Sex Differences in Estrogen Receptor Subcellular Location and Activity in Lung Adenocarcinoma Cells

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The role of estrogens in the increased risk of lung adenocarcinoma in women remains uncertain. We reported that lung adenocarcinoma cell lines from female, but not male, patients with non-small cell lung cancer respond proliferatively and transcriptionally to estradiol (E₂), despite equal protein expression of estrogen receptors (ER) α and β . To test the hypothesis that nuclear localization of ER α corresponds to genomic E₂ activity in lung adenocarcinoma cells from females, cell fractionation, immunoblot, and confocal immunohistochemical microscopy were performed. We report for the first time that E₂ increases phospho-serine-118-ER α (P-ser118-ER α) and cyclin D1 (CCND1) nuclear colocalization in H1793, but not A549 lung adenocarcinoma cells, derived from a female and male patient, respectively. ER β was primarily in the cytoplasm and mitochondria, independent of E₂ treatment, and showed no difference between H1793 and A549 cells. E₂ induced higher transcription of endogenous ER α -regulated CCND1 in H1793 than in A549 cells. Likewise, higher rapid, non-genomic E₂-induced extracellular signal-regulated kinase 1/2 activation was detected in H1793 compared with A549 cells, linking extracellular signal-regulated kinase activation to increased P-ser118-ER α . Furthermore, E₂ increased cyclin D1 and P-ser118-ER α nuclear localization in H1793, but not A549 cells. Together, our results indicate that nuclear localization of P-ser118-ER α provides one explanation for sex-dependent differences in E₂-genomic responses in lung adenocarcinoma cell lines.

Keywords: estrogen receptor; cyclin D1; phosphorylation; non-small cell lung cancer; sex differences

Lung cancer is the leading cause of cancer death in both women and men in the United States. However, the twofold-higher risk of lung cancer in female smokers or nonsmokers than in male smokers strongly suggests the involvement of sex-dependent factors in the etiology of lung cancer (1). In particular, women have higher incidence of lung adenocarcinoma, a type of non-small cell lung cancer (NSCLC), compared with men. The mechanisms underlying the sex difference in NSCLC risk are multifactorial, and include differences in phase I and II drug-metabolizing enzyme activities (1–3). The role of estrogens in lung adenocarcinoma is of particular research interest and debate (4, 5). A new clinical case study of 59 patients with NSCLC reported that 73% had higher estradiol (E₂) concentrations in the carcinomas compared with the corresponding nonneoplastic lung tissues from the same patient, regardless of sex, which correlated with aromatase mRNA, but not estrogen

CLINICAL RELEVANCE

Women have higher incidence of lung adenocarcinoma, a type of non-small cell lung cancer (NSCLC), compared to men. The mechanisms underlying the sex difference in NSCLC risk are multifactorial and complex. Here we show that nuclear localization of phospho-serine-118-estrogen receptor- α provides one explanation for sex-dependent differences in estradiol-genomic responses in lung adenocarcinoma cell lines.

receptor (ER) α or β staining (6). Thus, local estrogen production may play a role in NSCLC.

There are multiple mechanisms through which ER mediates estrogenic effects. Best characterized is the genomic action of ligand-activated ER mediated by the binding of ER directly to estrogen-responsive elements (EREs), or by tethering of the ER dimer to other transcription factors (e.g., AP-1 and Sp1) (7). These interactions recruit coregulators, chromatin-remodeling complexes, and RNA polymerase II to the target gene promoter (8). Serine phosphorylation is required for full activation of ER α (9), and phosphorylation of ER α on ser118 (phospho-serine-118-ER α [P-ser118-ER α]) activates N-terminal transcription activation function-1. Plasma membrane localization of ER activates rapid, membrane-initiated, “nongenomic” responses (e.g., activation of phosphatidylinositol-3-kinase [PI3K] and mitogen-activated protein kinase [MAPK]) (10). PI3K and MAPK phosphorylate ER α ser167 and ser118, respectively, thus linking rapid E₂ responses with later genomic actions (11). Approximately 20% of ER α in H23 lung adenocarcinoma cells was associated with the plasma membrane fraction, and E₂ rapidly activated MAPK (5). ER α and ER β have also been localized to mitochondria in human cell lines and tissues (12).

The expression and intracellular location of ER α and ER β have been examined by immunohistochemical (IHC) staining in normal and neoplastic human lung (reviewed in Refs. 6, 13–15). ER β was present in columnar epithelium, intermediate, basal, and smooth muscle lung cells, whereas ER α was expressed in basal and smooth muscle cells (16). One IHC study of tumors from 122 patients with NSCLC reported that ER α was primarily cytoplasmic, and ER α and epidermal growth factor receptor were correlated with poor prognosis, but not with sex (17). Nuclear ER α and ER β staining was detected in 45 and 52% of 65 archival NSCLC tumors, respectively, whereas 75 and 69% showed “extranuclear” ER α and ER β staining, respectively (18). This study also reported phosphorylation of nuclear ER α on ser118 and ser167 in the NSCLC tumors (18).

We reported that lung adenocarcinoma cell lines from female patients proliferated in response to E₂, and that growth was blocked by antiestrogens (i.e., 4-hydroxytamoxifen [4-OHT] and ICI 182,780 [ICI]) (13). In contrast, lung adenocarcinoma cell lines from male patients were not responsive to E₂ or antiestrogens (13). Similar results were obtained when the cell lines were transfected with an ERE-

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luciferase reporter and treated with E₂ (i.e., cell lines from females, but not males), showing genomic activation of ER α (13). Despite these phenotypic differences, all cell lines had equal expression of ER α and ER β at the mRNA and protein levels, with ER β expression being approximately two-times higher than ER α (13). These data indicate that the differences in the expression of ER α and ER β are not the mechanism accounting for the ability of adenocarcinoma cell lines from females to respond to E₂ and antiestrogens.

To test the hypothesis that nuclear ER α localization in lung adenocarcinoma cells from females accounts for their E₂-transcriptional responses, and to better understand the mechanism underlying the difference in the response of male- and female-derived lung adenocarcinoma cells to treatment with E₂, 4-OHT, and ICI (13), we examined the intracellular location of ER α , P-ser118-ER α , and ER β in normal lung fibroblasts and lung adenocarcinoma cells using confocal microscopy and subcellular fractionation, followed by Western blotting. Results were compared with MCF-7 human breast cancer cells as an established ER α /ER β , estrogen-responsive cell type. Our data demonstrate differences in the nuclear localization of ER α and P-ser118-ER α in lung adenocarcinoma cells derived from females and males that correspond to reported differences in the genomic activity of ER α in these cells (13).

