Integrin-linked kinase functions as a downstream mediator of endothelin-1 to promote invasive behavior in ovarian carcinoma

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Abstract

The endothelin-1 (ET-1) axis represents a novel target in several malignancies, including ovarian carcinoma. Upon being activated, the endothelin A receptor (ET_AR) mediates multiple tumor-promoting activities, including mitogenesis, escape from apoptosis, angiogenesis, metastasis-related protease activation, epithelial-mesenchymal transition, and invasion. Integrin-linked kinase (ILK) is a multidomain focal adhesion protein that conveys intracellular signaling elicited by $\beta1$-integrin and growth factor receptors. In this study, we investigate whether the signaling triggered by ET_AR leading to an aggressive phenotype is mediated by an ILK-dependent mechanism. In HEY and OVCA 433 ovarian carcinoma cell lines, activation of ET_AR by ET-1 enhances the expression of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins. ILK activity increases as ovarian cancer cells adhere to type I collagen through β_1 integrin signaling, and do so to a greater extent on ET-1 stimulation. ET-1 increases ILK mRNA and protein expression and activity in a time- and concentration-dependent manner. An ILK small-molecule inhibitor (KP-392) or transfection with a dominant-negative ILK mutant effectively blocks the phosphorylation of downstream signals, Akt and glycogen synthase kinase-3β. The blockade of ET-1/ET_AR-induced ILK activity results in an inhibition of matrix metalloproteinase activation as well as of cell motility and invasiveness in a phosphoinositide 3 kinase–dependent manner. In ovarian carcinoma xenografts, ABT-627, a specific ET_AR antagonist, suppresses ILK expression, Akt and glycogen synthase kinase-3β phosphorylation, and tumor growth. These data show that ILK functions as a downstream mediator of the ET-1/ET_AR axis to potentiate aggressive cellular behavior. Thus, the ILK-related signaling cascade can be efficiently targeted by pharmacologic blockade of ET_AR. [Mol Cancer Ther 2006;5(4):833–42]

Introduction

Ovarian cancer, the leading cause of death from gynecologic malignancy, 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 modalities strongly relays on improved knowledge of the molecular mechanisms controlling cancer invasion and metastasis.

Endothelin-1 (ET-1), which is present at high levels in ovarian tumor effusions, has a relevant role in ovarian cancer progression (2). The endothelin (ET) axis is composed of three isopeptides, ET-1, ET-2, and ET-3, and of 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 (3). ET-1 has been implicated in the pathophysiology of a wide range of human tumors (4), including ovarian carcinoma (5, 6). In the latter, ET-1 and the ET_AR are overexpressed in primary and metastatic lesions and their overexpression correlates with tumor grade (2, 6, 7). In ovarian tumor cells, ET-1 acts as an autocrine growth, survival, and angiogenic factor selectively through the ET_AR (2, 5, 8–10), triggering different signaling pathways (11). These include mitogen-activated protein kinase, phosphoinositide 3-kinase (PI3K)–dependent Akt activation, src-mediated epidermal growth factor receptor transactivation (10), which is partly responsible for mitogen-activated protein kinase phosphorylation (12), and p125 focal adhesion kinase and paxillin activation, which transduce signals mediated in tumor cell invasion (11). Thus, ET-1, after binding to ET_AR, consistently induces the activity of matrix metalloproteinases (MMP) as well as of the urokinase-type plasminogen activator system (13). Moreover, ET-1 inhibits gap junction intercellular communication by inducing the phosphorylation of connexin 43, allowing tumor cells to escape growth control, and invade (14). Recently, we showed that the ET-1/ET_AR autocrine pathway drives epithelial-to-mesenchymal

transition (EMT) in these tumor cells, by inducing a fibroblastoid and invasive phenotype, down-regulation of E-cadherin, increased levels of β-catenin, Snail and other mesenchymal markers (7).

Increased cell motility depends on the regulated expression of different integrins which mediate the attachment to an underlying extracellular matrix (ECM), as well as delivering intracellular messages to modulate cellular functions and behavior in association with growth factor–induced signals (15). The integrin/growth factor cross-talk involves the activity of integrin-linked kinase (ILK), an intracellular protein serine/threonine kinase that coordinates signaling elicited by integrins and growth factors (16–20), including insulin-like growth factor-I (21, 22), nerve growth factor (23), platelet-derived growth factor (24), and vascular endothelial growth factor (25, 26), in a variety of cell types. ILK, through direct binding to the cytoplasmic domains of β1 integrin subunits (20), connects integrins to the actin cytoskeleton and regulates actin polymerization by interacting directly with several proteins such as paxillin and parvin into the focal adhesion plaques, thus, coordinating cell spreading and actin organization. Because of its kinase activity, ILK activates a range of signaling pathways regulating anchorage-dependent cell growth and survival, cell-cycle progression, EMT, invasion and migration, cell motility, and contraction, vascular development and tumor angiogenesis (16).

Recent reports show that activated growth factor receptors promote oncogenic progression, at least in part, by activating ILK, whose expression, in fact, is increased in high-grade tumors including glioblastoma (27), melanoma (28), prostatic (29), gastric (30), thyroid (31), and ovarian carcinomas (32). In particular, in the latter, ILK expression correlates with transformation and tumor grading. Of interest, undefined components of ovarian ascites have been found to stimulate ILK levels (32). Consistent with the premise that ET-1 is present at high levels in ovarian tumor ascites, here we extended our studies on the mechanism of ET_AR-induced tumor cell invasion, demonstrating that ET-1 induces preferential adhesion of ovarian cancer cells to collagen type I by an enhanced expression of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins, which are involved in the adhesion process and in ILK activation. In this context, we tested whether ET-1 can promote the invasive phenotype of ovarian cancer cells via an ILK-dependent mechanism. In HEY and OVCA 433 ovarian cancer cells, ET-1 to ET_AR activation stimulates ILK expression and activity which is PI3K-dependent and regulates the phosphorylation of glycogen synthase kinase-3β (GSK-3β) and Akt, two important downstream effectors of ILK signaling. Inhibition of ILK activity decreases ET-1-induced MMP-2/MMP-9 activation and reduces tumor cell motility and invasion. Finally, a small-molecule ET_AR antagonist, ABT-627, suppresses tumor growth and expression of ILK and pGSK-3β in an ovarian xenograft tumor model. The present findings show that ILK functions as a down-

stream mediator of the $ET-1/ET_AR$ axis to potentiate the invasive phenotype in ovarian cancer cells; providing additional support to the potential use of ET_AR antagonists in ovarian cancer treatment.

