Gender difference in the activity but not expression of estrogen receptors α and β in human lung adenocarcinoma cells

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Abstract

The higher frequency of lung adenocarcinoma in women smokers than in men smokers suggests a role for gender-dependent factors in the etiology of lung cancer. We evaluated estrogen receptor (ER) α and β expression and activity in human lung adenocarcinoma cell lines and normal lung fibroblasts. Full-length ERα and ERβ proteins were expressed in all cell lines with higher ERβ than ERα. Although estradiol (E₂) binding was similar, E₂ stimulated proliferation only in cells from females, and this response was inhibited by anti-estrogens 4-hydroxytamoxifen (4-OHT) and ICI 182,780. In contrast, E₂ did not stimulate replication of lung adenocarcinoma cells from males and 4-OHT or ICI did not block cell proliferation. Similarly, transcription of an estrogen response element-driven reporter gene was stimulated by E₂ in lung adenocarcinoma cells from females, but not males. Progesterone receptor (PR) expression was increased by E₂ in two out of five adenocarcinoma cell lines from females, but none from males. E₂ decreased E-cadherin protein expression in some of the cell lines from females, as it did in MCF-7 breast cancer cells, but not in the cell lines from males. Thus, ERα and ERβ expression does not correlate with the effect of ER ligands on cellular activities in lung adenocarcinoma cells. On the other hand, coactivator DRIP205 expression was higher in lung adenocarcinoma cells from females versus males and higher in adenocarcinoma cells than in normal human bronchial epithelial cells. DRIP205 and other ER coregulators may contribute to differences in estrogen responsiveness between lung adenocarcinoma cells in females and males.

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Introduction

While the number of women dying as a result of metastatic breast and colon cancer is declining, the mortality associated with lung and bronchus cancer in females continues to rise (Greenlee et al. 2000). Lung cancer is the leading cause of cancer death in both women and men in the United States (Patel et al. 2004). Despite advances in chemotherapy for treating lung cancer, the 5-year survival rate has not increased significantly over the last 25 years, remaining at approximately 14% (Williams & Sandler 2001). The 2-fold higher frequency of lung cancer in women smokers than in men smokers (Shields 2000) strongly suggests the involvement of gender-dependent factors in the etiology of lung cancer (Omoto et al. 2001). Despite smoking for shorter periods of time, with fewer cigarettes per day and inhaling less deeply than men, women have a higher incidence of lung cancer, notably lung adenocarcinoma, a type of non-small cell lung cancer (NSCLC) (Stabile & Siegfried 2003). In addition, women non-smokers are at a 2.5-fold greater risk than male non-smokers for developing lung adenocarcinoma (Siegfried 2001).
The mechanisms underlying the gender difference in NSCLC incidence is likely to be multifactorial. For example, women are more susceptible to smoking-induced DNA damage than men (Stabile & Siegfried 2003). However, mutations of p53 and K-ras that are regarded as early events in carcinogenesis are rarely found in adenocarcinomas, which account for 75% of lung cancer in females (Hashimoto et al. 2000). Three of the nine adenocarcinoma cell lines used in this study have activating K-ras mutations, but no gender-dependent differences were apparent in this small sample. Differences in the expression, gene polymorphisms and activity of phase I (e.g. CYP1A1 (Mollerup et al. 1999)) and II drug metabolizing enzymes (e.g. glutathione-S-transferase (GST) and N-acetylttransferase (NAT) (Stabile & Siegfried 2003)), may play a role in gender differences. Interestingly, over-expression of the protooncogene c-erbB2/HER2/neu, a ligand-independent epidermal growth factor receptor, is associated with poor prognosis in NSCLC (Gatzemeier et al. 2004) as well as breast cancer (Pegram et al. 1999).

The gender differences in adenocarcinoma implicate hormones in lung cancer risk. Estrogens increase the risk of breast cancer (Wolff et al. 1996) and oral contraceptive therapy (OCT) is protective against ovarian and endometrial cancer (Boyle et al. 2000). However, the role of estrogen in lung cancer is unclear. Some studies, reviewed by Stabile and Siegfried (2003), indicate a role for estrogen in lung cancer risk. For example, one study noted a positive correlation between post-menopausal estrogen replacement therapy, smoking and lung adenocarcinoma (Taioli & Wynder 1994). A role for estrogen in the etiology of squamous cell carcinoma (SCC) in a Chinese population was suggested by a correlation between SCC and a higher number of menstrual cycles (Liao et al. 1996). Likewise, a large clinical trial in breast cancer patients showed that more women taking the anti-estrogen tamoxifen had a second primary non-breast cancer, including lung cancer, compared with those taking the aromatase inhibitor exemestane, although the differences were not statistically significant (Coombes et al. 2004). On the other hand, the higher survival rates for women than men with NSCLC in a study of 14676 women may indicate a protective effect of estrogen (Moore et al. 2003). Indeed, a recent study reported that post-menopausal users of hormone replacement therapy (HRT) were at lower risk of developing lung cancer and that the protective effect of HRT was mainly observed in current smokers who were also the ‘lightest smokers’, i.e. <22 pack-years (Schabath et al. 2004). Clearly, further studies are needed to investigate the role of estrogens in lung cancer risk.

