Regulation of Endogenous Gene Expression in Human Non–Small Cell Lung Cancer Cells by Estrogen Receptor Ligands

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Abstract

Estrogen receptor (ER) agonists and antagonists elicit distinct responses in non–small cell lung cancer (NSCLC) cells. To determine how such responses are generated, the expression of ERα, ERβ, and ER coregulators in human lung fibroblasts and human NSCLC cell lines was evaluated by immunoblot. Ligand-dependent estrogenic responses in NSCLC cells are probably generated via ERβ and the p160 coactivator GRIP1/TIF2, because expression of these proteins was detected, but not full-length ERα or the p160 coactivator SRC-1. ERβ and GRIP1/TIF2 are shown to interact *in vitro* in a ligand-dependent manner and thus may form functional transcription complexes in NSCLC cells. Furthermore, the capacity of ER ligands to regulate gene expression in NSCLC cells was explored using gene miniarrays. Expression profiles were examined after treatment with ER agonist 17-β-estradiol (E2), the pure ER antagonist ICI 182,780 (fulvestrant, Faslodex), or epidermal growth factor, which served as a positive control for an alternative growth stimulus. E-cadherin and inhibitor of differentiation 2 were differentially regulated by E2 versus ICI 182,780 in 201T and 273T NSCLC cell lines. Epidermal growth factor also stimulated proliferation of these cells but had no effect on expression of E-cadherin and inhibitor of differentiation 2, suggesting they are specific targets of ER signaling. These data show that NSCLC cells respond to estrogens/antiestrogens by altering endogenous gene expression and support a model in which ICI 182,780 reduces proliferation of NSCLC cells via its ability to disrupt ER signaling. ICI 182,780 may therefore have therapeutic benefit in NSCLC. (Cancer Res 2005; 65(4): 1598-605)

Introduction

The cellular response to estrogen is mediated by estrogen receptor α (ERα) and ERβ, which are encoded by distinct genes and show a differential tissue distribution (1, 2). Normal breast tissue shows expression of both ERα and ERβ, whereas in lung, ERβ seems to be the dominant form. These receptor proteins function as ligand-dependent transcription factors and regulate the expression of genes implicated in cell cycle control, signal transduction, and cell survival (3). There are multiple steps leading from estrogen exposure to a functional ER-dependent transcription response. These steps include binding of ligand to induce a receptor conformational change, release from an inhibitory complex with HSP90, and subsequent ER dimerization to bind estrogen response elements at estrogen-regulated genes (4). When bound to an agonist such as 17-β-estradiol (E2), the ER recruits p160 coactivator proteins, including SRC-1 (5, 6), GRIP1/TIF2 (7, 8), and AIB1 (9) as well as the coactivators p300 (10) and CBP (11). Each of these possesses histone acetyltransferase activity that opens chromatin and increases transcription. This sequence for ER results in formation of a complex that regulates gene expression. In addition to acting through estrogen response elements, ERs also regulate transcription from alternative DNA sites, including those of transcription factors SP1 and AP-1 (12). Consequently, the expression of genes lacking a classic estrogen response element may also be regulated in an estrogen-dependent manner. Differential cellular expression of ERα, ERβ, and ER coregulators underlies tissue specific responses to ER ligands. For example, the ability of tamoxifen to function as an ER agonist in uterus but an antagonist in breast results from differential tissue expression of SRC-1 (13).

Recent studies provide compelling evidence that estrogen-signaling pathways play an important role in normal lung biology and in controlling the growth of lung cancer (14). Transgenic mice harboring an estrogen-regulated luciferase reporter construct display a 5-fold increase in luciferase activity in the lung upon estradiol treatment, indicating that the lung is a hormone-responsive tissue (15). Furthermore, targeted inactivation of ERβ results in lung abnormalities in female mice, including a decrease in the number of alveoli and altered surfactant homeostasis (16). With regard to lung cancer, estradiol has been reported to stimulate proliferation of some but not all cell lines, whereas the pure antiestrogen ICI 182,780 consistently inhibits growth *in vitro* and *in vivo* (14, 17, 18). Estrogens may also affect lung cancer growth clinically as full estrogen blockade, achieved through inhibition of estrogen biosynthesis with the aromatase inhibitor, exemestane, reduces the occurrence of primary lung cancers in women with breast cancer as compared with tamoxifen, an agent that displays ER partial agonist activity in certain tissue and cellular contexts (19).

Whether ERα and/or ERβ mediate the ligand-dependent transcriptional response of NSCLC cells to estrogen remains controversial. In the cell lines we have examined to date, ERα transcripts are variably detected by reverse transcription-PCR, little to no full-length ERα protein is detected by immunoblot using whole cell extracts, and immunohistochemistry reveals an aberrant cytoplasmic localization that is inconsistent with its role as a nuclear transcription factor (14). In contrast, ERβ transcripts are detected in the majority of NSCLC cases examined, full-length protein is detected by immunoblot, and immunohistochemistry reveals a predominantly nuclear localization (14, 17, 18, 20). More specifically, ERβ immunostaining was found in 17 of 20 human adenocarcinomas, whereas it was found in only 3 of 10 squamous

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cell carcinomas; ERα was not detected in any of these 30 tumors (18). With regard to the p160 coactivators, AIB-1 is not detected in human lung tissue or the human lung cancer cell line A549 (21), suggesting that alternative p160 cofactors associate with the ER to regulate gene expression in response to estrogen exposure in these cells.

Prior studies employing exogenous estrogen-regulated reporter constructs suggest that NSCLC cells possess the components necessary to generate ER-mediated transcription responses (14). However, no data address whether ER ligands have the capacity to regulate endogenous gene expression in these cells. The studies presented here were conducted to address this issue and to identify the proteins that mediate transcriptional responses to estrogen in lung cancer cells.