Materials and Methods

Cell Culture

Human ovarian carcinoma cell lines, HEY and OVCA 433, were cultured as previously described (13). 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 μmol/L; kindly provided by Abbott Laboratories, Abbott Park, IL), BQ 123 and BQ 788 (1 μmol/L; Peninsula Laboratories), were added 15 minutes before the agonist. Pretreatment with KP-392 (10 μmol/L, Quadra Logic Technologies QLT, Vancouver, British Columbia, Canada), SB203580 (5 μmol/L), PD 98059 (10 μmol/L), wortmannin (200 nmol/L), or LY294002 (25 μmol/L; Calbiochem-Novabiochem Corporation, San Diego, CA) was done for 30 to 60 minutes prior to the addition of ET-1. When indicated, cells were transfected overnight with 1 μg of ILK cDNA (kinase dead) in pUSEamp (E359K mutant) or with empty vector (Upstate, Lake Placid, NY).

Flow Cytometry Analysis

Subconfluent HEY cells were treated with ET-1 (100 nmol/L) for 24 hours and incubated with primary antibodies to α_1, α_2, α_3, α_4, α_6, β_1, and β_2 integrin subunits (Chemicon International, Temecula, CA) for 40 minutes at 4°C. FITC-conjugated secondary antibodies were applied to the cell for 30 minutes at 4°C. Labeled cells were scanned on a FACScan cytometer (Becton Dickinson, Bedford, MA).

Adhesion Assay

Serum-starved HEY and OVCA 433 cells were treated with ET-1 (100 nmol/L) and/or BQ 123 (1 μmol/L) or BQ 788 (1 μmol/L; Peninsula Laboratories) for 24 hours and labeled by incubation with chromium-S1-labeled sodium chromate (50 μCi for 10^6 cells; Du Pont, New England Nuclear Research Products, Wilmington, DE) for 1 hour at 37°C. Labeled cells (5×10^4) were plated in 96-well plates precoated with fibronectin (25 μg/mL; Becton Dickinson), laminin (50 μg/mL; Becton Dickinson), type I collagen (10 μg/mL; Becton Dickinson) for 120 minutes at 37°C. To determine the effect of integrin blocking on adhesion, cells were preincubated with anti-$\alpha_2\beta_1$ and anti-$\alpha_3\beta_1$ antibodies (Chemicon) for 1 hour at 37°C. The percentage of cell adhesion was calculated as follows: cpm adherent cells / cpm adherent + cpm nonadherent cells × 100. The assay was done in sextuplicate and results were expressed as the mean of three separate experiments.

Reverse Transcription-PCR

Reverse transcription-PCR was done using a Superscript One-Step Reverse Transcription-PCR System (Invitrogen) according to the manufacturer's instructions. Briefly, 1 μg of RNA was reverse-transcribed. The primer sets were as follows: ILK, 5′-CATCAATGCAGTGAATGAGC-3′ and

5′-GACATTCCTCATTGAAGTCC-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-TGAAGGTCGGTGT-CAACGGA-3′and 5′-GATGGCATGGACTGTGGTCAT-3′. Thirty-five cycles of amplification were done under the following conditions: melting at 95°C for 30 seconds; annealing at 54°C for 45 seconds; and extension at 72°C for 30 seconds. The PCR products were analyzed by electrophoresis on a 2% agarose gel and the relative intensity of signals was quantified using NIH image (Scion Corporation, Frederick, MD).

ILK Immune Complex Kinase Assay

Cell lysates (0.25-1.0 mg of protein) were immunoprecipitated with 1 μg of affinity-purified rabbit anti-ILK (Upstate) overnight at 4°C with rotation. Protein A-Sepharose (Sigma, St. Louis, MI), pre-swollen in NP40 lysis buffer [150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 50 mmol/L HEPES (pH 7.4), 1 μg of leupeptin/mL, 1 μg of aprotinin/mL, 3 mmol/L phenylmethylsulfonyl fluoride] was added for 2 hours at 4°C to capture the antibodies. After two washes with NP40 lysis buffer and two washes with kinase wash buffer [10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 50 mmol/L HEPES (pH 7.5), 0.1 mmol/L sodium orthovanadate, 1 mmol/L DTT], assays were done directly on the protein A beads in a 25 μL reaction volume containing 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 50 mmol/L HEPES (pH 7.5), 0.1 mmol/L sodium orthovanadate, 2 mmol/L sodium fluoride, 5 μCi of γ-^{32}P (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and 2.5 μg of myelin basic protein (MBP) as substrate (Upstate). Incubation was for 30 minutes at 30°C. The reaction was stopped with 10 μL of SDS-PAGE nonreducing stop buffer and heated for 5 minutes at 95°C. Phosphorylated MBP bands were visualized by autoradiography of dried SDS-10% PAGE gels, followed by quantitation in a PhosphorImager (Bio-Rad Laboratories, Richmond, CA).

Immunoblotting

Total cell lysates were subjected to SDS-PAGE and processed by immunoblotting using antibodies specific to phospho-GSK-3β (pSer[9]), GSK-3β, phospho-Akt (Ser[473]), Akt (Cell Signaling, Beverly, MA), ILK (Upstate). The proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and quantified them using NIH image (Scion).

Short Interfering RNA Treatment

HEY cells were transfected with 100 nmol/L short 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 manufacturer's protocol. Cells were harvested 48 hours later and ET_AR mRNA and protein levels were determined.