MATERIALS AND METHODS

Chemicals

E₂, PD98059, 4-OHT, pertussis toxin (PTX), and methyl- β -cyclodextrin (β -CD) were from Sigma (St. Louis, MO). Src tyrosine kinase inhibitor PP2 and ICI were from Tocris (Ellisville, MO).

Cell Lines and Treatment

A549, NCI-H23, NCI-H1299, NCI-H1395, NCI-H1435, NCI-H1792, NCI-H1793, NCI-H1944, NCI-H2073, and MCF-7 cell lines were purchased from ATCC (Manassas, VA). Before treatment, cells were placed in phenol red-free media supplemented with 5% dextran-coated, charcoal-stripped FBS (DCC-FBS) for 48 hours. Ethanol (EtOH) was used as the vehicle control for all experiments. Lung adenocarcinoma cell line characteristics are listed in Table 1. NF1604, karyotyped as 46XY, were derived from human fetal lung tissue and immortalized by human telomerase (19).

Antibodies

Polyclonal antibodies HC-20 for ER α and H150 for ER β , and the HC-20 blocking peptide were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal ER α antibody AER320 was from LabVision/Neo-

markers (Thermo Fisher, Fremont, CA). HC-20 and AER320 were raised against peptides in the C terminus of ER α (aa 545–595 for HC-20 and aa 495–595 for AER320; see Figure E1A in the online supplement). HC-20 and H150 were demonstrated to be ER subtype-specific for IHC/confocal microscopy (20). HC-20 has been used for IHC staining of human lung tumors (14, 18), and AER320 has been used for IHC of human breast tumors (21, 22), but not, to our knowledge, for lung. The relative purity of subcellular fractions was analyzed by antibodies for α -tubulin (cytoplasm; LabVision), Tom20 (mitochondria, generously provided by Dr. Brian Wattenberg, University of Louisville), histone H1 or poly (ADP-ribose) polymerase (PARP) (nuclear; Santa Cruz Biotechnology). P-ser118-ER α antibody was from Cell Signaling (Danvers, MA). Cyclin D1 antibody was from Abcam (Cambridge, MA).

Western Blotting

Subcellular fractions were separated according to the protocol provided in the Mitochondrial Isolation Kit for Mammalian Cells from Pierce (Rockford, IL). The indicated amounts of protein (see figures) of cytoplasmic, mitochondrial, and nuclear protein extracts (CE, ME, and NE, respectively) or whole-cell extract (WCE) were separated on 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes, and blocked (13). The HC-20-blocking peptide was incubated at a 2:1 ratio with HC-20 antibody in 5% BSA-Tris-buffered saline-Tween for 1 hour at room temperature before incubation with the polyvinylidene fluoride membrane overnight at 4°C (Figure E2). Super Signal West Pico Chemiluminescent Substrate (Pierce) was used to detect protein bands as visualized on Kodak BioMaxML film (Eastman Kodak, Rochester, NY). Resulting immunoblots were scanned into Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA) using a Microtek ScanMaker III scanner (Microtek, Carson, CA). Un-Scan-It 5.1 for Windows (Silk Scientific, Orem, UT) was used to quantitate the integrated optical densities for each band and analyze the relative amounts of immunoreactive protein, based on pixel density.

Immunofluorescence Staining

Cells were grown on coverslips, and were incubated with 200 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) for 45 minutes, washed with PBS, then fixed with cold methanol:acetone (1:1) for 5 minutes and washed twice with cold PBS. After blocking with 1% goat serum and 0.3% Triton X-100 in PBS for 30 minutes, primary antibody (ER α , HC-20; ER β , H150) was added at a 1:300 dilution for 1 hour. After removing primary antibodies, the cells were stained with secondary antibodies labeled with Quantum Dot 525 (Quantum Dot Corp., Hayward, CA). The immunofluorescence analysis of P-ser118-ER α and cyclin D1 used combinations: (1) monoclonal (anti-mouse) P-ser118-ER α and anti-rabbit cyclin D1 antibodies; and (2) polyclonal (anti-rabbit) P-ser118-ER α and AER320 ER α monoclonal (anti-mouse) antibodies. The H1793 and A549 cells were grown on coverslips. Before fixation, the cells were incubated in phenol red-free media supplemented with 5% DCC-FBS for 48 hours (serum starva-

TABLE 1. CHARACTERISTICS OF THE CELL LINES USED IN THIS STUDY AND A SUMMARY OF ESTROGEN RECEPTOR α AND ESTROGEN RECEPTOR β SUBCELLULAR DISTRIBUTION BY CONFOCAL AND IMMUNOFLUORESCENT MICROSCOPY

Cell Line	Sex of Patient	Smoker/NS	ER α Subcellular Distribution		ER β Subcellular Distribution	
			Control	+ E ₂	Control	+ E ₂
NF1604 normal lung fibroblasts	Male	NS	C	C	N, C, M	N, C, M
A549	Male	Unknown	N < < C	C	N, C, M	N, C, M
NCI-H1792	Male	Smoker	C	C	C, M	C, M
NCI-H23*	Male	Smoker	C		C	
NCI-H1299*	Male	Smoker	C		N, C	
NCI-H1793	Female	NS	N, C, M	N, C, M	N > C, M	N > C, M
NCI-H1944	Female	Smoker	N, C	N, C	C, M	N < C, M
NCI-H1395*	Female	Smoker	N, C		N < < C	
NCI-H1435*	Female	NS	N, C		N, C	
MCF-7 human breast cancer	Female	Unknown	N, C	N, C	N < < C, M	N < C > M

Definition of abbreviations: C, cytoplasmic; E₂, estradiol; ER, estrogen receptor; M, colocalization with MitoTracker Red (mitochondria); N, nuclear; NS, nonsmoker. The data summarized here were taken from confocal images, including those shown in Figures 1–4.

* Cell lines examined by immunofluorescent microscopy; where indicated as +E₂, cells were treated with 10 nM E₂ for 45 minutes.

tion) and treated with 10 nM E₂ for 6 hours. Cells were washed with PBS, fixed with cold methanol:acetone (1:1) for 5 minutes, and washed twice with cold PBS. After blocking with 1% goat serum and 0.3% Triton X-100 in PBS for 30 minutes, primary antibodies (cyclin D1, P-ser118-ER α , or AER320) were added at a 1:300 and 1:1,000 dilution, respectively, for a 1-hour incubation. After removing primary antibodies, secondary antibody was added. The secondary anti-mouse antibody was labeled with Zenon Alexa Fluor 488 (green color), and the secondary anti-rabbit antibody was labeled with Zenon Alexa Fluor 594 (red color) labeling kits from Molecular Probes. Cells were then incubated with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Molecular Probes). Images were obtained using a confocal microscopy (Axiovert 100; Zeiss, Jena, Germany) and LSM510 software (Zeiss), or by immunofluorescence imaging using a Zeiss Axiovert 200 inverted microscope with a 63 $\times$ objective lens using AxioVision Release 4.3 software.