Materials and Methods

Cells. The MCF-7 breast cancer cell line and the A549 bronchioloalveolar lung cancer cell line were purchased from American Type Culture Collection (Manassas, VA). The human NSCLC cell lines 201T (adenocarcinoma), 273T (squamous cell carcinoma), and 128-88T (squamous cell carcinoma) were derived from pulmonary tumors (22). The 201T, 273T, and 128-88T cell lines were maintained in basal medium Eagles (Atlanta Biologicals, Norcross, GA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and 100 units/mL penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA). A549 cells were maintained in the same media except that the serum was reduced to a final concentration of 1%. MCF-7 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/mL penicillin-streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents and Antibodies. 17-β-Estradiol (E2) and genistein were purchased from Sigma (St. Louis, MO). ICI 182,780 and propyl pyrazole triol were purchased from Tocris (Ellisville, MO). Genistein was prepared as a 0.1 mol/L stock in DMSO. The remaining ER ligands were prepared as 1×10^{-2} mol/L stocks in 100% ethanol and stored at −20°C. Before use in an experiment, they were further diluted in ethanol. Epidermal growth factor (EGF) was the kind gift of Dr. Jennifer Grandis (Department of Otolaryngology, University of Pittsburgh), and was prepared as a 1.0 μg/mL stock in sterile, distilled water. The rabbit polyclonal antibodies used in these studies anti-ERα [sc-544 (hinge), sc-543 (COOH terminus), sc-7207 (NH_2 terminus)], anti-ERβ (sc-8974), anti-GRIP1 (sc-8996), anti-SRC-1 (sc-8995), and anti-inhibitor of differentiation 2 (Id-2; sc-489) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal anti-E-cadherin antibody was from BD Transduction Laboratories (San Diego, CA). Monoclonal anti-tubulin antibodies were kindly provided by Dr. Richard Steinman (Department of Medicine, University of Pittsburgh). Horseradish peroxidase–conjugated secondary antibodies (sheep anti-mouse immunoglobulin) and (donkey anti-rabbit immunoglobulin) were purchased from Amersham Biosciences (Piscataway, NJ).

Preparation of Whole Cell Extracts. Cells were harvested by use of a cell scraper and collected by centrifugation at 4°C (6 minutes at 200 × *g*). The resulting cell pellets were washed in ice-cold PBS and then lysed in Triton X-100/SDS buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-Cl (pH 8.0), 420 mmol/L NaCl] supplemented with protease and phosphatase inhibitors. Whole cell extracts were clarified by centrifugation at 4°C (10 minutes at 16,000 × *g*), and the proteins quantitated using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Immunoblot Analysis. Proteins were resolved on 7.5% (GRIP1/TIF2, SRC-1, and E-cadherin), 10% (ERα and ERβ), or 14% (Id-2) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 hour in a solution of 5% nonfat milk/TTBS [0.05% Tween 20, 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4)]. Blots were incubated for 1 hour with primary antibodies diluted in blocking solution and then washed four times (10 minutes per wash) in TTBS. Washed blots were subsequently incubated for 1 hour with horseradish peroxidase–conjugated secondary antibodies diluted in blocking solution. All washing and incubation steps were conducted at room temperature. Blots were washed in TTBS as described above and then immunoreactive complexes detected using Western Lightning Chemiluminescence Reagent (Perkin-Elmer Life Science, Boston, MA). Id-2 complexes were detected using SuperSignal Reagent (Pierce, Rockford, IL).

Radiolabeled ER. ^{35}S-methionine-labeled wild-type human ERβ was prepared using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's protocol. The DNA template used in the *in vitro* transcription/translation reaction, plasmid T7 ER HBD, encodes amino acids 180 to 530 of human ERβ. Translation products were routinely analyzed by gel electrophoresis to verify their integrity.

Preparation of Glutathione *S*-Transferase-GRIP1. Glutathione *S*-transferase (GST)-GRIP1 synthesis was induced in XL-1-Blue cells transformed with the plasmid pGEX-GRIP 563-1121 via the addition of isopropyl-L-thio-B-D-galactopyranoside to a final concentration of 0.1 mmol/L. After 2 hours, the cells were collected and resuspended in 5.0 mL ice-cold NETN buffer [100 mmol/L NaCl, 20 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA, 0.5% NP40, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail]. The cell suspension was sonicated until clear, and then Triton X-100 was added to a final concentration of 1%. The lysate was mixed for 30 minutes at 4°C and then clarified by centrifugation at 12,000 × *g* for 10 minutes at 4°C. The cell extracts were stored in 5.0-mL aliquots at −80°C until use. To prepare beads for the binding assay, glutathione-sepharose beads (Amersham Biosciences) were washed and equilibrated in NETN buffer; 250 μL of the washed beads were mixed with 5.0 mL of GST-GRIP cell extract for 1.5 hours at 4°C. The beads were collected by centrifugation (3 minutes at 1,000 rpm at 4°C) and washed thrice with NETN. The washed beads with bound GST-GRIP were resuspended in a final volume of 5.0 mL binding buffer [50 mmol/L NaCl, 50 mmol/L Tris-Cl (pH 8.0), 0.02% Tween 20, 100 μg/mL bovine serum albumin, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail] and used immediately.

GST Binding Assay. For each reaction, vehicle control or the appropriate ligand was diluted in 500 μL binding buffer. In a separate tube, 2 μL of radiolabeled ERβ was mixed with 500 μL of GST-GRIP1 beads. To measure the effect of each ligand on ERβ binding to GRIP1, the contents of each tube were mixed and the reactions incubated for 3.5 to 4.0 hours at 4°C on a rocking platform. The beads were collected by centrifugation and washed in 1.0 mL wash buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.05% Tween20]. The wash was completely removed and the beads resuspended in 100 μL fresh buffer. The suspension was transferred to a vial containing 3.0 mL scintillation fluid and the bound radioactivity measured using a scintillation counter. For each assay, each reaction was done in duplicate. In some experiments, the washed beads were boiled in Laemmli buffer, and the released radiolabeled ERβ protein was analyzed by SDS-PAGE (e.g., Fig. 1*C*).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxy-Methoxyphenyl)-2-(4-Sulfophenyl)-2*H*-Tetrazolium Inner Salt (MTS) Assays. 201T cells were seeded into 96-well plates at a density of 4×10^3 cells per well and allowed to attach overnight. The next day, the cells were washed with PBS and 100 μL of phenol-red free tissue culture medium without serum was added. After 72 hours, the cells were treated by adding an additional 100 μL of medium containing test ligands. Effects on cell growth were determined 72 hours later using the CellTiter Aqueous One assay system (Promega). Absorbance was read at 490 nm using an ELISA plate reader (model EL-340, Bio-Tek Instruments, Winooski, VT).