ELISA

Subconfluent HEY cells were serum-starved for 24 hours and incubated for the indicated times. The conditioned medium was then collected, centrifuged, and stored in aliquots at −20°C. Gelatinase activities in conditioned media were determined by a MMP Gelatinase Activity Assay Kit (Chemicon) according to the manufacturer's instructions. The sensitivity of the assay is <5 ng/mL MMP in a range of 10 to 200 ng/mL.

Gelatin Zymography

The ovarian tumor cell supernatants were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/mL gelatin as previously reported (13). Briefly, the cells were washed for 30 minutes at 22°C in 2.5% Triton X-100 and then incubated in 50 mmol/L Tris (pH 7.6), 1 mmol/L $ZnCl_2$, and 5 mmol/L $CaCl_2$ for 18 hours at 37°C. After incubation, the gels were stained with 0.2% Coomassie blue. Enzyme-digested regions were identified as white bands on a blue background.

Chemotaxis and Chemoinvasion Assay

Chemotaxis and chemoinvasion assays were done with a 48-well modified Boyden chamber (NeuroProbe, Pleasanton, CA) and 8 μmol/L pore size polyvinyl pyrrolidone-free polycarbonate Nucleopore filters (Costar, New York, NY) as previously reported (13). In chemoinvasion assay, the filters were coated with an even layer of 0.5 mg/mL Matrigel (Becton Dickinson). The lower compartment of the chamber was filled with ET-1 (100 nmol/L) and/or BQ 123 (1 μmol/L) or ABT-627 (1 μmol/L) or KP-392 (10 μmol/L) or LY294002 (25 μmol/L). Serum-starved HEY cells (5×10^5 cells/mL) were placed in the upper compartment (55 μL/well). BQ 123 and ABT-627 were previously added to the cells and preincubated for 15 minutes at 37°C. In the chemoinvasion assays done using ET_AR siRNA, HEY cells were transfected for 48 hours and then incubated in the upper compartment. After 4 hours (chemotaxis) or 6 hours (chemoinvasion) of incubation at 37°C, the filters were removed, stained with Diff-Quick (Merz-Dade, Dudingen, 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 (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 ABT627. Control mice were injected with drug vehicle. On day 40 after tumor injection, tumors were removed from control and treated mice, snap-frozen for immunohistochemical and immunoblot analysis.

Immunohistochemistry

Indirect immunoperoxidase stain of tumor xenografts was done on acetone-fixed cryostat sections (4 μmol/L) as previously described (33). ILK and pGSK-3β expression 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 or normal immunoglobulin served as negative control.

Statistical Analysis

Results are representative of at least three independent experiments each done in triplicate. All statistical analysis was assessed using a two-tailed Student's t test and by two-way ANOVA as appropriate.

Results

### ET-1 Induces the Expression of β_1 Integrin and ILK in Ovarian Cancer Cell Lines

Changes in expression of the integrin receptors mediate cell invasiveness through the ECM (34). To determine whether ET-1 is capable of modulating the integrin phenotype of ovarian cancer cells, flow cytometry analysis was done in HEY and OVCA 433 cancer cell lines previously characterized for ET-1 production and ET_AR expression (5, 7). Following ET-1 stimulation, the absolute levels of α_1, α_4, α_6, α_V and β_2 integrin subunits remained unaltered (data not shown), whereas the levels of α_2, α_3, and β_1 integrin subunits were significantly increased ($P < 0.001$; Fig. 1A). To evaluate the contribution of ET-1 to the adhesion of ovarian cancer cells on different ECM molecules, HEY and OVCA 433 cells were cultured with ET-1 for 24 hours and their adhesive profile was analyzed by determining cell adhesion on laminin, fibronectin, and type I collagen. As described in Fig. 1B, cell adherence to laminin and fibronectin remained unchanged, whereas ET1 up-regulated the attachment of both cell lines to type

I collagen. To confirm that $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are active in collagen binding, we assessed whether $\alpha_2\beta_1$ and $\alpha_3\beta_1$ inhibitory antibodies could block the cell attachment to type I collagen. Both antibodies strongly reduced cell adhesion induced by ET-1, demonstrating that a $\beta1$-dependent signaling is responsible for this effect (Fig. 1C). In order to identify the endothelin receptor subtype mediating this effect, we used BQ 123, a selective ET_AR antagonist, and BQ 788, a selective ET_BR antagonist, in the presence or in the absence of ET-1. Although BQ 123 was completely inhibitory, BQ 788 was ineffective, demonstrating that ETA is the receptor involved in the preferential change of cell adhesiveness to type I collagen promoted by ET-1 (Fig. 1C).