To examine the specific staining of HC-20 and H150 antibodies, A549, H1793, H1793, and H1944 cells were incubated with Mito-Tracker Red, stained with 4',6-diamidino-2-phenylindole, and incubated with the FITC secondary antibody without primary antibodies (data not shown). Figures E1C and E1D show the effect of E₂ on ER α and ER β subcellular distribution on MCF-7 cells, with normal lung fibroblast cells (NF1604) used as controls. In addition, the specificity of the ER α immunofluorescence was demonstrated by ablation of any green fluorescence signal by the HC-20-blocking peptide (Figure E2).

Cell Proliferation/Viability Assays

Cell proliferation/viability was determined using the Cell Titer 96 AQueous One MTT assay from Promega (Madison, WI), according to the manufacturer's instructions. Cells were plated in 96-well plates in growth medium and allowed to adhere for 24 hours, after which the medium was replaced with phenol red-free Improved Minimum Essential Medium supplemented with 5% DCC-FBS for 24 hours before treatment with vehicle control (i.e., EtOH), E₂, 4-OHT, or ICI, alone or in combination, for 4 days, with treatments and medium replenished after 48 hours. Where indicated, cells were preincubated with 50 μ M PD98059 (mitogen-activated protein kinase 1 [MEK1] inhibitor, 1 h) before adding the indicated cotreatments. MTT assays were performed on Day 4.

FACS of Cell Cycle Phase Using Propidium Iodide

A549 and H1793 cells were grown in six-well plates with 1×10^6 cells per well, and treated with EtOH (vehicle control) or 10 nM E₂ for 6 hours. For cell cycle analysis, cells were harvested, fixed in 70% EtOH, and incubated for 30 minutes in propidium iodide staining buffer (0.1 M PIPES [pH 6.8], 1.5 M NaCl, 0.5 M MgCl₂, and 0.1% Triton X100) containing 2.5 μ g/ml RNase DNA-free (Roche) and 10 μ g/ml propidium iodide (Roche). Cellular DNA content was analyzed by flow cytometry using a MoFlo Flow Cytometer (Beckman Coulter, Hialeah, FL) equipped with an argon laser tuned at 488 nm. BD CellQuest Pro Software (BD Bioscience, San Jose, CA) was used for acquisition and analysis of each cell sample (number of cells, 10,000) in the J. G. Brown Cancer Center Cell Sorting Core Facility ($n = 5$ replicate experiments).

RNA Isolation, RT-PCR, and Real-Time Quantitative RT-PCR

RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Gaithersburg, MD). Oligo(dT)₁₂₋₁₈ primers (Promega) were annealed to 2 μ g of total RNA and reverse transcribed with the High Capacity cDNA Archive kit (PE Applied Biosystems, Foster City, CA) using the manufacturer's protocol. The cDNA generated was used as a template for PCR with primers specific for ER α splice variants, $\Delta 2$ and $\Delta 7$ (23). Taqman primers and probes for cyclin D1 (*CCND1*) and 18S rRNA were purchased as Assays-on-Demand Gene Expression Products (PE Applied Biosystems). The expression of each target gene was determined in quadruplicate in two separate experiments and normalized using 18S. Real-time qRT-PCR was performed in the ABI PRISM 7900 SDS 2.1 (PE Applied Biosystems) using relative quantification real-time qRT-PCR for *CCND1*, and 18S was performed using absolute quantification. Analysis and fold differences were determined using the comparative CT method, and data are presented as relative to EtOH-treated cells.

P-Extracellular Signal-Regulated Kinase 1/2 and Extracellular Signal-Regulated Kinase 1/2 ELISA

A549 and H1793 cells were preincubated with 50 μ M PD98059 (MEK1 inhibitor, 1 h), 100 ng/ml PTX (a G α inhibitor, 17 h), 5 mM β -CD (a lipid raft disruptor, 30 min), or 10 μ M PP2 (Src kinase inhibitor, 30 min) before E₂ treatment. Cells were treated with 10 nM E₂ for 0–30 minutes and 1, 2, 4, 6, 8, and 24 hours. After treatment, the cells were fixed with fresh 4% formaldehyde in PBS and assayed as described in the manufacturer's protocol (FACE extracellular signal-regulated kinase [ERK] 1/2 ELISA kit; Active Motif, Carlsbad, CA). Each treatment was run in triplicate wells. Values were averaged, and three experiments performed.

Statistical Analysis

Statistical analyses were performed using Student's *t* test or one-way ANOVA, followed by Student-Newman-Keuls or Dunnett's *post hoc* tests using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

RESULTS

Subcellular Localization of ER α and ER β in Lung Adenocarcinoma Cell Lines

To test the hypothesis that nuclear localization of ER in lung adenocarcinoma cells from female, but not male, patients accounts for their observed E₂-dependent transcriptional and proliferative responses, the intracellular localization of ER α and ER β was examined (Figures 1–3 and summarized in Tables 1 and 2). Because all four adenocarcinoma cell lines from males, and all five cell lines from females, showed similar cell growth and transcriptional responses to E₂ (17), we selected A549 and H1793 as representative male- and female-derived lung adenocarcinoma cells for biochemical fractionation studies (Figures 1A and 1B). HC-20 ER α antibody detected the 66-kD ER α in MCF-7 cells, but not in A549 or H1793 cells (Figure 2A and Figure E3). The absence of the 66-kD ER α in A549 and H1793 is identical to the findings in a report on A549 and other lung cancer cell lines (14). In contrast, AER320 detected the 66-kD ER α band in cellular subfractions in which HC-20 failed to detect the 66-kD ER α , and demonstrated higher nuclear ER α in lung adenocarcinoma cell lines from females than males (Figures E1B and E4).

ER $\alpha\Delta 7$ in H1793 Lung Adenocarcinoma Cells

To examine the identity of the apparent 52- to 55-kD ER α protein(s) detected by HC-20 in H1793 cells, RT-PCR was performed using specific primers to detect the deletion (Δ) of exons 7 and 2 (23). As a control, MCF-7 cells were shown to express 319- and 482-bp products, corresponding to ER $\alpha\Delta 7$ (52 kD) (23, 24) and full-length ER α . Notably, ER $\alpha\Delta 7$ and wild-type (WT) ER α were detected in H1793 (Figure E5). The ER $\alpha\Delta 2$ primers did not detect the expected 365-bp product for ER $\alpha\Delta 2$ in H1793 cells, but, instead, detected a 292-bp product. MCF-7 did not express ER $\alpha\Delta 2$ (Figure E5). These data indicate that H1793 cells express ER $\alpha\Delta 7$, corresponding to the 52- to 55-kD ER α band detected by HC-20 (Figure 1A), and that a mutation(s) in exons 1 and/or 3 preclude amplification of WT ER α with the ER $\alpha\Delta 2$ primers.