Estrogens exert their molecular action by interaction with two subtypes of estrogen receptor (ER), ERα and ERβ. In the original characterization of ERα and ERβ mRNA tissue distribution, in rats, ERβ was predominant in lung (Kuiper et al. 1997). There was no reported lung phenotype for ERα null (αERKO) mice (Couse et al. 1997, Rubanyi et al. 1997), but a comparison of the lungs of wild-type (wt) versus ERβ null (βERKO) mice revealed decreased numbers of alveoli in adult female ERβ−/− mice (Patrone et al. 2003). Lungs of female βERKO mice also showed decreased surfactant, platelet-derived growth factor A (PDGF-A), and granulocyte-macrophage colony-stimulating factor (GM-CSF), indicating that ERβ plays a role in lung homeostasis in mice (Patrone et al. 2003). Immunohistochemical staining revealed ERβ expression in human lung in columnar epithelium and in intermediate, basal and smooth muscle cells whereas ERα was expressed in basal and smooth muscle cells (Taylor & Al-Azzawi 2000). ERα and ERβ have been proposed to play opposite roles, i.e. ‘yin and yang’, in cell proliferation with ERα being proliferative and ERβ anti-proliferative (Lindberg et al. 2003). For example, ERα expression is increased in breast cancer and over-expression of ERβ inhibits estradiol (E2)-stimulated breast cancer cell proliferation (Paruthiyil et al. 2004). Whether the ERα:ERβ ratio increases in lung cancer is unknown.

There are a limited number of studies with conflicting data regarding ERα and ERβ expression in human lung cancer and in lung cancer cell lines (Croxtall et al. 1994, Caltagirone et al. 1997, Omoto et al. 2001, Radzikowska et al. 2002, Stabile et al. 2002, 2005, Hershberger et al. 2005) and only two studies directly comparing ER expression in lung cancer specimens and cell lines in males and females (Fasco et al. 2002, Mollerup et al. 2002). One study reported similar levels of ERα and ERβ in females and males (Mollerup et al. 2002). In contrast, another group reported higher ERα expression in females than in males whereas ERβ expression was similar (Fasco et al. 2002). A general conclusion from these reports is that lung tumors from women are more likely to express ERα than tumors from men, with ranges from 7–97% by immunohistochemistry (IHC), and that ‘the potential role of estrogens in lung cancer is understudied’ (Patel 2005).

It is well-established that E2 stimulates the proliferation of estrogen-responsive tissues and cell lines, e.g. human breast and endometrial cells. In contrast to the many inconsistent reports on ER expression, there are only four reports examining the functional
consequences of ER expression in lung adenocarcinoma cell lines (Stabile et al. 2002, 2005, Hershberger et al. 2005, Pietras et al. 2005). The studies revealed that E2 stimulated proliferation of lung adenocarcinoma cell lines from males, e.g. NCI-H23 (Table 1), in vitro in an ER-dependent manner (Stabile et al. 2002, 2005, Pietras et al. 2005) and one study reported that E2 increased E-cadherin and Id-2 expression in 201T cells, also from males (Hershberger et al. 2005).

The variability of ERα and ERβ expression between studies and the paucity of functional studies on ER in lung adenocarcinoma indicate that more information is needed to determine the significance and role of these receptors in NSCLC. In particular, no one has directly compared the effect of E2 and anti-estrogens on the proliferation or estrogenic responses of lung adenocarcinoma cell lines from male versus females in a side-by-side comparison.

In this study, we used nine different human adenocarcinoma cell lines from male or female lung cancer patients to test their estrogen responsiveness. We demonstrate that the human adenocarcinoma cell lines, A549, NCI-H23, NCI-H1299, NCI-H1792, NCI-H1395, NCI-H1435, NCI-H1793, NCI-H1944, and NCI-H2073, express ERα and ERβ proteins. Although no differences in the levels of ERα and ERβ proteins or in [3H]E2 binding in cells from males or females were detected, there were significant differences in cellular biochemical responses to E2 and the anti-estrogens 4-hydroxytamoxifen (4-OHT) and ICI 182,780 between cell lines from males and females. Whereas the proliferation of the cell lines from females was stimulated by E2 and blocked by concomitant administration of 4-OHT or ICI 182,780, the cell lines from males were non-responsive to these treatments. Similar results were detected at the transcriptional level. Although other investigators have demonstrated responses of lung adenocarcinoma cell lines from males to selective ER modulators (SERMs) including fulvestrant (Stabile et al. 2002, 2005, Hershberger et al. 2005), our data support the possible use of SERMs such as tamoxifen or fulvestrant (ICI 182,780) for selectively treating women with lung adenocarcinomas, although further studies will be needed to extend these suggestions.