In view of the fact that ovarian cancer invasiveness is a β_1 integrin-driven event (35) which correlates with ILK overexpression (36), and that such expression is stimulated by ascitic fluid (32) known to contain high levels of ET-1 (2), we analyzed ILK expression following ET-1 treatment in both HEY and OVCA 433 cell lines. In both cell lines, ET-1 induced ILK mRNA and protein expression in a time- and concentration-dependent manner (Fig. 2A–C). The inhibitory effect produced by two different ET_AR antagonists, ABT-627, a nonpeptide ET_AR antagonist, and BQ 123, a peptide antagonist, as well as by RNA interference (siRNA) that target ET_AR (7) showed that ET_A is the relevant receptor (Fig. 2D) in inducing ILK expression. By using an immunocomplex kinase assay, we
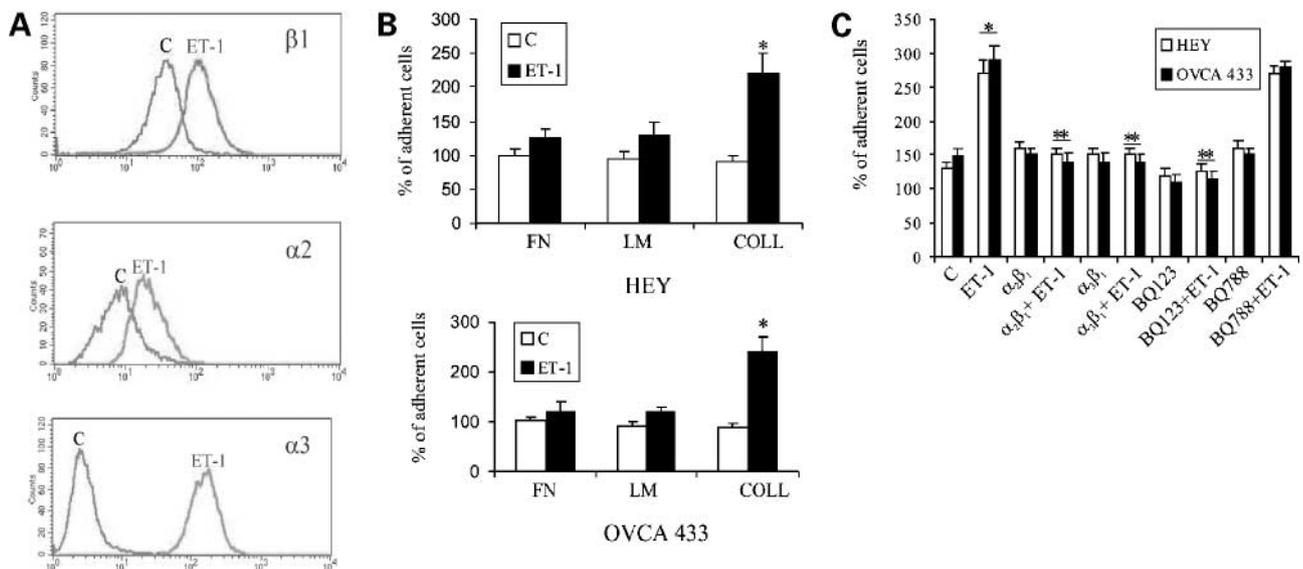
Figure 1. **A,** ET-1 up-regulates α_2, α_3, and β_1 integrin expression in ovarian cancer cells. Serum-starved HEY cells were treated with ET-1 (100 nmol/L) and analyzed by FACS analysis for the expression of α_2, α_3, and β_1 integrin subunits. **B,** ET-1 promotes adhesion of ovarian cancer cells on type I collagen. Serum-starved HEY and OVCA 433 cells were treated with ET-1 for 24 h and then plated in plates precoated with fibronectin (*FN*; 25 μg/mL), or laminin (*LM*; 50 μg/mL), or type I collagen (*COLL*; 10 μg/mL) and analyzed for cell adhesion. *Columns,* mean of three independent experiments each done in sextuplicate; *bars,* $\pm$SD; *, $P \leq 0.005$ compared with control. **C,** ET-1/ET_AR-dependent cell adhesion is mediated by $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins. Serum-starved HEY and OVCA 433 cells were treated with ET-1 (100 nmol/L) in the absence or in the presence of BQ 123 (1 μmol/L) or BQ 788 (1 μmol/L) for 24 h and analyzed for cell adhesion on type I collagen. As indicated, cells were preincubated with anti-$\alpha_2\beta_1$ and anti-$\alpha_3\beta_1$ integrin antibodies before the plating. *Columns,* mean of three independent experiments each done in sextuplicate; *bars,* $\pm$SD; *; $P \leq 0.0001$ compared with untreated cells (control, *C*); **, $P \leq 0.005$ compared with ET-1 alone.
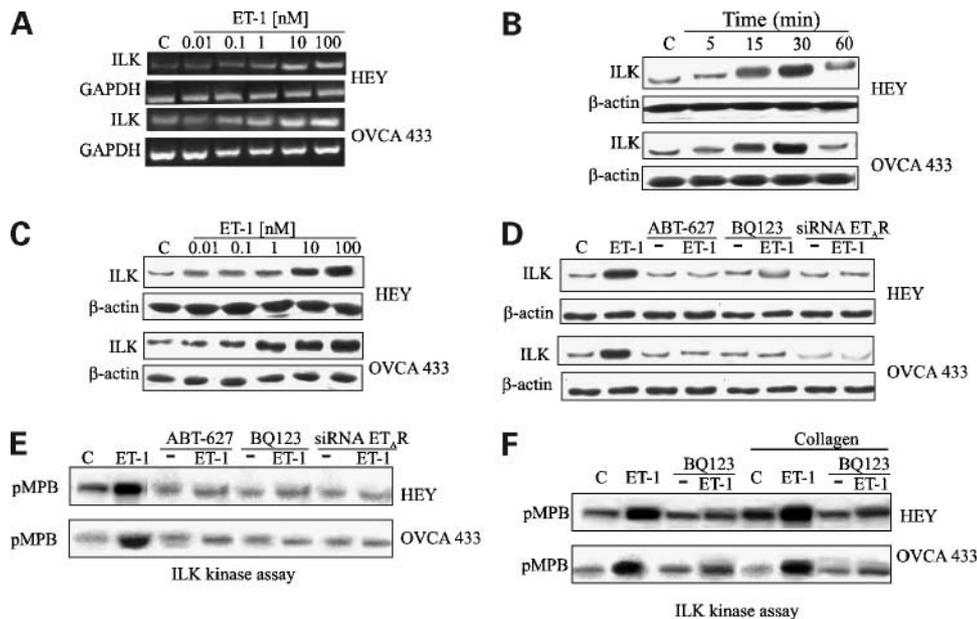
Figure 2. **A,** ET-1 enhances ILK mRNA transcript levels in a dose-dependent manner. Serum-starved HEY and OVCA 433 cells were treated with different concentrations of ET-1 for 30 min and subjected to reverse transcription-PCR analysis using ILK-specific primers. Primers for the amplification of the glyceraldehyde-3-phosphate dehydrogenase were used to normalize the loading of mRNA amount. **B,** ET-1 induces time-dependent ILK expression. Serum-starved HEY and OVCA 433 cells were incubated for different times with 100 nmol/L of ET-1. Cell lysates were immunoblotted with anti-ILK and for internal control with anti-β-actin. **C,** ET-1 induces dose-dependent ILK expression. Serum-starved HEY and OVCA 433 cells were incubated for 30 min with different concentrations of ET-1. Cell lysates were immunoblotted with anti-ILK and for internal control with anti-β-actin. **D,** ET-1 induces ILK expression through ET_AR. Serum-starved HEY and OVCA 433 cells were treated with ET_AR antagonists, ABT-627 or BQ 123, or transfected with ET_AR siRNA in the presence or absence of ET-1. Cell lysates were immunoblotted with anti-ILK and for internal control with anti-β-actin. **E,** ET-1 promotes ILK activity through ET_AR. Serum-starved HEY and OVCA 433 cells were treated with ET_AR antagonists, ABT-627 or BQ 123, or transfected with ET_AR siRNA in the presence or absence of ET-1 for 30 min. ILK activity was indicated by the amount of ^{32}P-labeling of MBP (*pMBP*) of the immunoprecipitates with ILK antibodies. **F,** ET-1 enhances ILK activity induced by type I collagen through ET_AR. Serum-starved HEY cells cultured on either regular tissue plates or type I collagen-coated plates were treated with ET-1 (100 nmol/L) in the absence or in the presence of BQ 123 (1 μmol/L) for 30 min. ILK activity was indicated by the amount of ^{32}P-labeling of MBP (*pMBP*) of the immunoprecipitates with ILK antibodies.