Subcellular Localization of ER β in Lung Adenocarcinoma Cell Lines

ER β 1 was predominantly in the nuclear fraction (Figure 1A, Table 2). In addition, a 46-kD ER β band was detected in all subcellular fractions from A549 and H1793 cells. The amount of 46-kD ER β was reduced in the nuclear fraction and enriched in the mitochondrial fraction of H1793 cells (Figure 1A, Table 2).

Figure 1. Subcellular localization of estrogen receptor (ER) α and β in lung adenocarcinoma cell lines. (A) Western blot analysis of subcellular fractions from MCF-7 breast cancer cells, A549 and H1793 lung adenocarcinoma cells. Nuclear (N), cytoplasmic (C), and mitochondrial (M) extracts. Loading of equal amounts of protein from each subcellular fraction allowed a direct comparison of the relative amount of each ER α and ER β variant expressed in each cellular compartment and between cell lines. The membranes were stripped and reprobed for histone H1 and Tom20 as a measure of the relative purity of the nuclear and mitochondrial fractions, respectively. (B) The graphs show the quantitation of the percent of total immunoreactive ER α and ER β proteins in nuclear (N), cytoplasmic (C), and mitochondrial (M) extracts. Values are the average of three to six separate experiments ($\pm$ SEM). * $P < 0.001$, nuclear ER α values were significantly different. (C) Confocal microscopic imaging of lung adenocarcinoma cells: A549 and H1792 from male patients and H1793 and H1944 from female patients. As indicated, cells were treated with either ethanol (EtOH; vehicle) of 10 nM estradiol (E₂) for 45 minutes. Each set of two side-by-side images shows ER (ER α or ER β as indicated, green color) on the left and a merged image of 4',6-diamidino-2-phenylindole (DAPI; blue), ER (green), and Mitotracker Red CMXRos (red) on the right (labeled at the top as ER and Merge, respectively). The yellow color indicates overlap of Mitotracker red

and ER α or ER β signaling in mitochondria. Scale bars, 20 μ m. The yellow arrows indicate the absence or presence of ER α or ER β in the nucleus, as described in the text.

A 36-kD ER β band was predominantly in the nuclear fraction of A549 and H1793 cells (Figure 1A, Table 2).

MCF-7 has proportionally more ER α and ER β in the nucleus than either A549 or H1793 cells (Figure 1B). Within

the nuclear fraction, A549 had significantly lower total ER α (~6%) than H1793 (~17%) cells ($P = 0.0012$). A549 cells had approximately 2.5 times more ER β in the nuclear fraction compared with ER α . H1793 cells had similar amounts (~17%)

Figure 2. Immunofluorescence microscopic imaging of ER α and ER β in lung adenocarcinoma cells. (A) Immunofluorescent staining with ER α antibody HC-20 (green) in untreated H23, H1299, H1395, and H1495 cell lines, counterstaining with DAPI (blue), and the merged images (Merge) are shown. (B) Fluorescence microscopic imaging of ER β in lung adenocarcinoma cells. Immunofluorescent staining with ER β antibody H150 (green) is shown for untreated H23, H1299, H1395, and H1435 lung adenocarcinoma cells. Cells were counterstained with DAPI (blue), and the merged images (Merge) are shown. All images were captured with same time exposure. Scale bars, 20 μ m.

Figure 3. Phospho-serine-118-ERα (P-ser118-ERα) in lung cancer cells. (A) Western blot of whole-cell extract from H1793 and A549 cells using P-ser118-ERα, ERα 66-kD using monoclonal antibody AER320, and β-actin antibodies. Note that AER320 detects full-length, wild-type (WT) ERα (Figure 1 and Ref. 13). The cells were treated for either 4 or 6 hours with EtOH or 10 nM E₂ as indicated. (B) The relative levels of P-ser118-ERα/ERα were quantitated. Values are the average of three to six separate experiments (± SEM). The P-ser118-ERα/ERα 66-kD ratio in EtOH-treated H1793 cells was set to 1 within each experiment and at each time point (4 or 6 h) for comparison between experiments. Values are significantly different from the P-ser118-ERα/ERα 66-kD ratio in EtOH-treated H1793 cells: **P* < 0.05, ***P* < 0.001. (C) Western blot of cytoplasmic (C) and nuclear (N) extracts from H1793 cells for P-ser118-ERα, and subfraction controls (histone H1, nuclear; α-tubulin, cytosol) in EtOH-treated H1793 cells. (D) Dual-label fluorescence immunostaining of control (EtOH)- and E₂-treated H1793 and A549 cells. ERα AER320 antibody (green) and P-ser118-ERα antibody (red) were used as described in MATERIALS AND METHODS. Images of nuclei (DAPI) and ERα, and the merged image were captured at identical times of exposure (16 and 230 ms, respectively) for each cell line. To examine differences in the level of P-ser118-ERα in EtOH- versus E₂-treated cells, images of P-ser118-ERα were captured at 100, 200, and 300 ms of exposure, as indicated at the bottom of the photos. Scale bars, 20 μm.

of total ERβ and ERα in the nuclear fraction. Differences in the relative expression of the ERβ isoforms in the cellular sub-fractions and between cell lines are summarized in Table 2.

Confocal Imaging of ERα and ERβ in Lung Adenocarcinoma Cells

ERα was cytoplasmic in both untreated and E₂-treated A549 cells, results that are in agreement with biochemical fractionation and Western blot analysis (Figure 1C). E₂ did not increase nuclear ERα in A549 cells (Figure 1C, yellow arrow). ERβ was located in both nuclear and cytoplasmic compartments, and overlapped with Mitotracker Red, indicating that ERβ is in the mitochondria in the perinuclear area of A549 cells (Figure 1C). These observations support the biochemical fractionation/Western data in Figure E1B. E₂ did not alter ERβ localization, but increased colocalization of ERβ with Mitotracker Red (Figure 1C).

ERα was localized in the cytoplasm, and not in the nucleus, of H1792, another male-derived lung adenocarcinoma cell line, and did not change with E₂ treatment (Figure 1D). ERβ was

detected in the cytoplasm and mitochondria of H1792 cells, but not in the nucleus, and did not change with E₂ (Figure 1D).