Treatment of Cells for Gene Array Studies. MCF-7 cells (8×10^5 cells per T25 flask) or human NSCLC cell lines 201T and 273T (4.5×10^5 cells per T25 flask) were seeded in phenol-red free RPMI medium (Mediatech Cellgro, Herndon, VA) containing 10% charcoal-dextran stripped fetal bovine serum (Hyclone) and allowed to attach overnight. The following day, the medium was removed and the cells treated with fresh medium containing

ethanol vehicle control (0.3%, final concentration) or test ligand. The final concentration of each ligand was E2 (30 nmol/L), ICI 182,780 (100 nmol/L), and EGF (30 ng/mL). Twenty-four hours post-treatment, the cells were harvested and RNA extracted using TRIzol (Invitrogen Life Technologies). RNA was dissolved in sterile, diethylpyrocarbonate-treated water and quantitated spectrophotometrically. The integrity of each RNA preparation was confirmed by agarose gel electrophoresis.

Gene Arrays. For these studies, the GEA Q Series Human p53 Signaling Pathway Gene Array and the GEA Q Series Human Breast Cancer and Estrogen Signaling Pathway Gene Array from SuperArray (Bethesda, MD) were used. Each array profiles the expression of a panel of 96 genes. For each array, 4 μg RNA was reverse transcribed into cDNA in the presence of gene-specific oligonucleotide primers and ^{33}P-dCTP (30 μCi per reaction) as described in the manufacturer's protocol. Arrays were prehybridized individually in 2.0 mL of CG hybridization buffer [250 mmol/L sodium phosphate (pH 7.2), 7% SDS, 10 μg/mL bovine serum albumin, 1 mmol/L EGTA], for 1 to 2 hours at 60°C. The ^{33}P-dCTP-labeled cDNA probes were diluted with 0.75 mL hybridization solution, added to the arrays, and hybridized overnight at 60°C. The following day, unbound probe was removed and the arrays washed in CG wash solution [20 mmol/L sodium phosphate (pH 7.2), 1% SDS, and 1 mmol/L EDTA]. Washes were done at 60°C (four washes, 15 minutes each). The arrays were briefly air-dried, wrapped in Saran, and exposed to a Molecular Dynamics low energy phosphor screen.

Data Analysis. To determine whether E2, ICI, and EGF stimulate the proliferation of 201T human lung cancer cells, the MTS assay was used. The relative growth was calculated by dividing the absorbance obtained at 490 nm for treated cells by that obtained for cells treated with vehicle only. ANOVA was then done to estimate the effect of each dose level on relative growth. For analysis of the arrays, the radioactivity bound to each spot on the array was quantitated using the Molecular Dynamics Phosphorimager and ImageQuant software. The resulting raw data were imported into an Excel spreadsheet. To identify treatment-induced changes in gene expression, hybridization intensity ratios were calculated by dividing the signal for a given gene on one array by its signal on a second array. The intensity ratios for all genes were then log-transformed and averaged to derive the mean hybridization intensity for the arrays. A Normal distribution was then fit to the data. Genes whose expression was above the 95th percentile or below the 5th percentile of the distribution were considered differentially regulated by treatment. Visual inspection of the arrays was used to confirm differential regulation.

Results

Some lung cancer cells grow in response to estrogen exposure, whereas antiestrogens consistently inhibit growth (14, 17, 18). To identify proteins that may mediate these biological responses, the expression of ERα, ERβ, and the p160 coactivators GRIP1/TIF2 and SRC-1 was evaluated by immunoblot (Fig. 1*A-B*), using a panel of human normal lung fibroblasts (NLFB; ref. 14) and lung cancer cell lines. Although full-length ERα (66 kDa) was detected in MCF-7 breast cancer cells, none was observed in either NLFB or NSCLC cell lines using an antibody directed against the hinge region of the protein (Fig. 1*A*). Similar results were obtained using antibodies raised against either the NH_2 terminus or COOH terminus of ERα (Fig. 1*B*). Thus, full-length ERα protein is either not present at all or is present in amounts that are below the limit of detection of this assay. In contrast, full-length ERβ protein was detected in each of the samples analyzed (Fig. 1*A-B*). These results agree with those previously reported, using a partially overlapping set of NSCLC cell lines (14). With regard to the p160 coactivators, GRIP1/TIF2 but not SRC-1 was detected in both NLFB and NSCLC cell lines (Fig. 1 and data not shown). Whereas GRIP1/TIF2 migrated as a single protein of 160 kDa in NLFB, it migrated as a doublet in MCF-7 cells and in the NSCLC cell lines. These data show that NSCLC cells express proteins necessary to generate a transcriptional response to estrogen and suggest that ERβ and GRIP1 are likely mediators of this response.

To test whether a ligand-regulated interaction between ERβ and GRIP1/TIF2 occurs, a GST pull-down assay was used. Recombinant GST-GRIP1 was incubated with radiolabeled ERβ in the presence of various ER ligands and binding measured after 4 hours. ERβ binding was detected by gel electrophoresis (Fig. 1*C*) or by quantitating the radioactivity bound to glutathione beads in the presence of GST-GRIP1 (Fig. 1*D*). The ERβ agonist, genistein, but not the ERα agonist, propyl pyrazole triol, increased the GRIP1/ERβ interaction, confirming the receptor specificity of the assay (Fig. 1; refs. 23, 24). The ERβ/GRIP1 interaction was also stimulated by E2, as expected. Thus, E2 may promote the association of ERβ and GRIP1/TIF2 in lung cancer cells that express both proteins, thereby modulating gene expression.