also documented that ILK kinase activity was up-regulated by ET-1, as shown by the MBP phosphorylation levels. This event is mediated by ET_AR, as shown by the inhibitory effect induced by ET_AR antagonists and ET_AR siRNA (Fig. 2E). Because there is no evidence that the interaction of β_1 integrin with type 1 collagen would result in ILK activation in ovarian cancer cells, we assessed whether this can occur differentially on cells seeded on plates uncoated or coated with type I collagen. As shown in Fig. 2F, ILK activity increased as ovarian cancer cells adhered to type I collagen. Similar to the interaction of β_1 integrin with this matrix, ET-1 induced ILK activity. Enforced activation of β_1 integrin by ET-1 promoted a more pronounced ILK activity in collagen-coated plates to an ever greater extent than that induced by cell adhesion to collagen (Fig. 2F), indicating that in ovarian cancer cells, ILK activity is under the control of ET-1, which can regulate ILK either directly or indirectly through β_1 integrin signaling, which in turn, can be enhanced by ET-1. Interestingly, the addition of BQ 123 blocked the basal and ET-1-induced ILK activity of adherent cells on collagen, indicating that ET-1 regulates β_1 signaling in the stimulation of ILK activity via ET_AR binding.

Activation of ET_AR by ET-1 Induces ILK-Dependent GSK-3β Phosphorylation and Akt

Because previous studies have shown that substrates of ILK include Akt and GKS-3β (16), we investigated whether ET-1-induced ILK activity mediated the signaling of these two downstream kinases. By immunoblotting with phosphospecific antibodies, we observed that in HEY cells, ET-1 induced a dose-dependent inhibition of GSK-3β, through phosphorylation at Ser[9], and activated Akt, through phosphorylation at Ser[473] (Fig. 3A). Moreover, the ET-1-induced ILK activity, as well as GSK-3β and Akt phosphorylation, were time-dependent. The detection of phosphorylated MBP bands was observed after 10 minutes and reached the maximum after 30 minutes (Fig. 3B). A similar kinetic of phosphorylation was observed for ET-1-induced GSK-3β and Akt phosphorylation. Both ET_AR antagonists and transfection with ET_AR siRNA blocked these effects, indicating ET_AR as the receptor involved in these signaling pathways (Fig. 3C). To determine whether ILK is critical for the regulation of ET-1-induced GSK-3β and Akt phosphorylation, 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 (16).
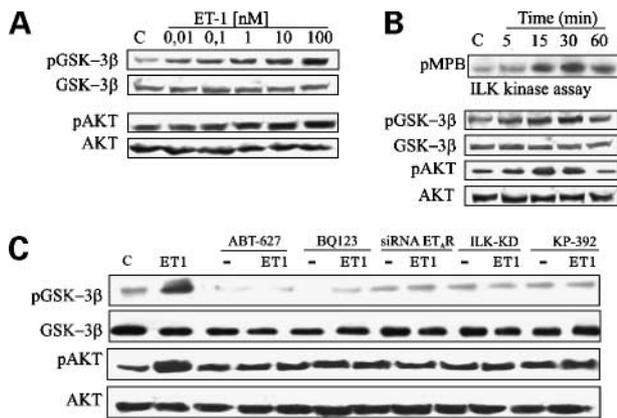
Figure 3. ET-1 induces dose-dependent (**A**) and time-dependent (**B**) phosphorylation of GSK-3β and Akt. Serum-starved HEY cells were stimulated with ET-1 with different doses (**A**) and time periods (**B**). Cell lysates were immunoblotted for phosphorylated GSK-3β (Ser[9]; *pGSK-3β*) and total GSK-3β and for phosphorylated Akt (Ser[473]; *pAkt*) and total Akt. ILK activity was indicated by the amount of ^{32}P-labeling of MBP (*pMBP*) of the immunoprecipitates with ILK antibodies. Phosphorylation of GSK-3β and Akt by ET-1/ET$_A$R is mediated by ILK (**C**). Serum-starved HEY cells were treated with ET$_A$R antagonists, ABT-627 or BQ 123, or ILK inhibitor, KP-392, or transfected with ET$_A$R siRNA or ILK-KD in the presence or absence of ET-1 (100 nmol/L) for 30 min. Cell lysates were immunoblotted for phosphorylated and total GSK-3β and for phosphorylated and total Akt.

As shown in Fig. 3C, the phosphorylation of GSK-3β and Akt is inhibited upon the knockdown of ILK activity, whereas the level of GSK-3β and Akt remained unchanged. These results collectively indicate that ET-1 through ET$_A$R promotes ILK-dependent phosphorylation of its downstream targets, GSK-3β and Akt, in ovarian cancer cells.