H1793 cells showed diffuse cytoplasmic ERα staining, and the overlap with Mitotracker Red indicates mitochondrial colocalization (Figure 1E). ERα was also detected in the nucleus, and distribution was unaffected by E₂ (Figure 1E). These observations are congruent with the biochemical fractionation/Western data for H1793 (Figure E1B). ERβ was in both cytoplasm and nucleus, and E₂ increased nuclear and perinuclear fluorescence (Figure 1E, yellow arrow). In both control and E₂-treated H1793, ERβ colocalized with Mitotracker Red (Figure 1E).

ERα was diffusely distributed throughout the cytoplasm and nucleus in H1944, another female-derived lung adenocarcinoma cell line (Figure 1F). E₂ increased ERα signal intensity in the perinuclear region, and resulted in a punctuate ERα signal within the nucleus (Figure 1F). ERβ was distributed throughout the cytoplasm, and not the nucleus, and overlapped with Mitotracker Red in the perinuclear region of H1944 cells (Figure 1F). E₂ increased ERβ in the nucleus of some H1944

TABLE 2. SUBCELLULAR DISTRIBUTION OF ESTROGEN RECEPTOR α AND ESTROGEN RECEPTOR β SPLICE VARIANTS AS DETERMINED BY BIOCHEMICAL FRACTIONATION

Localization	MCF-7		A549		H1793	
	ERα	ERβ	ERα	ERβ	ERα	ERβ
Cytoplasmic	80, 66, 52–55	64, 60, 46, 36	80, 52–55	64, 60, 46, 36	80, 52–55	64, 46, 36
Mitochondrial	80, 66	64, 60, 46, 36	80, 52–55	64, 60, 46, 36	80, 52–55	64, 60, 46, 36
Nucleus	80, 66, 52–55	64, 60, 46, 36		64, 60, 46, 36	80, 52–55	64, 60, 46, 36

Definition of abbreviation: ER, estrogen receptor.

MCF-7 human breast cancer cells, A549 and H1793 lung adenocarcinoma cells, from male and female patients respectively, were separated into cytosolic, nuclear, and mitochondrial extracts as described in Materials and Methods. Conclusions summarized in this table are from Western blots with HC-20 ERα and H150 ERβ antibodies including those shown in Figures 1 and 2. Bold numbers are highest expression; all values presented are expressed in kilodaltons.

cells (see yellow arrow in Figure 1F). Together, biochemical fractionation/Western and confocal imaging data indicate that the two lung adenocarcinoma cell lines from females have more nuclear ER α compared with the cell lines from males (Tables 1 and 2). The subcellular localization of ER β was independent of cell line and donor sex.

Fluorescence Imaging of ER α and ER β in Lung Adenocarcinoma Cell Lines

Fluorescence microscopy was used to examine ER α and ER β in other lung adenocarcinoma cell lines from males (H23 and H1299) and females (H1395 and H1435) (Figure 2). ER α was in the cytoplasm of H23, H1299, H1395, and H1435 cells (not all data shown). Nuclear ER α was detected in H1395 and H1435 cells (Figure 2A). In H1299 cells, ER α was excluded from nucleoli (Figure 2A). ER β was cytoplasmic in H23 cells, and showed higher cytoplasmic than nuclear signal intensity in H1299, H1395, and H1435 cells (Figure 2B). Each experiment was repeated using at least four independent cell cultures at different times, and are summarized in Table 1. Taken together with the confocal imaging and cellular subfraction studies, we conclude that lung adenocarcinoma cells from females have more nuclear ER α than those from males.

P-ser118-ER α in H1793 and A549 Cells

Because P-ser118-ER α was reported in NSCLC tumors (18) and is a marker of functional, ligand-dependent ER α signaling in breast tumors (25), we tested the hypothesis that P-ser118-ER α levels correspond to estrogenic responses in H1793 cells. Basal P-ser118-ER α was approximately 4.3-fold higher in H1793 compared with A549 cells, and E₂ increased P-ser118-ER α in H1793, but not in A549 cells (Figures 3A and 3B). P-ser118-ER α was exclusively nuclear in H1793 cells (Figure

3C). Dual-label immunofluorescence staining for ER α (green) and P-ser118-ER α (red) revealed more P-ser118-ER α in H1793 than in A549 cells (Figure 3D and Figure E6), confirming conclusions from the subcellular fractionation/Westerns: H1793 cells have more nuclear P-ser118-ER α than A549 cells. ER α was primarily in the perinuclear region of H1793, and showed diffuse staining throughout the cytoplasm and nucleus of A549. The detection of nuclear ER α in A549 cells using antibody AER320 is different from the lack of ER α nuclear staining using antibody HC-20 (Figure 1C). In addition, E₂ increased the intensity of bright nuclear punctuate “speckles” of ER α that overlapped with P-ser118-ER α in H1793, but not in A549 cells (see arrows in Figure E6).

Cyclin D1 in Lung Adenocarcinoma Cell Lines

If E₂ in up-regulation of cyclin D1 (*CCND1*) transcription by genomic ER α activity allows G1-S cell cycle progression in lung adenocarcinoma cells, as reported for breast tumors (26), then E₂ should stimulate higher *CCND1* transcription in H1793 than in A549 cells. Indeed, E₂ increased higher *CCND1* in H1793 than in A549 cells, commensurate with our previous report of greater E₂-transcriptional responsiveness of the female-derived cells (Figure 4A and Ref. 13). The maximum induction was detected at 3 hours of E₂ treatment in H1793 cells, and preincubation with the MAPK inhibitor, PD98059, blocked E₂-induced *CCND1* transcription (Figure 4B). These data are in agreement with a report that MAPK activity is required for E₂-induced cyclin D1 expression and cell cycle progression in MCF-7 cells (27).

To determine if pharmacological inhibition of E₂-stimulated *CCND1* transcription and P-ser118-ER α and cyclin D1 nuclear localization by PD98059 (Figure E7) affected cell proliferation, we compared the effect of ER ligands in the presence or

Figure 4. E₂ induces higher *CCND1* transcription in H1793 than in A549 lung adenocarcinoma cells. (A) A549 and H1793 cell lines were treated with EtOH or E₂ for the indicated time, and *CCND1* expression was determined by real-time qRT-PCR, and is expressed relative to time zero EtOH for each cell line, as described in MATERIALS AND METHODS. Values are the means ($\pm$ SEM) of three to four separate experiments in which each sample was run in triplicate. The maximum induction of *CCND1* was detected after 3 hours of E₂ treatment in H1793 cells. **P* < 0.05, significantly different from EtOH time zero for that cell line. (B) A549 and H1793 cell lines were pre-treated with 50 μ M PD98059 for 1 hour and treated with EtOH or E₂ for 3 hours. *CCND1* is expressed as fold relative to EtOH for each cell line. Values for PD98059 pretreatment are the

average of triplicate determinations. (C) A549 and H1793 cells were treated as indicated with EtOH, 10 nM E₂, 100 nM 4-hydroxytamoxifen (4-OHT), 100 nM ICI 182,780 (ICI) (alone or in combination). Where indicated, cells were pretreated with 50 μ M PD98059 for 1 hour before adding the other indicated treatments. MTT assays were performed as described in MATERIALS AND METHODS. Values are the means ($\pm$ SEM) of three to six separate experiments in which each sample was run in quadruplicate. **P* < 0.05, significantly different from EtOH time zero for that cell line; ***P* < 0.05, significantly different from E₂ alone value for that cell line.

