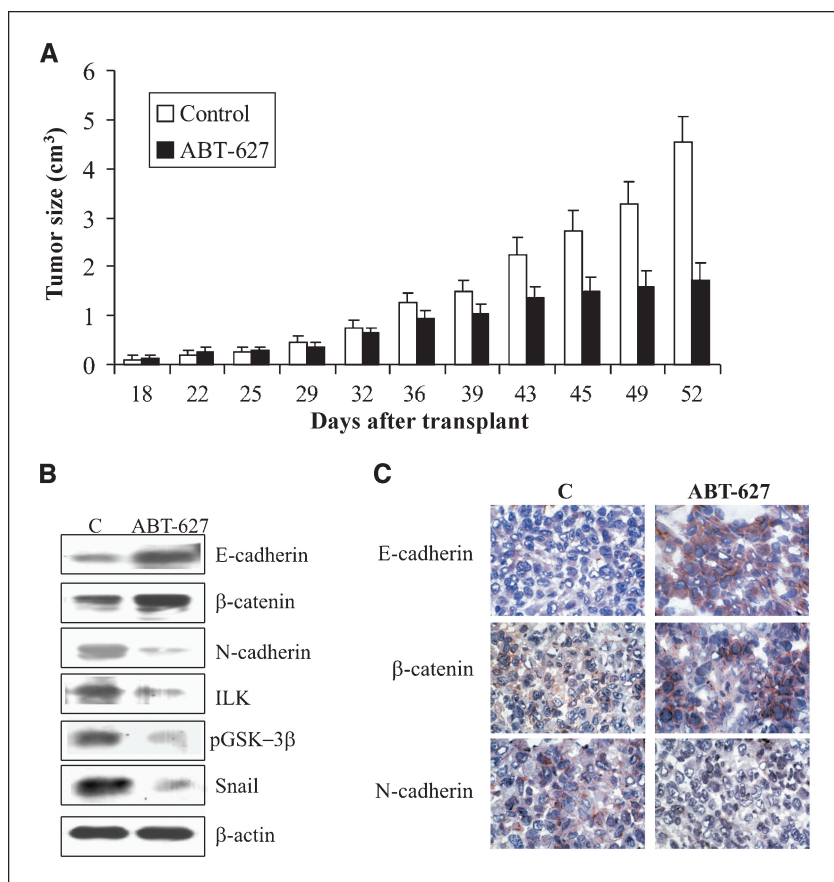


Figure 4. Blockade of ET_AR by ABT-627 reverses EMT *in vivo* and inhibits tumor growth. **A**, antitumor activity of ABT-627 treatment on established HEY human ovarian carcinoma xenografts. Mice were given injections of 1.5×10^6 HEY cells s.c. in the dorsal flank. After 7 days, the groups of 10 mice were treated i.p. for 21 days with drug vehicle or with ABT-627 (2 mg/kg/d). Three different experiments with a total of 20 mice for each experiment were done; bars, SD. ABT-627 caused a significant inhibition of HEY tumor growth in treated animals over a 21-day dosing regimen. The comparison of time course of tumor growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant ($P < 0.001$). **B**, immunoblotting for E-cadherin, N-cadherin, β -catenin, *Snail*, pGSK-3 β , and ILK expression in HEY tumor xenografts. **C**, comparative immunohistochemical analysis of E-cadherin, N-cadherin, and β -catenin expression in HEY tumor xenografts (original magnification, $\times 320$; N-cadherin, $\times 400$).

Treatment with ABT-627 produced a 65% inhibition of HEY tumor growth (Fig. 4A). ABT-627 was generally well tolerated with no detectable signs of acute or delayed toxicity. Analysis of EMT molecule expression in ovarian carcinoma xenografts revealed a marked reduction of N-cadherin, *Snail*, pGSK-3 β , and ILK and an increase of E-cadherin and β -catenin expression in ABT-627-treated mice compared with controls (Fig. 4B). Immunohistochemical evaluation of tumors confirmed increased expressions of E-cadherin and β -catenin and a concomitant decrease of N-cadherin expression following ABT-627 treatment (Fig. 4C).

E-cadherin is down-regulated in endothelin-1/endothelin A receptor-expressing primary ovarian tumor tissues. To explore the pathophysiologic function of ET_AR in human ovarian cancer, a cohort of 50 primary ovarian cancer tissues were stained by immunohistochemistry for ET_AR, E-cadherin, and N-cadherin expression. Consistent with our observations in cell cultures and HEY xenografts, expression of ET_AR was significantly correlated with the down-regulation of E-cadherin ($P < 0.008$) and with enhanced expression of N-cadherin ($P < 0.007$; Fig. 5A and B). The ET_AR expression was grade dependent, with grade 3 and 4 cancers showing higher levels than early-grade cancers ($P < 0.01$; Fig. 5B). These results are consistent with our previous findings wherein the expression of ET_AR significantly correlates with neovascularization ($P < 0.0002$) and vascular endothelial growth factor (VEGF) expression (6). The tumor-staining data indicate the biological relevance of ET_AR in the regulation of EMT *in vivo*, resulting in down-regulation of epithelial marker E-cadherin and concomitant up-regulation of N-cadherin.