### ET-1-Induced ILK Activity and GSK-3β Involves PI3K Signaling

In ovarian cancer cells, ET-1 is known to activate the p42/44 and p38 mitogen-activated protein kinase pathway as well as the PI3K/AKT pathway through the ET$_A$R (4, 8, 11, 12). We therefore investigated the signaling pathways involved in ET-1-mediated ILK expression and activity in HEY cells by using specific inhibitors. The selective inhibitors of mitogen-activated protein kinase/extracellular signal-regulated kinase (PD98059) and p38 mitogen-activated protein kinase (SB203580) did not significantly inhibit ET-1-stimulated ILK expression (Fig. 4). Because the primary ILK sequence indicated a structural basis for PI3K-dependent regulation, we investigated whether an ET-1-activated PI3K pathway was involved in the regulation of ILK. HEY cells were treated with two different PI3K inhibitors, LY294002, and wortmannin, and ILK activity was examined. As shown in Fig. 4B, both the specific PI3K inhibitors suppressed the ET-1-induced ILK kinase activity in ovarian cancer cells. As expected, GSK-3β and Akt phosphorylations were also blocked by LY294002 and wortmannin, indicating that ET-1-induced ILK activity, as well as GSK-3β and Akt phosphorylation, are mediated through a PI3K-dependent signaling.

ET$_A$R-Driven ILK Signaling Is Required for Invasive Ovarian Cancer Cell Behavior

Several studies have implicated either ET-1 or ILK in the expression and activity of different MMPs, such as MMP-2 and MMP-9 (13, 16, 27). To establish whether ILK activity is necessary for ET-1-induced MMP activity, HEY cells were treated for 24 hours with ET-1 in the presence or absence of KP-392, or LY294002, or after transfection with a kinase-deficient, dominant-negative form of ILK, and changes in MMP processing were evaluated by gelatin zymography. As shown in Fig. 5A and B, in both cell lines, ET-1-induced gelatinase activation was reduced after inhibition of ILK activity, indicating ILK as a mediator of ET-1-induced MMP activation. ET-1 has been previously reported to potentiate the motility and invasiveness of ovarian cancer cells (7, 10, 13). Thus, we analyzed whether the ET-1-induced PI3K-dependent ILK activation could affect the invasive behavior of ovarian cancer cells. Treatment with the ET$_A$R antagonist, ABT-627, resulted in a significant decrease in ET-1-induced cell migration and invasion in HEY cells, confirming the involvement of ET$_A$R in this effect (Fig. 5C and D). Moreover, pretreatment of ovarian cancer cells with small-molecule inhibitor of ILK activity, KP-392, or with PI3K inhibitor, LY294002, or transfection with a dominant-negative form of ILK, resulted in a reduction of the ET-1 capacity to induce cell migration (Fig. 5C) or invasion (Fig. 5D), as measured by chemotaxis and chemoinvasion assay, placing PI3K-dependent ILK activity at a crossroads of ET-1/ET$_A$R signaling to activate an invasive program in ovarian cancer cells.
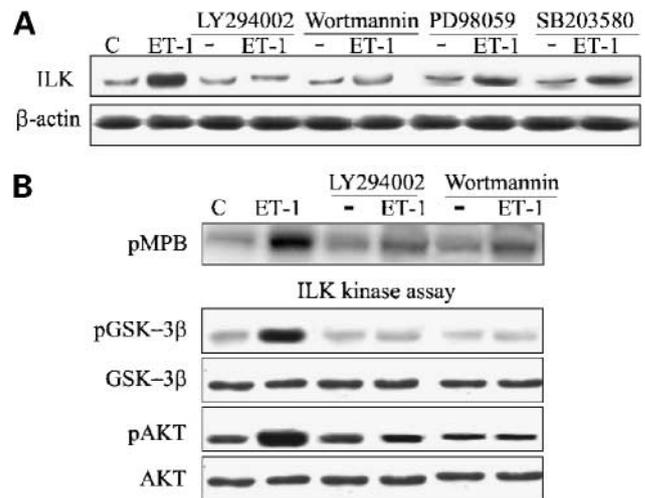
Figure 4. ET-1-induced ILK expression, activity, and downstream phosphorylation of GSK-3β and Akt requires PI3K signaling. **A,** serum-starved HEY cells were stimulated with ET-1 (100 nmol/L) in the absence or in the presence of LY294002 (25 μmol/L), wortmannin (200 nmol/L), PD98059 (10 μmol/L), or SB203580 (5 μmol/L). Cell lysates were immunoblotted with anti-ILK and for internal control with anti-β-actin. **B,** HEY cells treated with ET-1 (100 nmol/L) for 30 min in the absence or presence of LY294002 (25 μmol/L) or wortmannin (200 nmol/L). ILK activity was indicated by the amount of ^{32}P-labeling of MBP (*pMBP*) of the immunoprecipitates with ILK antibodies. Cell lysates were immunoblotted for phosphorylated and total GSK-3β and for phosphorylated and total Akt.
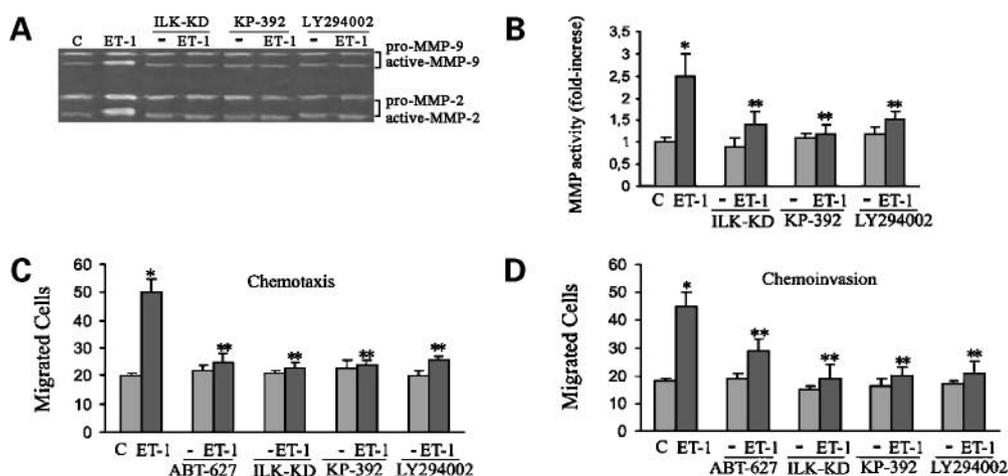
Figure 5. Effect of ET-1-induced ILK signaling on MMP secretion and activation in ovarian carcinoma cell lines. Enzymatic activity of MMP-2 and MMP-9 was studied by SDS-PAGE gelatin zymography in conditioned media from ovarian cancer cells (**A**) or MMP gelatinase activity assay kit (**B**). Serum-starved cells transfected with ILK-KD or treated with KP-392 (10 μmol/L) or LY294002 (25 μmol/L). MMP gelatinase activities were measured in conditioned media of HEY cells treated with ET-1 (100 nmol/L) for 24 h. *Columns,* mean of results from three experiments each done in duplicate; *bars,* ±SD; *, $P < 0.005$ compared with control; **, $P < 0.001$ compared with ET-1. ET-1-induced cell migration (**C**) and invasion (**D**) involves the ILK-dependent PI3K pathway. Serum-starved HEY cells (5×10^5 cells/mL) untreated or transfected with ILK-KD or treated with ABT-627 (1 μmol/L), KP-392 (10 nmol/L), or LY294002 (25 μmol/L) were seeded on a 48-well Boyden chamber for 4 and 6 h for migration and invasion assays, respectively. ET-1 and/or inhibitors were added in the lower wells. *Columns,* mean of three independent experiments each done in triplicate; *bars,* ±SD; *, $P \leq 0.001$ compared with control; **, $P \leq 0.01$ compared with ET-1.