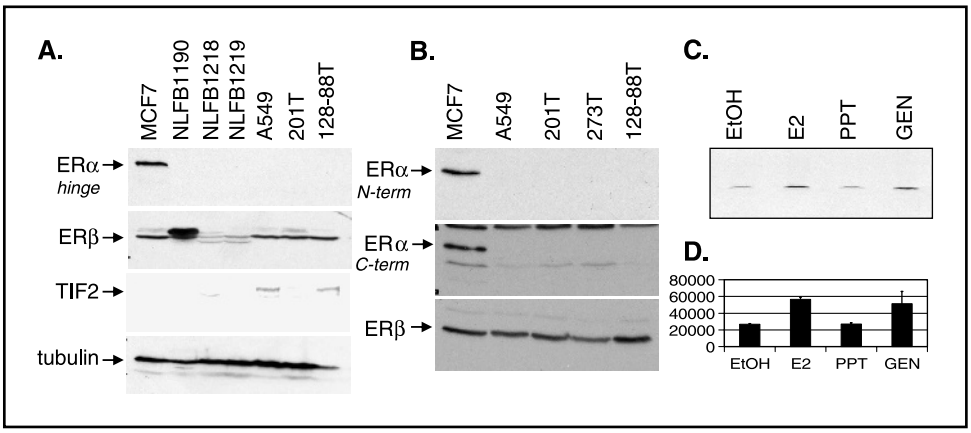

Figure 1. Human lung cancer cell lines express ERβ and GRIP1/TIF2, which interact *in vitro*. *A,* protein extracts (30 μg per sample) from NLFB and NSCLC cells were analyzed by immunoblot for ERα, ERβ, and GRIP1/TIF2. NLFB were previously shown responsive to estrogen (14). Blots were reprobed for tubulin as a loading control. *B,* independently isolated set of extracts was prepared from NSCLC cell lines and analyzed for ER expression using additional ERα-specific antibodies. *C,* ability of ER ligands to modulate the interaction between ERβ and the p160 coactivator GRIP1/TIF2 was measured using GST pull-down assays as described in Materials and Methods. GST-GRIP1/ER complexes were collected and radiolabeled ERβ protein was detected after SDS-PAGE. *D,* bound radioactivity was quantitated. Each of the ligands was used at a final concentration of 1×10^{-7} mol/L. *Columns,* mean for duplicate determinations; *bars,* ±SD. Similar results were obtained in a second experiment.

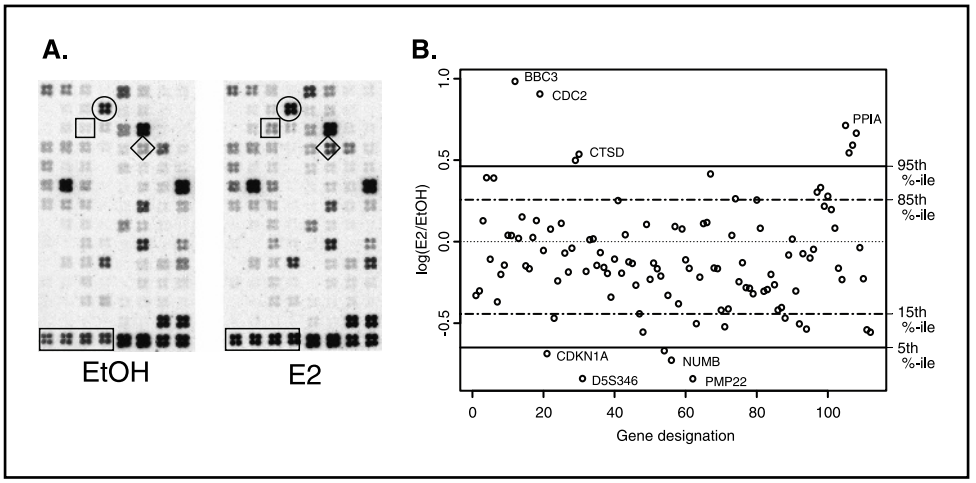

Figure 2. Gene arrays allow for detection of estrogen-regulated genes. MCF-7 cells were treated with either ethanol (*EtOH*, vehicle control) or E2 for 24 hours. RNA was extracted and used to generate cDNA as outlined in Materials and Methods. Radiolabeled cDNAs were hybridized overnight to the Human p53 Signaling Pathway Gene Array. Data were collected using a Molecular Dynamics phosphorimager and analyzed in Excel. *A,* images of arrays obtained for cells treated with either EtOH or E2. Genes whose expression increased significantly in response to treatment are *BBC3* or Bcl-2 binding component 3 or PUMA (*circles*), *cdc 2* (*squares*), *CTSD* or cathepsin D (*diamonds*), and *PP1A* or cyclophilin A (*rectangle*). *B,* analysis of array data. For data analysis, a hybridization ratio for each gene was calculated by dividing its signal intensity on the E2 array by its intensity on the EtOH array, then applying a logarithmic transformation. The resulting values were fit to a normal distribution. *Heavy dashed lines,* 15th and 85th percentiles of the distribution. *Solid lines,* 5th and 95th percentiles. *Spot,* a distinct gene on the array. Genes whose expression was <5th percentile or >95th percentile were considered significantly modulated by treatment and are identified with text (where space allowed).

To determine whether ER ligands have the capacity to regulate endogenous gene expression in NSCLC cells, gene arrays were used for expression profiling studies. This approach was validated by measuring the effects of a 24 hours of exposure of E2 on MCF-7 cells, a known estrogen-responsive breast cancer line, using the GEA Q Series Human p53 Signaling Pathway Gene Array. The p53 array contains some E2-regulated genes that were identified in large scale gene expression profiling studies of MCF-7 cells (3).