### Materials and methods

#### Chemicals

E2 and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma. ICI 182,780, 4,4’,4’-4-propyl-[1H]-pyrazole-1,3,5-triyltriphenol (PPT, an ERα-selective agonist) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, an ERβ-selective agonist) were purchased from Tocris (Ellisville, MO, USA). The R,R enantiomer of 5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (R,R-THC) was generously provided by Dr J A Katzenellenbogen of the University of Illinois (Sun et al. 1999).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sex of patient</th>
<th>Smoker/non-smoker (NS)</th>
<th>Reported ER status (reference)</th>
<th>K-ras mutation (codon GGT-GTT; aa G12C) (<a href="http://www.sanger.ac.uk">http://www.sanger.ac.uk</a>, unless otherwise indicated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>male</td>
<td>unknown</td>
<td>• no ERα (Croxtall et al., 1994)</td>
<td>K-ras point mutation (Okudela et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Low ERα and high ERβ</td>
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<td></td>
<td></td>
<td></td>
<td>(Stabile et al., 2002)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• No ERα, but ERβ (Hershberger et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>NCI-H23</td>
<td>male</td>
<td>smoker</td>
<td>Low ERα and high ERβ (Stabile et al., 2002)</td>
<td>K-ras point mutation (ATCC information)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ERα and ERβ (Pietras et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>NCI-H1299</td>
<td>male</td>
<td>smoker</td>
<td>• ERβ &gt; ERα (Mollerup et al., 2002)</td>
<td>No K-ras mutation, but mutant N-ras</td>
</tr>
<tr>
<td>NCI-H1792</td>
<td>male</td>
<td>smoker</td>
<td>• ERα; &lt; 10% of the level of ERα; in MCF-7 cells (Mollerup et al., 2002)</td>
<td>No K-ras mutation</td>
</tr>
<tr>
<td>NCI-H1395</td>
<td>female</td>
<td>smoker</td>
<td></td>
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</tr>
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<tr>
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<tr>
<td>NCI-H1944</td>
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<td>K-ras mutation</td>
</tr>
<tr>
<td>NCI-H2073</td>
<td>female</td>
<td>smoker</td>
<td></td>
<td>No K-ras mutation</td>
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</table>
Cell lines
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 (Manasas, VA, USA) and were maintained in the recommended media and supplements. The characteristics of the lung adenocarcinoma cell lines are listed in Table 1. The primary fibroblast cell strain GM1604 (Coriell Institute, Camden, NJ, USA) was originally derived from human fetal lung tissue, and was immortalized by expression of the catalytic subunit of human telomerase (hTERT), under a license from Geron Corporation (Menlo Park, CA, USA), by Dr L McDaniel (University of Texas Southwestern Medical Center, Dallas, TX, USA) and named NF1604. The cells were karyotyped and found to be 46XY (Ouellette et al. 2000). They were provided to WGM by Dr McDaniel under the terms of Material Transfer Agreement 3025 between WGM and Geron Corporation. NHBE cells were purchased from Cambrex (Walkersville, MD, USA) and maintained in bronchial epithelial growth medium supplemented with BulletKit growth factors (Cambrex).

Cell proliferation/MTT assay
Cell proliferation was determined using the Cell Proliferation Kit 1 (MTT) (Roche) and Cell Titer 96 AQueous One solution cell proliferation assays (Promega) according to the manufacturers’ protocols. Briefly, 2 × 10^3 cells were plated per well in 96-well plates in phenol-red-free media containing 10% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS) for 24 h prior to treatment. The cells were treated with ethanol (EtOH), ICI 182,780, E2, trans-resveratrol, 4-OHT or other compounds (see figure legends for details) for 4 days. Treatments were replenished after 48 h. The absorbance of solubilized formazan product was measured at 570 nm (MTT) and 490 nm (Cell Titer 96) directly in each well. Within each experiment, each treatment was performed in quadruplicate and values were averaged. Values were compared with those in the wells treated with vehicle (EtOH) control which was set to 100%. At least four separate experiments were performed for each cell line.

RNA extraction and quantitative real time RT-PCR
Cells were grown to 50–70% confluency in 100 mm ×20 mm cell culture dishes in phenol-red-free media containing 10% DCC-FBS for 24 h prior to treatment with EtOH (vehicle control), E2 or other compounds indicated in the text for 6 h. Cells were washed three times in PBS, and RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol followed by purification on RNeasy columns (Qiagen, Valencia, CA, USA). RNA quality was determined using the RNA 6000 Nano Assay kit on the Agilent 2100 Bioanalyzer (Wilmington, DE, USA). RNA concentration was determined by absorbance at 260 nm. Total RNA was reverse-transcribed using random hexamers and the High Capacity cDNA archive kit (PE Applied Biosystems (ABI), Foster City, CA, USA). The QIAquick PCR purification kit (Qiagen) was used to purify cDNA.