ET$_A$R Antagonist – Induced Inhibition of Tumor Growth *In vivo* Is Associated with the Suppression of ILK Expression

We next determined whether ET$_A$R blockade *in vivo* resulted in the inhibition of ET-1-induced ILK expression associated with tumor growth inhibition. We treated mice carrying HEY tumors with the potent ET$_A$R antagonist, ABT-627, an orally bioavailable ET$_A$R antagonist that potently (K_i = 34 pmol/L) and selectively binds to the ET$_A$R, blocking signaling pathways relevant in cancer cell proliferation and tumor angiogenesis (33). We showed that a significant and long-lasting tumor growth inhibition occurs in ABT-627-treated mice over a 21-day dosing regime (Fig. 6A), which was well tolerated, as judged by the absence of weight loss or other signs of acute or delayed toxicity. Western blot analysis of ILK, pAkt, and pGSK-3β expressions done on HEY tumor xenografts at day 40 after the tumor cell injection revealed a marked reduction of ILK, pAkt, and pGSK-3β expression in ABT-627-treated animals compared with control animals (Fig. 6B). Immunohistochemical evaluation done on the same tumors confirmed these results (Fig. 6C). These data indicate that ET-1 is a critical activator of ILK signaling and that blockade of ET$_A$R inhibits tumor growth and progression by also affecting ET-1-induced ILK activity.

Discussion

Extensive experimental evidences have documented that in a variety of neoplastic cells, the acquirement of the transformed phenotype requires integrin-mediated attachment to an underlying ECM substrate (37). Recently, ILK has been identified as an important effector of integrin and growth factor signaling to regulate cancer cell adhesion, migration, and invasion. The development of selective inhibitors of ILK therefore clearly adds a novel potential pharmacologic target in cancer treatment. In view of this, studies aimed at identifying signaling pathways involved in ILK induction are of major relevance from a biological as well as a clinical point of view. Different growth factors that bind tyrosine kinase have been shown to induce ILK expression (16). Among the network of signals operating in tumors, in this study, we provide ample evidence that in human ovarian carcinoma cells, the activation of ILK contributes to cancer invasion and progression as a crucial mediator of ET-1/ET$_A$R pathway. We first showed that ET-1 enhances the adhesion of ovarian cancer cells on the collagen via up-regulation of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins. Secondly, we show that ET-1 is capable of inducing ILK activation and overexpression at mRNA and protein levels in ovarian carcinoma cells. Interaction of β_1 integrin with type I collagen increases ILK activity, and ET-1 may mimic this signal and synergize with β_1 integrin to activate ILK. Furthermore, transfection with dominant-negative ILK and ILK small-molecule inhibitor have revealed the critical role of ILK in the stimulation of phosphorylation of GSK-3β and Akt, the major downstream components of ET$_A$R-mediated ILK signaling pathways. In addition, we observed that ILK activation is implicated in ET-1-enhanced migratory and invasive ability of ovarian cancer cells, which correlates with the increased secretion and activation of tumor-associated MMP-2 and MMP-9. Finally, interruption of ET-1/ET$_A$R signaling by ET$_A$R antagonists or by RNA interference

inhibited these effects and treatment of ovarian carcinoma xenografts with a selective ET_AR antagonist caused the *in vivo* tumor growth inhibition associated with a reduced expression of ILK and downstream Akt and GSK-3β phosphorylated expression in mice bearing established HEY tumors.

Several studies have shown the preferential adhesion of ovarian carcinoma cells to type I collagen, which represents the unique protein composition of the mesothelial ECM (38–40). Moreover, it has been shown that adhesion of ovarian carcinoma cells is a $β_1$ integrin-mediated event (35). According to these data, we showed that ET-1 up-regulates $α_2β1$ and $α_3β_1$ integrin expression and promotes the preferential adhesion of ovarian cancer cells to type I collagen via the $β_1$ integrin.