As expected, the expression of the majority of the genes included on the array did not change in response to treatment (Fig. 2*A*). To identify genes that were specifically affected, a Normal distribution was fit to the mean hybridization intensity data (E2/ethanol) for the array set, as outlined in Materials and Methods. Those genes whose expression was above the 95th percentile of the distribution were considered significantly increased by treatment, and those genes whose expression was below the 5th percentile of the distribution were considered significantly decreased by treatment. Bcl-2 binding component 3 (PUMA), cdc 2, cathepsin D, and cyclophilin A were increased in MCF-7 cells by E2 (Fig. 2*B*). The cyclin-dependent kinase inhibitor, p21 (*CDKN1A*), was among the genes significantly decreased by treatment. *Cathepsin D*, *cdc 2*, and *p21* were identified as E2-regulated genes in prior studies (3, 25), indicating that the array approach was sufficiently sensitive to allow the detection of E2-induced changes in gene expression in a breast tumor cell line.

Before conducting the array studies in NSCLC cells, biologically active concentrations of each ligand were determined by measuring effects on cell growth. 201T cells were seeded in phenol-red free tissue culture medium without serum and then treated with increasing concentrations of E2, or EGF, which was included as a positive control for a growth stimulus. As shown in Fig. 3, both E2 and EGF increased cell growth. E2-stimulated relative growth was greater than one ($P < 0.04$) at all dose levels. EGF-stimulated relative growth was significantly greater than one ($P < 0.003$) at dose levels $\geq$10 ng/mL. Concentrations of 30 nmol/L E2 and 30 ng/mL EGF were selected for further study and gave comparable stimulation. In the absence of exogenous E2, ICI 182,780 had no significant effect on cell growth at concentrations ranging from 1 nmol/L to 1 μmol/L (with *Ps*

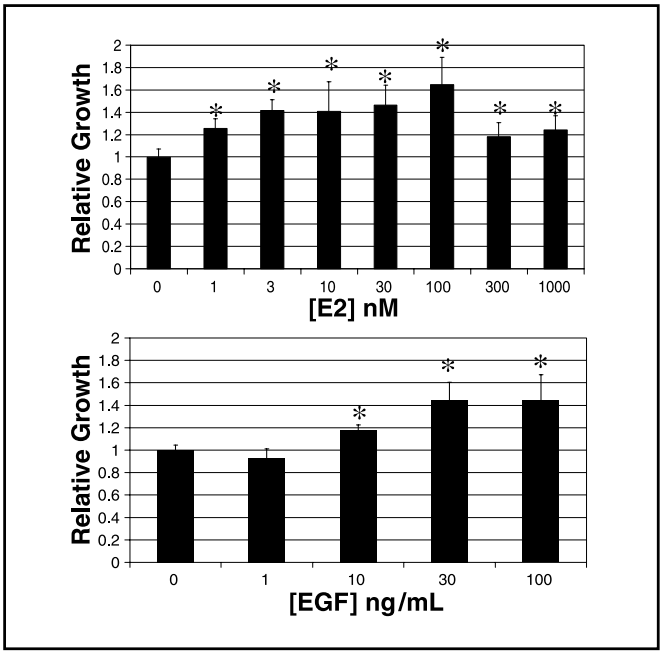

Figure 3. E2 and EGF stimulate growth of 201T human lung cancer cells. 201T cells were seeded into 96-well plates. The next day, the cells were washed and fresh medium lacking serum was added. The cells were serum-deprived for a total of 72 hours and then treated for 72 hours with the indicated ligands. Effects on cell growth were determined by MTS assay. The relative growth was calculated by dividing the absorbance obtained at 490 nm for treated cells by that obtained for cells treated with vehicle only. The concentration of vehicle was the same for all treatment groups. *Columns,* mean for 4 to 6 determinations at each ligand concentration; *bars,* ±SD. *, values that are significantly different than the control value.

ranging from 0.16 to 0.98; data not shown). A concentration of 100 nmol/L ICI 182,780 was selected for subsequent studies as it was expected to block ER function based on its known binding affinity (26).

The effect of the ER ligands E2 and ICI 182,780 on endogenous gene expression in human lung cancer cells was investigated using 201T adenocarcinoma cells that express detectable levels of full-length ERβ but not full-length ERα (Fig. 1). Cells were treated for 24 hours with ethanol, 30 nmol/L E2, 100 nmol/L ICI 182,780, or 30 ng/mL EGF. To detect E2-regulated genes, expression profiles obtained following treatment with E2 were compared with those obtained following treatment with the pure antiestrogen, ICI 182,780 using the GEA Q Series Human Breast Cancer and Estrogen Signaling Pathway Gene Array. As compared with ICI 182,780, treatment of 201T cells with E2 resulted in increased expression of E-cadherin, Id-2, and cyclophilin A (Fig. 4*A* and *B*). Cyclophilin A expression also increased following treatment with EGF, suggesting that its modulation may reflect a common component of proliferation (Fig. 4*C*). Topoisomerase 2A, keratin 18, thromboplastin, and the ribosomal proteins L13a and L27 were also increased in 201T cells treated with EGF as compared with ICI 182,780 (Fig. 4*C*).