ERα, ERβ and 18S rRNA Taqman primers and probes were purchased as Assays-on-Demand Gene Expression Products (ABI). Expression of genes was determined for each target gene in triplicate using 20 ng of purified sample cDNA in each experiment and replicated twice for a total n = 3. Each sample was normalized using 18S rRNA. The efficiency of each primer/probe set was evaluated by concentration optimization of primers, probes and input cDNA generating a standard curve with serial dilutions of untreated RNA. The efficiencies of real-time PCR of ERα, ERβ and 18S were 99.5, 99.5 and 97.9% with correlations of 0.986, 0.968 and 0.942 respectively. Real-time PCR was performed in the ABI PRISM 7700 SDS 2.1 using relative quantification with the following thermal cycler conditions: hold at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in a 20 μl reaction in 96-well plates. Each 96-well plate also included minus RT and reaction mix without cDNA as negative controls. Analysis and fold differences were determined using the comparative cycle threshold (Ct) method as described in ABI Technical Bulletin 2 (Bustin 2002, Ginzinger 2002). Fold change was calculated from the ΔΔCt values with the formula 2−ΔΔCt and is given as percentage relative expression compared with MCF-7 cells.

Preparation of whole-cell extracts (WCEs)
Each cell line was grown in its corresponding culture medium supplemented with 10% DCC-FBS, 1% penicillin/streptomycin plus the indicated concentrations of chemicals for 24 h prior to harvest. When cell treatment experiments were performed, cells were grown in phenol-red-free media supplemented as above plus the treatment(s) indicated in the figure legends and text. WCEs were prepared in RIPA buffer (50 mM Tris–HCl, pH 7.4; 1% Nonidet P-40; 0.25%
Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); aprotinin, leupeptin and pepstatin, each at 1 µg/ml; 1 mM Na3VO4; 1 mM NaF). Protein concentrations were determined using BioRad’s DCC assay (Hercules, CA, USA).

Specific [3H]E2 binding assay

Nuclear and cytoplasmic fractions (NE and CE) were prepared from each cell line using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer’s recommendations. Specific [3H]E2 binding to baculovirus-expressed recombinant human ERα (rhERα) and rhERβ (Kulakosky et al. 2002, Kulakosky & Klinge 2003) or to the NE, CE and WCE from the lung cell lines was measured in the presence of 200-fold molar excess of ‘cold’ E2 by adsorption to hydroxyapatite (HAP) as previously described (Kulakosky et al. 2002).

Western blotting

Identical amounts of whole-cell lysate extract (25–40 µg protein) were separated on 10% SDS-PAGE gels and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation, Pensacola, FL, USA). As positive and negative controls, known amounts (in fmols from HAP assays) of baculovirus-expressed rhERα and rhERβ, purchased from Panvera or produced in Sf21 cells (Kulakosky et al. 2002, Kulakosky & Klinge 2003) were separated in parallel with WCE samples. The transfer was monitored by pre-stained molecular weight markers (Precision Standard, BioRad). Following transfer, membranes were blocked in 5% milk Tris-buffered saline (TBS)–Tween then probed with antibodies: anti-ERα monoclonal antibody AER320 recognizes aa 495–595 (LabVision-Neomarkers, Fremont, CA, USA); anti-ERβ polyclonal antibody H-150 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognizes aa 1–150 of hERβ and N-19 (Santa Cruz Biotechnology) also recognizes the N-terminus of hERβ; anti-progesterone receptor (PR) monoclonal antibody AB-8 recognizes the N-terminal half of hPR (LabVision-Neomarkers). E-cadherin antibody (no. 4065) was purchased from Cell Signaling (Beverly, MA, USA). Blots were stripped and re-probed with anti-β-actin (Sigma) or α-tubulin (LabVision-Neomarkers) to normalize for protein loading. The antibody to DRIP205 was kindly provided by Dr M Garabedian (Pineda Torra et al. 2004). Super Signal West Pico Chemiluminescent Substrate (Pierce) was used to detect protein bands. Resulting immunoblots were scanned into Adobe Photoshop 7.0 using a Microtek ScanMaker III scanner (Carson, CA, USA). Un-Scan-It (Silk Scientific, Orem, UT, USA) was used to quantitate the integrated optical densities (IOD) for each band. The IOD for ERα, ERβ, PR, and E-cadherin were divided by concordant β-actin IOD in the same blot.

Transient transfection assay

For transient transfection, cells were plated in 24-well plates at a density of 1.5 × 10^4 cells/well in phenol-red-free OPTI-MEM I reduced serum medium (Gibco/Invitrogen) supplemented with 10% DCC-FBS, 1% penicillin/streptomycin. Transient transfection was performed using FuGene6 (Roche). Each well received 250 ng of a pGL3-pro-luciferase reporter (Promega) containing two tandem copies of a consensus estrogen response element (ERE; i.e. EREc38 (Tyulmenkov et al. 2000)) and 5 ng Renilla luciferase reporter (pRL-tk) from Promega. At 24 h after transfection, triplicate wells were treated with EtOH (vehicle control), E2, 4-OHT, DPN, PPT, ICI 182,780, resveratrol or other compounds as indicated in the figures. The cells were harvested 30 h post-treatment using Promega’s Passive Lysis buffer. Luciferase and Renilla luciferase activities were determined using Promega’s dual-luciferase assay in a Plate Chameleon luminometer (BioScan, Washington, DC, USA). Firefly luciferase was normalized by Renilla luciferase to correct for transfection efficiency. Fold induction was determined by dividing the averaged normalized values from each treatment by the EtOH value for each transfection condition within that experiment. Values were averaged from multiple experiments as indicated in the figure legends.