Discussion

One hallmark of epithelial cancer progression is EMT in which tumor cells undergo loss of polarity and cell-cell junctions and acquire a mesenchymal phenotype and the ability to invade the extracellular matrix and to migrate to distant sites (14, 47). In this study, we provide ample evidence that in human ovarian carcinoma cells, ET_AR activation by ET-1 contributes to tumor progression by acting as a crucial mediator of EMT. This conclusion is supported by several lines of evidence. First, ET-1 through the ET_AR induces a spectrum of key events including down-regulation of the epithelial adherens, such as E-cadherin and β -catenin, induction of mesenchymal markers, such as N-cadherin and vimentin, and enhanced cell invasiveness. Second, ET-1/ET_AR regulates *Snail* and β -catenin, the major contributors to E-cadherin suppression, at multiple levels. Thus, ET-1 increases both *Snail* and β -catenin protein stability and transcriptional activity. Third, ILK is a downstream check point of the signaling pathways activated by ET-1/ET_AR that, via GSK-3 β inactivation, promotes *Snail* and β -catenin signaling and acquisition of an invasive phenotype. Fourth, interruption of ET-1/ET_AR autocrine signaling by two selective ET_AR antagonists or by RNA interference reverts EMT, increases the transcription level of E-cadherin, as well as inhibits *Snail* transcription activity and basal cell invasion rate. Furthermore, pharmacologic blockade of ET_AR inhibits tumor xenograft growth and EMT molecular determinants. Finally, a significant correlation between ET_AR and E-cadherin down-regulation and N-cadherin expression was observed in human ovarian cancer tissues, indicating the relevant role of ET_AR in the EMT process.

Loss of normal tissue architecture and microenvironmental interactions are signatures of epithelial cancer progression (16).

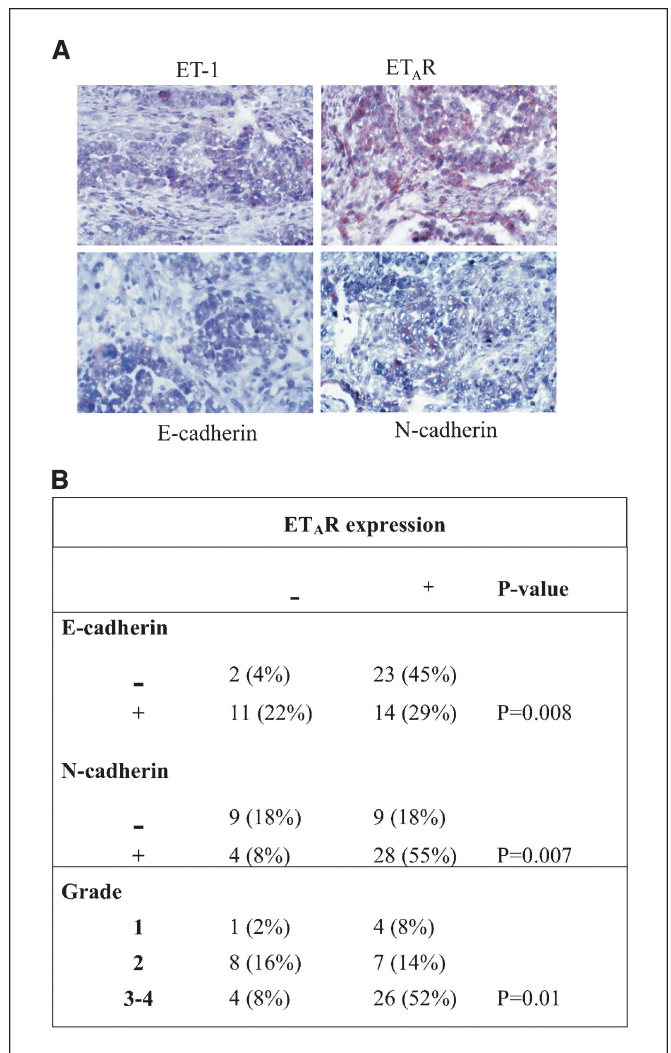


Figure 5. Correlation between E-cadherin, N-cadherin, and ET_AR expression in human ovarian tumor tissues. A, immunohistochemical staining of representative human primary ovarian carcinoma tissue samples for expression of ET-1, ET_AR, E-cadherin, and N-cadherin (original magnification, $\times 200$). B, relationship between ET_AR expression and E-cadherin and N-cadherin in human primary ovarian carcinomas. The expression patterns of ET_AR, E-cadherin, and N-cadherin in the 50 ovarian cancer samples were determined by immunohistochemistry and summarized. The correlation between ET_AR and E-cadherin and N-cadherin was analyzed using Fisher's exact test.