To determine whether the effects of E2 and ICI 182,780 on gene expression could be generalized to other NSCLC cells, the array experiments were repeated using the human lung squamous cell carcinoma line, 273T. 273T cells express ERβ and EGF receptor and

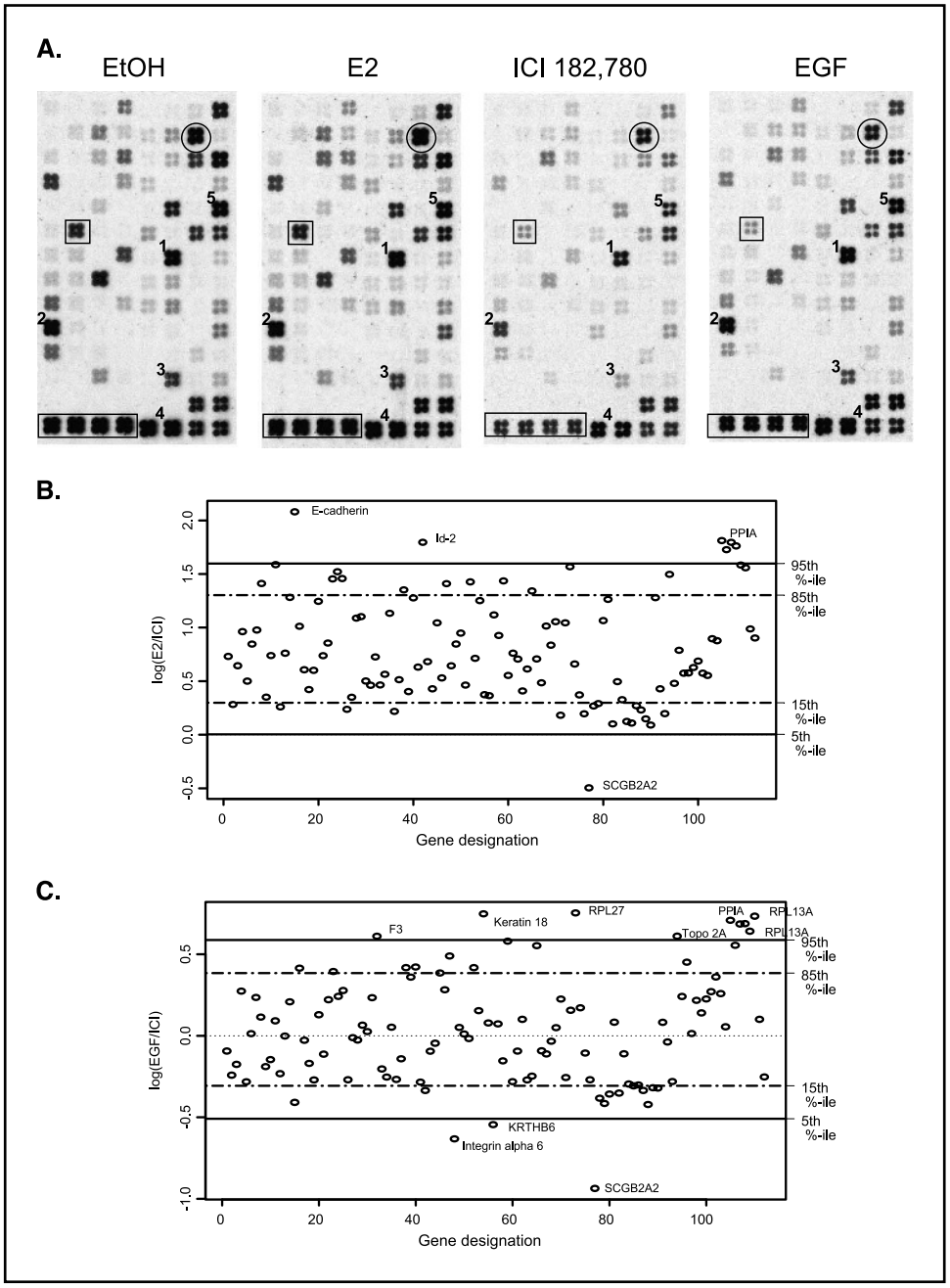

Figure 4. ER ligands modulate gene expression in 201T human lung cancer cells. 201T cells were treated with ethanol (*EtOH*, vehicle control), E2, ICI 182,780, or EGF for 24 hours. RNA was extracted and used to generate cDNA. Radiolabeled cDNAs were hybridized to the Human Breast Cancer and Estrogen Signaling Pathway Gene Array. Data were analyzed as outlined in Fig. 2. *A,* miniarray images. Shapes identify genes whose expression changed significantly in response to treatment with E2 versus ICI 182,780: *E-cadherin* (*circles*), *Id-2* (*squares*), and *PP1A* or cyclophilin A (*rectangle*). Numbers identify genes whose expression changed significantly in response to treatment with EGF versus ICI 182, 780: (*1*) keratin 18, (*2*) ribosomal protein L27, (*3*) topoisomerase 2A, (*4*) ribosomal protein L13a, (*5*) thromboplastin (F3). *B,* data analysis for E2-treated cells versus ICI 182,780-treated cells. *C,* data analysis for EGF treated cells versus ICI 182,780-treated cells.

are induced to proliferate in response to either E2 or EGF exposure (Fig. 1 and ref. 27). In these cells, E2 but not EGF increased expression of E-cadherin, Id-2, Cyclin D1, Integrin β4, and SERPINE1 (PAI-1; Fig. 5*A-C*). In contrast, EGF but not E2 increased the expression of IL-6, CD44, Kruppel-like factor 5, and topoisomerase 2A (Fig. 5*A-C*).

In both 201T and 273T cells, E2 and ICI 182,780 had opposite effects on E-cadherin and Id-2 expression, as compared with ethanol control, where E2 increased and ICI 182,780 decreased expression. To confirm these results, proteins were extracted from treated NSCLC cells and E-cadherin or Id-2 levels assessed by immunoblot. Consistent with the results of the array studies, treatment with E2 resulted in an increase in E-cadherin and Id-2 protein levels whereas treatment with ICI 182,780 decreased levels of both proteins (Fig. 6). In cells treated simultaneously with E2 and ICI 182,780, the level of E-cadherin expression was comparable to that seen in cells treated with ICI 182,780 alone. These data support the conclusion that E-cadherin and Id-2 represent endogenous targets of estrogen signaling in lung cancer and that ICI 182,780 has the capacity to block E2 signaling in these cells.