absence of PD98059 pretreatment on A549 and H1793 cell viability/proliferation using the MTT assay (Figure 4C). E_2 selectively increased H1793, but not A549, cell proliferation, and concomitant treatment with 4-OHT or ICI blocked the E_2 stimulation, indicating that the E_2 -induced increase in cell proliferation was ER mediated. These data are in agreement with previous bromodeoxyuridine incorporation data on these cell lines (13). Pretreatment with PD98059 reduced cell viability of both A549 and H1793, although the effect was approximately 20% greater in H1793 than in A549, and blocked E_2 stimulation of H1793 cell proliferation.

Nuclear localization of cyclin D1 is important for cell cycle progression (28), and cyclin D1 is an ER α coactivator (29). Dual-immunofluorescence staining detected nuclear cyclin D1 and P-ser118-ER α colocalization in H1793, but not in the A549 cells (Figure 5A). The intensity of nuclear cyclin D1 and P-ser118-ER α was highest in E_2 -treated H1793 cells in the prometaphase or metaphase stages (Figure 5B). In agreement with our report that E_2 increases proliferation of H1793 cells, but not A549 cells (13), E_2 increased the number of H1793 cells in metaphase, and these cells appeared to have higher levels of cyclin D1 and P-ser118-ER α around the mitotic plate (Figure 5). FACS was used to quantify cell cycle distribution after 6-hour treatment of H1793 and A549 cells with vehicle control (EtOH) or 10 nM E_2 (Table 3). E_2 specifically increased the proportion of H1793 cells in the S and G2/M phases of the cell

cycle, but had no effect on cell-phase distribution of A549 cells (Table 3). Together, these data indicate that E_2 stimulates cell cycle progression in H1793 cells, and not in A549 cells.

E_2 Rapidly Activated MAPK via Nongenomic Signaling in Lung Adenocarcinoma Cells

E_2 rapidly (in 2 min) activates the phospho-p42/p44 MAPK (ERK1/2) pathway in H23 lung adenocarcinoma cells (5, 18), and MAPK phosphorylates ER α on ser118 (9). If the higher P-ser118-ER α in H1793 versus A549 cells (Figure 3) corresponds to differences in E_2 -induced ERK1/2 activation, then more P-ERK1/2 should be detected in E_2 -treated H1793 cells than A549 cells. Indeed, E_2 increased the P-ERK1/2:ERK1/2 ratio in H1793 cells by approximately threefold in 5 minutes, which returned to basal levels after 24 hours (Figure 6A). A549 showed an approximately 1.9-fold increase in P-ERK1/2, peaking at 10 to 60 minutes. Basal P-ERK1/2 was not different in these cell lines (0.25 ± 0.03 in H1793 cells; 0.24 ± 0.02 in A549 cells). The higher E_2 -induced P-ERK1/2 in H1793 cells agrees with an E_2 -induced increase in P-ser118-ER α in H1793 cells, but not in A549 cells. Preincubation with PD98059 (a MEK1 inhibitor), PTX (a G α inhibitor), β -CD (a lipid raft disruptor), or PP2 (a Src kinase inhibitor) before E_2 treatment blocked E_2 -induced ERK1/2 phosphorylation (Figure 6B), indicating functional nongenomic ER-mediated signaling. The reason why PP2

Figure 5. E_2 increases nuclear P-ser118-ER α and cyclin D1 in H1793 lung adenocarcinoma cells. (A) Immunofluorescence staining of control (EtOH)- and E_2 -treated H1793 (top two rows) and A549 (bottom two rows) cells for P-ser118-ER α (green) and cyclin D1 (red). Cells were counterstained with DAPI (blue) to image nuclei. Scale bar, 80 μ m. The red and green scales with Max 5 and Min 1 indicate variation in staining intensity with P-ser118-ER α and cyclin D1 antibodies. (B) Examination of P-ser118-ER α (green) and cyclin D1 (red) in individual cells, counterstained with DAPI (blue) during the indicated phases of the cell cycle after 6 hours of treatment with 10 nM E_2 . Images were captured using identical exposure times. Scale bar, 20 μ m.

TABLE 3. CELL CYCLE ANALYSIS OF ESTRADIOL-TREATED A549 AND H1793 LUNG ADENOCARCINOMA CELLS

Cells and Treatment	G ₀ /G ₁	S	G ₂ /M
A549 (EtOH control)	79.9 ± 0.6	4.9 ± 0.1	12.1 ± 0.3
A549 + 10 nM E ₂	80.2 ± 0.8	5.1 ± 0.1	11.6 ± 0.3
H1793 (EtOH control)	80.4 ± 3	5.1 ± 0.6	9.9 ± 1.6
H1793 + 10 nM E ₂	74.2 ± 0.8*	6.3 ± 0.2*†	14.2 ± 0.5*‡

Definition of abbreviations: E₂, estradiol; EtOH, ethanol.

Briefly, 1×10^6 cells were precultured for 48 hours in Ham's F12 for A549 or serum-free media with hydrocortisone, insulin, transferrin, estrogen, and selenium (HITES) for H1793 cells. Media were supplemented with 5% dextran-coated, charcoal-stripped FCS. Cells were treated with EtOH (vehicle control) or 10 nM E₂ for 6 hours to examine cell cycle distribution at the same time used in immunohistochemical analyses of estrogen receptor α and cyclin D1 subcellular localization. After treatment, the cells were harvested, permeabilized, stained with propidium iodide, and analyzed by FACS as detailed in MATERIALS AND METHODS. All phases are given in percentages; values are the means ($\pm$ SEM) of five independent cultures.

* $P < 0.005$, significantly different between the same treatment between A549 and H1793.

† $P < 0.005$, significantly different from EtOH in the same cell line.

‡ $P < 0.05$, significantly different from EtOH in the same cell line.

stimulated basal ERK activity in H1793 is unknown, but PP2 increased basal MAPK in GH3/B6/F10 rat pituitary cell (30). In contrast, both β -CD and PD98059 inhibited basal ERK activation, results commensurate with the disruption of plasma membrane domains (e.g., caveolae) (5) and inhibition of MEK1 activity, respectively.