Here we describe a mechanism whereby ET-1/ET_AR signaling orchestrates transcriptional programs that regulate E-cadherin down-regulation, EMT, and progression to invasive and metastatic ovarian carcinoma.

Recent data (34–36) show that inhibition of GSK-3 β promotes nuclear localization and transcription of *Snail*, as well as *Snail* and β -catenin protein stabilization, revealing that many oncogenic pathways could control cell adhesion, cell fate, and invasion during metastasis. Consistent with these findings, we show that ET-1 acts through ET_AR-mediated pathway to stabilize *Snail* and β -catenin proteins and activate a *Snail*- and β -catenin-driven transcriptional program that regulates EMT determinants. Thus, phosphorylation of GSK-3 β by ET-1 resulting in the *Snail* and β -catenin stabilization shows that inhibition of GSK-3 β remains central to ET-1-induced key hallmarks of EMT. These results were consistent with the previous observation that ET-1 increases cytosolic β -catenin in

cardiomyocytes to a level equivalent to that induced by the GSK-3 β inhibitor (48).

In view of its position at the crossroads connecting integrins and the actin cytoskeleton, ILK was hypothesized to be a candidate signaling molecule that functions at the convergence point of cell adhesion- and growth factor-triggered signal transduction, thus regulating the EMT process (39). Here we show that ET-1-driven ILK signaling is necessary for GSK-3 β phosphorylation and related Snail and β -catenin protein stability, transcriptional activity, and invasion, implicating for the first time ILK and its downstream substrate GSK-3 β as check points of finely tuned interconnected signals induced by ET-1/ET_AR to modulate EMT. Thus, we propose a complex scheme wherein activation of ET_AR pathway by ET-1 drives inhibition of GSK-3 β by an ILK-mediated signaling pathway to stabilize Snail and β -catenin proteins in a coordinate fashion so as to cooperatively engage transcriptional programs leading to EMT.

Because all the molecular effectors of EMT are triggered by ET_AR activation, blockade of this receptor results in inhibition of tumor growth and progression of ovarian carcinoma xenografts. We also show that ET_AR blockade restores E-cadherin and β -catenin expression and suppresses *Snail*, ILK, and N-cadherin expression *in vivo*, further identifying the mechanisms through which the autocrine ET-1/ET_AR loop sustains EMT process and, in turn, creates a tumor microenvironment more permissive to progression.

In this context, ET-1/ET_AR induces the disruption of normal host-tumor interactions regulating changes in cadherin, connexin, and MMP expression (13, 43). Indeed, the immunohistochemical and immunoblot analysis of HEY xenografts provides *in vivo* evidence for this concept showing that treatment with ABT-627 induces a significant reduction of microvessel density, expression of VEGF, cyclooxygenase-2, and MMP-2, and increased tumor apoptosis and connexin 43-based gap junctional intercellular plaques (9, 13, 43).

It is becoming increasingly clear that E-cadherin acts as a late-stage microenvironmental tumor suppressor in ovarian carcinoma (19, 20, 49). This observation was supported by the immunohistochemical profile of cadherin phenotype in primary ovarian cancer samples, indicating that expression of ET-1/ET_AR axis correlates with the switch of cadherin expression associated with advanced-stage tumors. These findings complement and extend the recent analysis of a genome-wide expression profile of late-stage ovarian cancer whereby ET-1 has been identified as a key gene that activates cell signaling controlling cell migration, spread, and invasion (50).

Although further studies are required to examine the complex connection network that regulates EMT in tumor metastasis, a scheme is emerging in which we identify for the first time that mimicry of the Wnt pathway, through ET_AR-driven molecular events, is required for the cooperative regulation of *Snail* and β -catenin signaling, which occurs via ILK and GSK-3 β .

Understanding the regulatory loops that allow close coordination of EMT program may provide a novel strategies aimed at preventing the development of metastasis. In this regard, the ability of ET_AR antagonists to induce concomitant suppression of tumor cell proliferation and/or survival (7, 43) and simultaneously control EMT provides a rationale for developing a more effective therapeutic intervention in aggressive ovarian cancer.

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