Statistics

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 (San Diego, CA).

Results

E2 stimulates, and 4-OHT and ICI 182,780 inhibit, the proliferation of female lung adenocarcinoma cell lines. We examined the effect of E2, 4-OHT, ICI 182,780 and R,R-THC (a selective ERα agonist/ERβ antagonist (Sun et al. 1999)) alone or in combination on the proliferation of lung adenocarcinoma cell lines from females (Fig. 1) and males (Fig. 2). Of the cell lines from females, H1395 showed the most stimulation by E2 and H2073 showed the least. The magnitude of
E2-induced proliferation is consistent with that seen in MCF-7 breast cancer cells (Fig. 1F) and reported for the 201T adenocarcinoma cell line (Hershberger et al. 2005). E2 stimulation was blocked by 4-OHT — which is an active metabolite of the drug tamoxifen, used clinically to block ER action in breast cancer (Jordan & Pappas 2003) — and ICI 182,780 (Faslodex), a pure steroidal ER antagonist. 4-OHT inhibited proliferation in all cell lines from females. The inhibition of E2-induced proliferation by R,R-THC, an ER\(\beta\)-selective antagonist/ER\(\alpha\) agonist (Sun et al. 1999), was similar to that with ICI 182,780, but less than 4-OHT, suggesting the possibility that ER\(\beta\) may have a role in cell replication.

In contrast to the results in the lung adenocarcinoma cell lines from females, E2 had no effect on the proliferation of NF1604 (Fig. 2A) or any of the adenocarcinoma cells from males (Fig. 2B–E). The lack of E2-induced proliferation of H23 cells contradicts the stimulatory effect of E2 reported previously...
The reason for this discrepancy may result from the 24-h serum-free incubation of the H23 cells prior to E2 treatment in the previous report (Stabile et al. 2002). In contrast, we maintained all cell lines in 10% DCC-FBS for 24 h prior to hormone treatment. Thus, the hormone/ligand effects we measured may be reduced in magnitude compared with serum-deprived cells.

ICI 182,780 (Faslodex) had no effect on the proliferation of lung adenocarcinoma cell lines from males. 4-OHT inhibited the proliferation of H1299 and H1792 cells by ~20% (Fig. 2C and D). In the case of H1299, the combination of E2 and 4-OHT also inhibited proliferation by approximately 25%. Notably, 4-OHT alone or combined with E2 had a greater inhibitory effect on the proliferation of adenocarcinoma cells from females (Fig. 1) than males; i.e. reducing proliferation by 50–75% versus 20% respectively. R,R-THC did not affect the proliferation of any of the cell lines from males, whether alone or in combination with E2. We conclude that lung adenocarcinoma cell lines from females are more responsive to E2, 4-OHT, ICI 182,780 and R,R-THC compared with cell lines from males.

**Figure 2** ER ligands do not affect the proliferation of NF1604 normal lung fibroblasts or adenocarcinoma cell lines from males. NF1604 (A), H23 (B), H1299 (C), H1792 (D) and A549 (E) lung adenocarcinoma cells, all from males, were treated with the indicated concentrations of E2, 4-OHT, ICI 182,780 and R,R-THC; or the indicated combinations thereof for 4 days as described in the Materials and methods section and Fig. 1. Cell proliferation was measured by MTT assay as described in the Materials and methods. Values are expressed as relative to EtOH control. Each bar represents the mean ± S.E.M. of at least six independent experiments; a, significantly different from control (EtOH) values, P<0.05.
Expression of ERα and ERβ mRNA and protein in lung adenocarcinoma cells

One possible explanation for the lack of estrogen responsiveness in the lung adenocarcinoma cells from males is that they do not express ER or express ER at reduced levels compared with the cells from females. Of the nine adenocarcinoma cell lines used in this study, the ER status has been reported in A549, H23 and H1435 (Table 1). It is noteworthy that contradictory findings were reported (Table 1).

Quantitative real-time RT-PCR was used to determine ERα and ERβ mRNA expression in NHBE, NF1604 and the lung adenocarcinoma cell lines (Fig. 3A). Fold differences in gene expression were determined by the comparative \( C_T \) method using 18S rRNA as the endogenous control. MCF-7 cells were used as the calibrator and expressed more ERα mRNA than NHBE, NF1604 or any of the lung adenocarcinoma cell lines. ERβ mRNA expression was higher than ERα expression in all the lung cell lines. There was no difference in ERα mRNA expression between NHBE, NF1604 and the lung adenocarcinoma cell lines. H23, H1299, H1395, H1792, H1793, H2073 and NF1604 expressed higher levels of ERβ mRNA than MCF-7 cells.