Inhibition of MAPK Blocks E₂-Induced Nuclear Accumulation of P-ser118-ER α and Cyclin D1 in H1793 Cells

If nuclear accumulation of P-ser118-ER α and cyclin D1 in E₂-treated H1793 cells, but not A549 cells, is mediated by nongenomic E₂ activation of the MAPK pathway, then pretreatment of H1793, but not A549, with MEK inhibitor, PD98059, should reduce nuclear P-ser118-ER α and cyclin D1. Indeed, this anticipated result was observed: PD98059 blocked the appearance of P-ser118-ER α and cyclin D1 in the nuclei of H1793 cells

and not A549 cells (Figure 7 and Figure E7). These results indicate that nongenomic E₂-signaling through the MAPK pathway leads to nuclear P-ser118-ER α , and thus genomic ER signaling in H1793 cells, but not in A549 cells.

The Antiestrogens, ICI and 4-OHT, Block Nuclear Accumulation of P-ser118-ER α and Cyclin D1 in H1793 Cells

ICI and tamoxifen are well established antagonists of genomic ER that are clinically useful in the treatment of ER-positive breast tumors (31). ICI inhibits cyclin D1 activity, and 4-OHT decreases cyclin D1 expression (32). In contrast to the nuclear colocalization of P-ser118-ER α and cyclin D1 in E₂-treated cells, dual-immunofluorescence staining detected diffuse intracellular distribution of cyclin D1 and P-ser118-ER α in H1793 cells treated with ICI alone or with E₂, and 4-OHT alone or with E₂ (Figure 8). Although the intensity of P-ser118-ER α staining in ICI-treated cells was similar to E₂ treatment, no nuclear P-ser118-ER α was seen, even in cells treated with E₂ and ICI (Figure 8). Although the combined treatment of H1793 cells with E₂ and either ICI or 4-OHT did not completely inhibit nuclear localization of cyclin D1 in all cells, it is noteworthy that ICI or 4-OHT treatment blocked nuclear colocalization of cyclin D1 with P-ser118-ER α , as these two ER antagonists clearly blocked nuclear P-ser118-ER α accumulation.

DISCUSSION

There is increasing evidence that estrogens, including those produced locally by aromatase, stimulate lung adenocarcinoma cell proliferation (5, 6, 14, 33). Recently, the first mouse model demonstrating that E₂ stimulates lung adenocarcinoma tumor formation in both ovariectomized females and intact males was reported (34). Here, we demonstrate, for the first time, that nuclear P-ser118-ER α is higher in E₂-transcriptionally responsive H1793 lung adenocarcinoma cells from females than in A549 non-E₂-responsive lung adenocarcinoma cells from males. Phosphorylation of ER α on ser118 is required for transcriptional activity (9), and indicates an intact E₂-dependent ER α signaling pathway in primary breast tumors *in vivo* (25). Ser-118-ER α

Figure 6. E₂ rapidly induces phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in lung adenocarcinoma cells. (A) H1793 and A549 cells were treated with EtOH (t₀) or 10 nM E₂ for the indicated times. (B) H1793 and A549 cells were pretreated with 50 μ M PD98059 (MEK1 inhibitor, 1 h), 100 ng/ml pertussis toxin (PTX; a G α inhibitor, 17 h), 5 mM methyl- β -cyclodextrin (β CD; a lipid raft disruptor, 30 min), or 10 μ M PP2 (Src kinase inhibitor, 30 min), before EtOH (t₀) or 10-minute E₂ treatment. The values are the relative levels of P-ERK1/2:total ERK1/2 compared with the EtOH t₀ for each cell line, and are the means ($\pm$ SEM) from three experiments. * $P < 0.05$ compared with t₀ values for that cell line; * $P < 0.05$ compared with t₁₀ E₂ values for that cell line.

Figure 7. MEK1 inhibitor blocks nuclear accumulation of P-ser118-ER α and cyclin D1 in H1793 cells, but not in A549 cells. H1793 and A549 cells were pretreated with 50 μ M PD98059 for 1 hour and treated with EtOH (t_0) or 10 nM E₂ for 6 hours. The cells were stained with P-ser118-ER α antibody (green) and cyclin D1 antibody (red) for dual-label fluorescence immunostaining, as described in MATERIALS AND METHODS. Images of nuclei (DAPI), ER α , cyclin D1, and the merged image were captured at identical times of exposure for each cell line and treatment.

phosphorylation is dependent on E₂ activation of the Ras-Raf-MAPK (ERK) pathway (9). Here, we show that nuclear P-ser118-ER α corresponds to higher nongenomic E₂-activation of MAPK activity in H1793 cells than A549 cells. Furthermore, we demonstrate that the MEK inhibitor, PD98059, blocked E₂-induced nuclear localization of P-ser118-ER α and cyclin D1 in H1793 cells, while having no effect on ER α or cyclin D1 in A549 cells. The increase in nuclear P-ser118-ER α offers a mechanism to explain, in part, the observed differences in E₂-induced ERE-luciferase activity in H1793 cells, but not in A549 cells, as we reported previously (13). Furthermore, data from the MTT assay, showing that pharmacologic inhibition of MAPK signal-

ing with PD98059 inhibits E₂-induced cell proliferation in H1793 cells, indicate a connection(s) between E₂-induced MAPK signaling with ER α phosphorylation, *CCND1* transcription, cyclin D1 nuclear localization, and cell proliferation in H1793 cells, but not in A549 cells. The inhibition of E₂-activated ERK by PP2, PD98059, and β -CD agrees with previous reports of rapid E₂-induced MAPK activation via nongenomic ER signaling (reviewed in Ref. 11) in H23 (5, 18) and 201T (33) NSCLC cell lines. Our H1793 data agree with a report showing that the maximum P-ser118-ER α levels occurred after 4–6 hours of E₂ treatment in MCF-7 cells (35).

E₂ increased higher levels of *CCND1* gene transcription in H1793 cells compared with A549 cells, data corresponding with the higher E₂-induced MAPK activation, nuclear P-ser118-ER α , and nuclear localization of cyclin D1 in H1793 cells. In addition, we detected colocalization of P-ser118-ER α and cyclin D1 in the nucleus of actively dividing H1793 cells, but not A549 cells, supporting a role for this interaction for cell replication (36, 37). Importantly, these data correspond to those of our previous report that E₂ selectively, and in an ER-dependent manner, increased proliferation, ERE-driven reporter gene transcription, and induction of selected E₂ target genes in H1793 cells and other lung adenocarcinoma cell lines from females, but not in A549 cells and other cell lines from males (13). These studies support a role for estrogens and other growth factors, the pathways of which intersect with ER signaling mechanisms in promoting the growth of NSCLC cells in female patients.