Discussion

Previous studies have documented that lung cancer cells express ERs, are induced to proliferate in response to estrogen exposure, and modulate expression of an exogenous estrogen-regulated reporter construct (14). To understand these responses, we examined the expression of proteins with the potential to affect estrogen signaling in human lung cancer cell lines and used gene

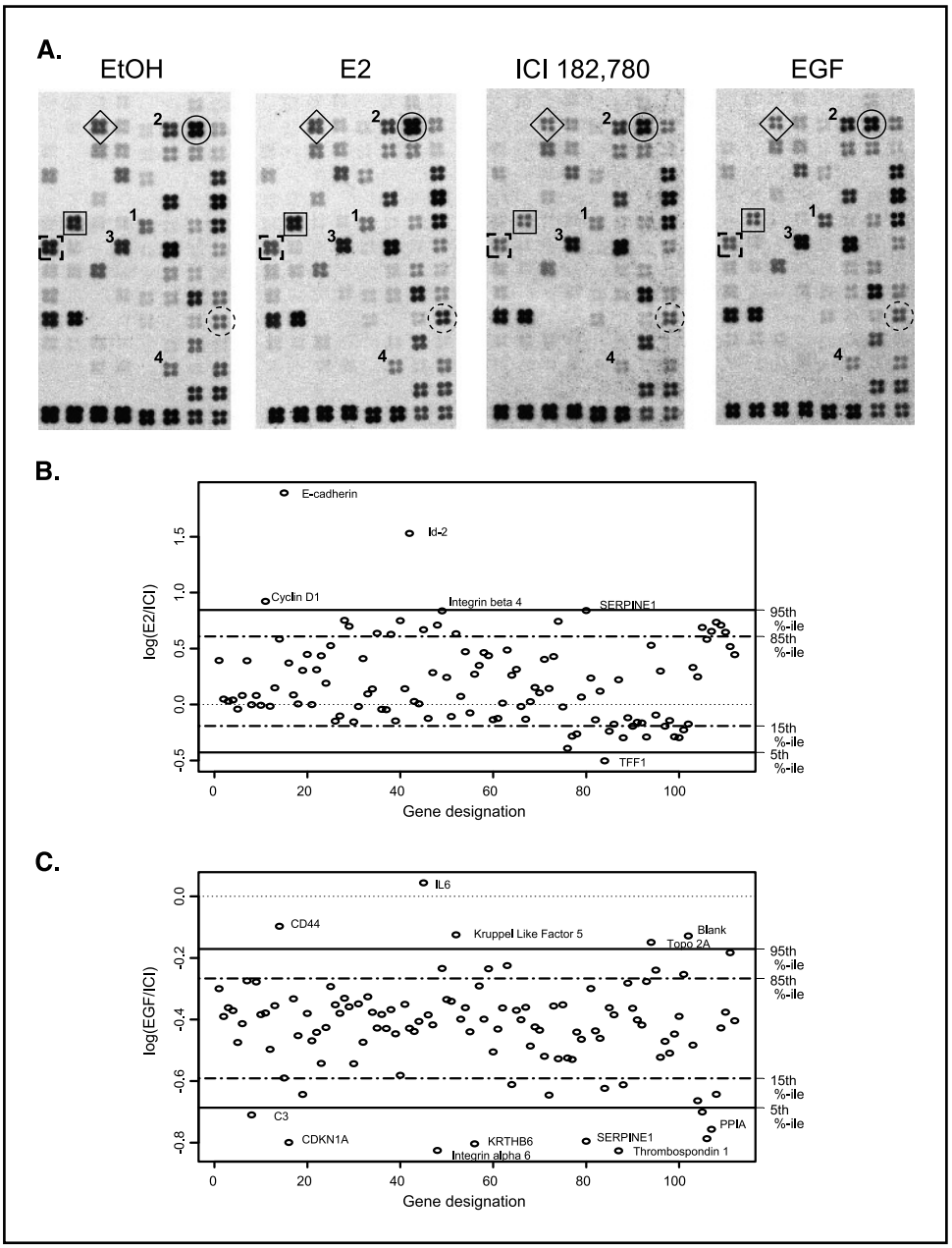

Figure 5. ER ligands modulate gene expression in 273T human lung cancer cells. 273T cells were treated and array studies conducted as in Fig. 4. *A,* Miniarray images. Shapes identify genes whose expression changed significantly in response to treatment with E2 versus ICI 182,780: *E-cadherin* (*circles*), *Id-2* (*squares*), *cyclin D1* (*diamonds*), integrin β4 (*hatched squares*), SERPINE1 or PAI-1 (*hatched circles*). Numbers identify genes whose expression changed significantly in response to treatment with EGF versus ICI 182, 780: (*1*) IL-6, (*2*) CD44, (*3*) KLF5, (*4*) topoisomerase 2A. *B,* data analysis for E2-treated cells versus ICI 182,780-treated cells. *C*, data analysis for EGF-treated cells versus ICI 182,780-treated cells.

arrays to determine whether estrogens regulate endogenous gene expression in these cells.

Our immunoblot data support a model in which transcriptional responses to estrogen in NSCLC cells are generated via ERβ and the p160 coactivator, GRIP1/TIF2. We were able to confirm this interaction using GST-pull down assays, consistent with other reports (24, 28), so the coexpression of these proteins in lung cancer cells is expected to be significant. The other p160 coactivators, AIB-1 and SRC-1, are less likely to be important since human lung cancer cells do not express AIB-1 (21) and we did not detect SRC-1 by immunoblot (data not shown). Although not detected in human lung cancer cells, SRC-1 transcripts and protein are detected in the normal mouse lung (29). It is not clear whether these differences in SRC-1 expression are species specific, reflect differences between normal lung and lung tumor cells, or are reflective of the different methodologies used to assess coactivator expression.

The SDS-PAGE migration of GRIP1/TIF2 from extracts prepared from lung tumor cells is different than GRIP1 from extracts of normal human lung fibroblasts. It is possible that this difference results from a post-translational modification of GRIP1/TIF2 that occurs in lung cancer cells but not normal cells. GRIP1 is post-translationally modified by mitogen-activated protein kinase–mediated phosphorylation (30) and by sumoylation (31). Phosphorylation potentiates GRIP1 transcriptional activity (30) and sumoylation increases GRIP1 nuclear colocalization with the androgen receptor and enhances its coactivator activity (31). If the altered mobility we observe results from such modifications, the coactivator function of GRIP1/TIF2 is probably enhanced in lung tumor cells compared with normal cells.