To determine ERα and ERβ protein expression, known concentrations, as assayed by [\(^3\)H]E2 binding (Kulakosky et al. 2002), of baculovirus-expressed rhERα and rhERβ protein were separated in parallel with the WCEs of the lung adenocarcinoma cell lines and used as calibrators to estimate ERα and ERβ expression levels by Western blot (Fig. 3B–E). All cell lines expressed full-length ERα and no shorter ERα products were detected (Fig. 3B and C). These data indicate that intact ERα is expressed in the adenocarcinoma cell lines. Our data differ from a report that MCF-7, H23 and A549 expressed ERα as 80 and 54 kDa bands, but that only MCF-7 expressed 67 kDa ERα (Stabile et al. 2002). ERα expression in NHBE and all adenocarcinoma cell lines was similar to MCF-7 cells whereas NF1604 was lower (Fig. 3F).

As antibodies have a history of showing cross-reactivity with ERα, we used two different antibodies to examine ERβ expression. We have demonstrated the specificity of the AER320 ERα and H150 ERβ antibodies for their respective ER subtype (Klinge et al. 2005). The N-19 antibody only recognized the
ERβ1 60 kDa band (Fig. 3D), i.e. the ‘long’ isoform, and not the ERβ1s ‘short’ isoform of ERβ (Scobie et al. 2002). Western blots using the H150 ERβ antibody indicated that most cells express the ERβ1 and ERβ1s isoforms as 61–64 and 50–53 kDa sizes respectively (Fig. 3E). A summary of the estimated ERα and ERβ expression in the cells based on Western blotting is presented in Fig. 3F. ERβ expression was greater than ERα expression in all lung adenocarcinoma cell lines, and in NHBE and NF1604 cells than in MCF-7 cells. NHBE cells showed higher ERβ expression than NF1604, MCF-7 or any of the lung adenocarcinoma cell lines. Others have reported ERβ1 migrating as 62–64 kDa in human tissues (Choi et al. 2001) and ERβ from mouse lung was 65 kDa (Patrone et al. 2003). As ERβ splice variants ERβ2/cxL and ERβ2/cxS are expressed in human tissues as 55–51 kDa proteins, it is possible that the lower ERβ band detected in the Western blots shown here may represent more than one isoform of ERβ. Further testing will be

**Figure 4** Lack of estrogen responsiveness of NF1604 and male lung adenocarcinoma cells in transient transfection assay. NF1604 (A), A549 (B), H23 (C), H1299 (D) and H1792 (E) male adenocarcinoma cells were transiently transfected with an ERE-luciferase reporter as described in the Materials and methods section. The cells were treated with the indicated concentrations of E2, 100 nM 4-OHT and 1 μM ICI 182,780, alone or in combination as indicated for 24 h. Luciferase and Renilla luciferase were assayed as described in the Materials and methods. Luciferase values were normalized by Renilla luciferase values and the relative light unit (RLU) value for EtOH (vehicle control) was set to 1. Values are the means ± S.E.M. of three separate transient transfection assays; a, significantly different from EtOH control (P<0.05, Student’s t test).
required to examine this possibility. There was no correlation between the expression of the shorter ERβ protein and estrogen responsiveness or gender from which the cells were obtained. These data indicate that it is unlikely that over-expression of the dominant negative ERβ2/cx isoform accounts for differences in estrogen responsiveness between cells.

Figure 5 E2 stimulates endogenous ER transcriptional activity in female human lung adenocarcinoma cells as determined by transient transfection assay. H1395 (A), H1435 (B), H1793 (C), H1944 (D) and H2073 (E) female adenocarcinoma cells and MCF-7 breast cancer cells (F) were transiently transfected with an ERE-luciferase reporter; they were then treated, harvested and assayed as described in the Materials and methods section, and Fig. 4. Note that these cells were not transfected with ER. a, Significantly different from EtOH control; b, significantly different from the 10 nM E2 alone value (P < 0.05, Student’s t test).

[^3]H]E2 binding to endogenous ER in lung adenocarcinoma cells

Another explanation for the lack of E2 response in the adenocarcinoma cells from males is that the expressed ER is non-functional. One functional measure of ER expression is specific[^3]H]E2 binding.
Lung adenocarcinoma cell lines expressed 0.11–0.16 fmol ER/μg WCE protein, as measured by specific [3H]E2 binding, and no statistical difference was detected between cell lines from males or females (Fig. 3G). [3H]E2 binding in the lung samples was significantly lower than in MCF-7 cells. Since a possible explanation for the lack of E2 response in lung adenocarcinoma cells from males is that ER is excluded from the cell nucleus, we prepared NEs and CEs and measured [3H]E2 binding to each fraction (Fig. 3H). [3H]E2 binding was higher in NEs than in CEs, but no difference was detected between samples from females or males.