Cyclin D1 is a negative prognostic factor in lung cancer (38). Here, we demonstrate, for the first time, nuclear colocalization of cyclin D1 and P-ser118-ER α in female-derived H1793 cells, whereas cyclin D1 was perinuclear in male-derived A549 cells. Furthermore, A549 cells had significantly lower P-ser118-ER α compared with H1793 cells. These data indicate that a potential mechanism by which E₂ selectively stimulates proliferation of H1793 and not A549 cells (as shown in Ref. 13) may involve cyclin D1 acting as a coactivator (29) for P-ser118-ER α

Figure 8. The antiestrogens, ICI and 4-OHT, block nuclear accumulation of P-ser118-ER α and cyclin D1 in H1793 lung adenocarcinoma cells. H1793 cells were pretreated with either EtOH or 100 nM ICI for 6 hours, and then treated with EtOH, 10 nM E₂, ICI alone or with 10 nM E₂, or 100 nM 4-OHT alone or with 10 nM E₂ for 6 hours. The cells were stained with P-ser118-ER α antibody (green) and cyclin D1 antibody (red) as described in MATERIALS AND METHODS. Images of nuclei (DAPI), ER α , cyclin D1, and the merged image were captured at identical times of exposure for each treatment. Scale bar, 20 μ m.

activation of *CCND1* (and other) genes involved in cell cycle progression.

Our findings, that NF1604 and male-derived lung adenocarcinoma A549 and H1792 cells have no or lower nuclear ER α compared with H1793, H1944, H1395, and H1435 lung adenocarcinoma cells from females and MCF-7 cells, indicate that proliferative and genomic activity of E₂, which were specific for lung adenocarcinoma cell lines from females (13), correlate with more nuclear ER α in female-derived cells. Moreover, these observations also agree with higher nuclear ER α in NSCLC tumors from female than from male patients (49 and 35%, respectively) (18).

ER α splice variants have been detected in various normal and neoplastic tissues, including lung cancer (39). We observed discrepancies in ER α recognition between HC-20 and AER320 ER α antibodies. HC-20 revealed 80- and 52- to 55-kD ER α bands in Western blots, but did not recognize full-length (66-kD) ER α in NF1604 or the lung cancer cell lines. Band specificity was demonstrated (Figure E3). In contrast, as seen here in Western blots and reported previously (13), AER320 detected full-length ER α in WCE and NE of the lung adenocarcinoma cell lines and NF1604 lung fibroblasts. Discrepancies in ER α immunoreactivity in NSCLC have been attributed to variability in epitope recognition (reviewed in Ref. 6).

Similar to our findings, 80- and 52- 55-kD ER α , but no full-length ER α , were reported in WCE from lung tumor cell lines, including A549 and H23 cells using HC-20, and were attributed to ER α exons 6 and 7 duplication and exon 4 deletion (80 and 55 kD, respectively) (14). In agreement with reports that ER α Δ 4 is cytoplasmic and unable to bind ligand or DNA (24), we observed a band corresponding to ER α Δ 4 in the cytoplasm of A549 and H1793 cells, but not in the nucleus of H1793 or MCF-7 cells. HC-20 was reported to recognize ER α Δ 2, Δ 2–3, and Δ 7 (58, 54, and 52 kD, respectively) (24). Here, PCR confirmed ER α Δ 7 mRNA in H1793 and MCF-7 cells. Although not previously identified in lung adenocarcinomas, ER α Δ 7 is the most common variant in breast and other cancers, and lacks ligand-dependent activation function–2, resulting in “dominant-negative activity” (24). ER α Δ 7 is nuclear, binds EREs *in vitro* and *in vivo*, and may act as a monomer (40). Because ER α Δ 7 competes with WT ER α , an increase in ER α Δ 7 may result in an E₂-resistant phenotype (40).

Although we have not excluded the possibility that the ER α Δ 2 and ER α Δ 2–3 splice variants may be in H1793 nuclei, these variants lack DNA-binding and dimerization domains, and are unable to affect WT ER α signaling (24). Therefore, ER α Δ 2 or Δ 2–3 would not be expected to affect ER genomic activity in the lung adenocarcinoma cells.

The 80-kD ER α protein detected in lung adenocarcinoma cell lines by HC-20 here, and as reported by others (14), has a duplication of exons 6 and 7, binds E₂ or tamoxifen, and was originally identified in MCF-7 cells (24). We suggest that the 80-kD and Δ 7 ER α splice variants may contribute to estrogen signaling in lung adenocarcinoma cells, but further studies will be necessary to probe the identity and parse the roles that ER α splice variants play in normal lung and in lung adenocarcinoma.

Because we detected full-length ER α using AER320, but not HC-20 (Figure 1), an alternative to epitope availability differences in ER α splice variants is that there may be post-translational modification of the F domain that precludes HC-20 interaction. The F domain contributes to ER α activity, is required for tamoxifen's agonist activity, and modulates ER α interaction with coregulators, including SRC-1 (41). Further studies will be required to identify post-translation modification(s) of the F domain of ER α in normal lung and in lung adenocarcinoma.

The data reported here demonstrate that, whereas ER β is largely cytoplasmic, A549 cells had proportionally more ER β in the nucleus than ER α when compared with H1793 and MCF-7 cells. These observations offer another possible mechanism for the lack of E₂-induced reporter gene activity and gene transcription detected in the A549 cell line (13). Because heterodimerization of ER β with ER α inhibits ER α -mediated transcriptional activity (42), an increase in ER β relative to ER α could block ER α 's transcriptional activity in lung adenocarcinoma cells. A new study reported that ER β is cytoplasmic, and not phosphorylated in response to E₂ in NSCLC cell lines, including 201T, Calu-6, and A549 cells, but did not examine ER α phosphorylation (43).

In conclusion, our results demonstrate nuclear localization of WT and splice variants of ER α in female-derived, but not male-derived, lung adenocarcinoma cells. E₂-induced activation of ERK1/2 signaling results in higher nuclear P-ser118-ER α and cyclin D1 in E₂-transcriptionally responsive H1793 compared with E₂-nonresponsive A549 lung adenocarcinoma cells. Importantly, these data correlate with our previous report of intact E₂-activated ER α genomic signaling in the H1793 female-derived, but not A549 male-derived, lung adenocarcinoma cells (13). Our findings imply that estrogens may affect lung adenocarcinoma cells through integration of nongenomic and genomic pathways in a cell line- and sex-dependent manner.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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