Our inability to detect full-length ERα by immunoblot using antibodies specific for three different epitopes of the protein suggests that ERα is not the primary mediator of transcriptional responses to E2 in NSCLC cells. The absence of ERα protein in whole cell extracts of human NSCLC cell lines has been observed by others (19). However, ERα staining has been detected in the cytoplasm of NSCLC cases by immunohistochemistry, suggesting that the protein, or a variant thereof, is not completely absent in lung tumors (14). In breast cancer cells, a pool of ERα associates with the plasma membrane. The membrane ER can use growth factor receptors, such as EGF receptor, to signal through downstream kinases (32). If a similar pathway exists in NSCLC cells, cytoplasmic ERα may contribute to the proliferative response to E2 by a means other than as a transcription factor. Evidence of cross-talk between the EGF receptor and ER pathways in NSCLC cells has recently been presented (27).

Expression of Id-2 was conversely affected by E2 and ICI 182,780, in both 201T and 273T cells, suggesting it is a downstream target of estrogen signaling in lung cancer cells. Id proteins are antagonists of basic-helix-loop-helix transcription factors that restrict cell cycle progression (33). Id-2 positively drives cell cycle progression by disrupting retinoblastoma Rb function (34). Id-2 is a direct transcriptional target of c-*myc*; the Id-2 promoter contains three *myc*-binding sites and c-*myc* increases Id-2 transcription (34). C-*myc* is a direct transcriptional target of E2 treatment (35, 36). Thus, E2 may secondarily increase Id-2 transcription via its effects on c-*myc*. The E2-mediated upregulation of Id-2 transcripts and protein observed in these studies provides a mechanistic explanation for its stimulatory effect on lung cancer proliferation.

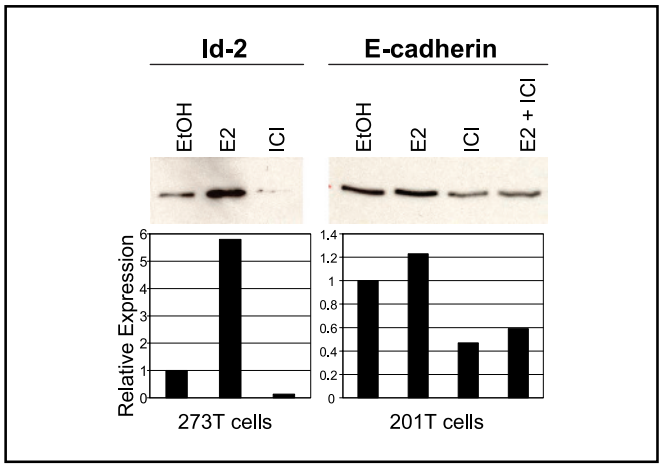

Figure 6. ER ligands modulate protein expression of E-cadherin and Id-2 in NSCLC cells. Cells were seeded into T75 flasks in complete medium and allowed to attach overnight. The following day, the cells were washed with PBS and cultured in phenol-red free RPMI 1640 for 72 house in the absence of serum. Cells were then treated with ethanol (*EtOH*, vehicle control), E2, ICI 182,780, E2 plus ICI 182,780, or EGF for 24 hours. Whole cell extracts were prepared and analyzed by immunoblot. E-cadherin and Id-2 expression was assayed using 7.5 or 30 μg of protein, respectively. The normalized relative expression levels, determined by densitometry, are plotted below each of the treatment groups.

An additional gene whose expression was significantly modulated by E2 but not EGF in both lung cancer cell lines was *E-cadherin*. Cadherins are calcium-dependent cell adhesion proteins that mediate linkages with the actin cytoskeleton (37). E-cadherin is reported to be a direct transcriptional target of ER, although the effects of estrogen on its expression differ in a cell-type specific manner. In MCF-7 breast cancer cells, ER binds directly to the E-cadherin promoter, recruits corepressors, and suppresses E-cadherin expression (38). In contrast, *in vivo* administration of estradiol increases E-cadherin transcription in the mouse ovary (39), as it does here in lung cancer cells *in vitro*. It is possible that the effect of estradiol exposure on E-cadherin expression depends on which ER subtype predominates in a given tissue, and which of the coactivators and corepressors are expressed in that tissue.

Treatment of 273T cells with E2 as compared with ICI 182,780 also resulted in a significant increase in expression of cyclin D1, a well-defined target of estrogen action in breast cancer cells (40, 41). Cyclin D1 expression was also increased by E2 in 201T cells, although it fell just below the 95% limit of the normal distribution we set in the array studies. A correlation exists between estrogen-induced proliferation and increased cyclin D1 expression in breast cancer cells, and blockade of cyclin D1 function inhibits estrogen-induced proliferation (42). Our data suggest that the ability of estrogen to regulate cyclin D1 expression may also be important in mediating its proliferative effects in lung cancer cells. The significance of modulating the expression of the estrogen target genes identified in this study to the growth of lung cancer cells remains to be determined.

Treatment with E2 resulted in an increase in E-cadherin and Id-2 protein levels whereas treatment with ICI 182,780 decreased levels of both proteins. The ability of ICI 182,780 to decrease the expression of estrogen-regulated genes and block E2-stimulated proliferation of NSCLC cells *in vitro* and *in vivo* (14, 17, 18) is consistent with a model in which ICI 182,780 reduces the

proliferation of NSCLC cells via its ability to disrupt ER transcriptional signaling. ICI 182,780 may therefore have therapeutic benefit in NSCLC. Furthermore, the ability of both E2 and EGF to regulate gene expression in NSCLC cells suggests that either of these pathways may promote growth. Consequently, blockade of both signaling pathways may be of greater therapeutic benefit than blocking either pathway individually. The effect of combining ER and EGF receptor inhibition on lung cancer growth is under active investigation both preclinically and clinically.

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