Transcriptional activity of endogenous ER in lung adenocarcinoma cells

A second function of ER, in addition to E2 binding, is to activate gene transcription. Most responses to E2 are mediated by the genomic ER pathway in which E2 stimulates or inhibits the transcription of target genes (Klinge 2000). To compare the transcriptional activity of endogenous ER in the adenocarcinoma cell lines, we performed transient transfection assays using a luciferase reporter driven by two tandem copies of an ERE (Klinge et al. 2004). E2 did not increase ERE-driven luciferase activity in NF1604 cells (Fig. 4A) or in A549, H23, H1299 or H1792 cells from males (Fig. 4B–E). Thus, similar to results seen in the proliferation studies, the adenocarcinoma cell lines from males were non-responsive to E2 in these transient transfection assays. ICI 182,780 suppressed basal transcription in H23, but not in any other cell line from males. Since ICI 182,780 targets ERa to the 26S proteasome for degradation (Marsaud et al. 2003), these results suggest a possible role for ERa in basal transcription of the transfected ERE-luciferase reporter in H23 cells.

In contrast, E2 stimulated ERE-driven luciferase activity in the adenocarcinoma cell lines from females (Fig. 5) and this stimulation was inhibited by concomitant treatment with 4-OHT or ICI 182,780, indicating that the effect was ER mediated. Further, these results are concordant with results from the MTT assays indicating E2 responsiveness in lung adenocarcinoma cell lines from females but not males, despite equivalent ERa and ERβ protein expression and specific [3H]E2 binding activity. 4-OHT and ICI
E-cadherin expression is not stimulated by E$_2$ in lung adenocarcinoma cell lines

E-cadherin is a calcium-dependent cell–cell adhesion molecule that is a marker for epithelial differentiation and is considered to be a tumor suppressor gene (Merot et al. 2004). The mRNA and protein levels of E-cadherin were recently reported to be stimulated by E$_2$ in 201T adenocarcinoma and in 273T squamous cell carcinoma lung cancer cell lines (Hershberger et al. 2005). Thus, we investigated whether E$_2$ stimulates E-cadherin expression in the cell lines used in this study (Fig. 6C). As anticipated, E-cadherin was not expressed in NF1604 cells. E$_2$ decreased E-cadherin expression in MCF-7 cells (Fig. 6D). This agrees with previous reports on E$_2$–ER-mediated inhibition of E-cadherin expression in MCF-7 and other breast cancer cell lines (Oesterreich et al. 2003). E-cadherin was expressed in NHBE cells and its expression was not altered by E$_2$. Not all the adenocarcinoma cell lines expressed E-cadherin and in the cell lines expressing E-cadherin — i.e. A549, H1944, H1395, H1435, H1944 and H2073 — E$_2$ either had no effect or decreased E-cadherin expression (Fig. 6D). These results contradict the conclusion that E-cadherin is an E$_2$-up-regulated target gene in NSCLC cells (Hershberger et al. 2005); however, because different cell lines were used, no generalizations can be made. We suggest that the cell lines expressing little or no E-cadherin represent more aggressive lung adenocarcinoma cell lines — i.e. H23, H1299, H1792 and H1793 — since functional loss of E-cadherin is associated with an epithelial-to-mesenchymal transition in carcinogenesis that results in invasion (Kumar & Hung 2005). Indeed, H1299 is from lymph node metastases, and H1792 from a pleural effusion from a patient with stage 4 disease; H2073 is from a patient with stage 3A disease, according to the ATCC web site (http://www.atcc.org/).