These findings clearly identify ET-1 as a critical upstream mediator of ILK activation through the capacity to up-regulate its expression and activity at different levels. Recent studies on integrin outside-in signaling indicate that upon ligation of $β_1$ integrin by ECM, ILK is recruited into the $β_1$ integrin-associated focal adhesion complex, thereby activating Akt (16). Because the activity of ILK induced by ET-1 is more effective during ovarian cancer cell spread and adhesion on type I collagen, it is likely that ET-1-mediated stimulation of ILK potentiates β1-integrin, signaling amplifying ILK activity. The $β_1$ integrin-mediated activation of

Figure 6. Blockade of ET_AR by ABT-627 inhibits ILK signaling and tumor growth *in vivo*. **A,** antitumor activity of ABT-627 treatment on HEY human ovarian carcinoma xenografts. Mice were given injections of $1.5 × 10^6$ HEY cells s.c. in the dorsal flank. After 7 d, groups of 10 mice were treated i.p. for 21 d with drug vehicle or with ABT-627 (2 mg/kg/d). *Points,* mean of three different experiments for a total of 20 mice in each experiment; *bars,* ±SD. ABT-627 caused a significant inhibition of HEY tumor growth in treated animals over a 21-d dosing regime. 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 ILK, pGSK-3β, and pAkt expression in HEY tumor xenografts. **C,** comparative immunohistochemical analysis of ILK expression. ABT-627 causes a significant inhibition of ILK expression in HEY tumor xenografts compared with tumors of control animals (original magnification, ×250).

ILK by ET-1 therefore points to a complex mechanism through which integrins and growth factors could synergize to expand the cellular communication signaling network leading to metastatic dissemination of ovarian carcinoma cells.
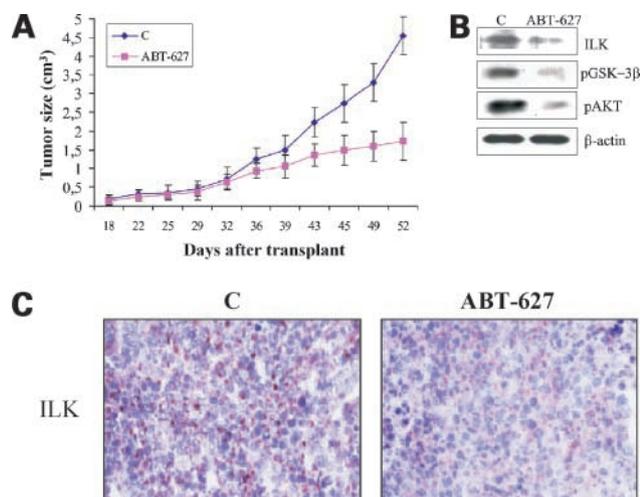
Moreover, by further dissecting the signaling pathways mediating ILK activity by ET-1, through the use of different pharmacologic inhibitors, we showed that the PI3K inhibitor blocked ET-1-induced ILK expression and activity, indicating that ET-1-mediated invasive effects are likely to be dependent on the ILK/Akt/GSK-3β pathway in a PI3K-dependent manner.

Akt is frequently activated in human epithelial cancer (41). Interestingly, in ovarian carcinomas, Akt activation has been linked to a loss of differentiation and an aggressive clinical behavior (42). These findings are consistent with the ability of Akt to directly affect epithelial cell morphology, tumorigenicity, cell motility, invasiveness, and EMT (43). EMT is an important biological process during malignant transformation and tumor progression which endows cancer cells to acquire mesenchymal and invasive properties (44).

In ovarian cancer cells, we recently showed that autocrine ET-1/ET_AR pathway mimics Wnt signaling to drive EMT. In particular, we have shown that ET-1 induces Snail and β-catenin stabilization and transcriptional activation that regulate EMT molecular determinants through an ILK-dependent mechanism (7). ILK has an essential role in EMT by connecting the cell-adhesion molecules, integrins and growth factors to the actin cytoskeleton, and to a wide range of signaling pathways. Overexpression of ILK induces down-regulation of E-cadherin and activation of β-catenin and Snail transcriptional activity (45). According to these data, we can postulate that ET-1 directly or through $β_1$ integrin drives a PI3K-mediated ILK signaling that is necessary for GSK-3β and Akt phosphorylation and the associated increase in MMP activity, motility, and invasion, identifying ILK and its downstream substrate GSK-3β and AKT as checkpoints of finely tuned interconnected signals induced by ET-1/ET_AR to modulate invasive EMT process.

In highly invasive human glioblastoma cells, increased ILK expression has been shown to stimulate the expression and activity of MMP-9, whereas inhibition of ILK activity resulted in the inhibition of invasion by blocking Akt signaling (27). Similarly, in this study, we showed that targeting ILK in ovarian carcinoma cells inhibited the ET-1-induced MMP-2/MMP-9 activation, cell migration, and invasion through the down-regulation of GSK-3β and Akt signaling.

Gaining a better understanding of the complexities of tumor context can improve the development of more effective antitumor treatments (46). In this regard, the ET-1 axis seems of clinical relevance because it regulates the interactions between tumor cells and the surrounding microenvironment by modulating changes in cell surface adhesion and communication molecules, integrins, and tumor proteinases. Because all these molecular events are triggered by ET_AR activation blockade of this receptor by the specific antagonist ABT-627 results in the inhibition of tumor growth in murine xenografts of HEY ovarian

carcinoma and in the suppression of ILK and pGSK-3β expression associated with a significant reduction of microvessel density, vascular endothelial growth factor, cyclooxygenase-2, MMP-2, N-cadherin, and Snail expression, and increased tumor apoptosis, connexin 43–based gap junctional intercellular plaques as well as E-cadherin and β-catenin expression. Altogether, these findings indicate that ET-1 coordinates these orchestrated events involved in the disruption of normal host-tumor interactions (10, 14, 33). It should be underlined that these data are now supported by a genome-wide expression profile of advanced stage serous ovarian cancer. From microarray results and bioinformatic analyses, ET-1 has been identified as a key gene that activates signaling pathways leading to ovarian cancer cell migration, spread, and invasion (47). In conclusion, the present study delineates the mapping of ET_AR-triggered molecular events resulting in the activation of other signaling molecules, such as ILK and its downstream targets, to expand the cellular communication network responsible for the invasive phenotype. Thus, targeting ILK and related signaling cascade via ET_AR blockade may expand our potential in the treatment of ovarian carcinoma.

Acknowledgments

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Integrin-linked kinase functions as a downstream mediator of endothelin-1 to promote invasive behavior in ovarian carcinoma

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