**PBP/TRAP220/DRIP205 expression is higher in lung adenocarcinoma cell lines than NHBE cells**

A difference in ER expression does not appear to be responsible for the proliferative activity of E$_2$ and the anti-proliferative activity of 4-OHT and ICI 182,780 in lung adenocarcinoma cells from females versus males. Thus, we hypothesized that the expression or activity of some critical component(s) of the ER signaling pathways, e.g. coactivators and/or corepressors, may be different between lung adenocarcinomas from male and female patients, a possibility that we are currently investigating. Previous studies showed that ER$_{\alpha}$ interacts directly with coactivator peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP)/thyroid receptor-associated protein (TRAP)220/Vitamin D receptor interacting protein (DRIP)205/Mediator 1 (MEDI) and that DRIP205 is recruited to estrogen target genes in an E$_2$-dependent, cyclic manner in MCF-7 breast cancer cells (Burakov et al. 2000, 2002, Kang et al. 2002). Further, since PBP/TRAP220/DRIP205 is an essential mediator of ER-mediated transcription at estrogen target genes (Zhang et al. 2005), we examined the expression of DRIP205 protein in lung adenocarcinoma cells from females versus males (Fig. 7). DRIP205 expression was higher in the lung adenocarcinoma cell lines and in MCF-7 cells compared with NHBE or NF1604 cells. The nature of the identity of the lower molecular weight bands recognized by the DRIP205 antibody in
the mean response to E2 and this proliferation is blocked by Here, we report that lung adenocarcinoma cell lines a role in this difference (Stabile & Siegfried 2003). Biochemical studies indicating that estrogen may play known, although there are epidemiological as well as males, whether smokers or non-smokers, are un- incidence of lung adenocarcinoma in females versus males, whereas there was no gender-dependent difference in ERα or ERβ mRNA expression levels between adenocarcinoma cells. Specific [3H]E2 binding and the estimated expression levels of full-length ERα and ERβ, based on immunoblotting with known concentrations of rhERα or rhERβ as calibrators, were also similar between the genders. Thus, a difference in ER expression does not appear to be the mechanism accounting for the proliferative activity of E2 and the anti-proliferative activity of 4-OHT and ICI 182,780 in lung adenocarcinoma cells from females versus males. Accordingly, we speculate that the expression or activity of some critical component(s) of the ER signaling pathways, e.g. coactivators and or corepressors, may be different between lung adenocarcinomas from male and female patients. Indeed, we observed that the average expression of the coactivator PBP/TRAP220/DRIP205, which is an essential ER coactivator that interacts directly with ERα and components of the RNA polymerase II holoenzyme/mediator complex (Burakov et al. 2000, Shang et al. 2000, Kang et al. 2002, Saville et al. 2002, Lee et al. 2005, Zhang et al. 2005), was higher in lung adenocarcinoma cell lines from females than males and higher in the lung cancer cell lines compared with NHBE, NF1604 or MCF-7 cells. PBP/TRAP220/ DRIP205 was reported to be over-expressed in breast tumors and cell lines relative to normal human mammary gland (Zhu et al. 1999) but, to our knowl- edge, no one has evaluated its expression in lung adenocarcinoma. There is one report on coactivator GRIP1 (TIF2) expression in NSCLC cell lines showing that GRIP1 is expressed in A549 adenocarcinoma and 128-88T squamous cell carcinoma cell lines, but not in the 201T adenocarcinoma cell line (Hershberger et al. 2005). Since there are currently 49 known ER-interacting coactivators and 18 known ER-interacting corepressors (Klinge 2000, Smith & O’Malley 2004), more experiments will be needed to address the possible different expression of ER coregulators in cells (Bowers et al. 2000). In addition to E2-induced cell proliferation, the cell lines from females, but not males, showed transcriptional activation of an ERE-luciferase reporter by endogenous ER in response to E2.

Surprisingly, despite the proliferative and transcriptional response to E2 in female versus male adenocarcinoma cells, ER expression (as measured by three independent tests) was similar between the genders. Real-time PCR revealed that mRNA for ERβ in all the lung adenocarcinoma cell lines was expressed at a level comparable with that of ERβ in MCF-7 cells and at a higher level than ERα in MCF-7 or any of the normal or neoplastic lung cells. There was no gender-dependent difference in ERα or ERβ mRNA expression levels between adenocarcinoma cells. Specific [3H]E2 binding and the estimated expression levels of full-length ERα and ERβ, based on immunoblotting with known concentrations of rhERα or rhERβ as calibrators, were also similar between the genders. Thus, a difference in ER expression does not appear to be the mechanism accounting for the proliferative activity of E2 and the anti-proliferative activity of 4-OHT and ICI 182,780 in lung adenocarcinoma cells from females versus males. Accordingly, we speculate that the expression or activity of some critical component(s) of the ER signaling pathways, e.g. coactivators and or corepressors, may be different between lung adenocarcinomas from male and female patients. Indeed, we observed that the average expression of the coactivator PBP/TRAP220/DRIP205, which is an essential ER coactivator that interacts directly with ERα and components of the RNA polymerase II holoenzyme/mediator complex (Burakov et al. 2000, Shang et al. 2000, Kang et al. 2002, Saville et al. 2002, Lee et al. 2005, Zhang et al. 2005), was higher in lung adenocarcinoma cell lines from females than males and higher in the lung cancer cell lines compared with NHBE, NF1604 or MCF-7 cells. PBP/TRAP220/ DRIP205 was reported to be over-expressed in breast tumors and cell lines relative to normal human mammary gland (Zhu et al. 1999) but, to our knowl- edge, no one has evaluated its expression in lung adenocarcinoma. There is one report on coactivator GRIP1 (TIF2) expression in NSCLC cell lines showing that GRIP1 is expressed in A549 adenocarcinoma and 128-88T squamous cell carcinoma cell lines, but not in the 201T adenocarcinoma cell line (Hershberger et al. 2005). Since there are currently 49 known ER-interacting coactivators and 18 known ER-interacting corepressors (Klinge 2000, Smith & O’Malley 2004), more experiments will be needed to address the possible different expression of ER coregulators in

H1793 is unknown, but only the full-length DRIP205 value was included in the calculations (Fig. 7B). Although, H1435 cells (female) showed expression similar to that of A549 (male) and H1299 (male), the average DRIP205 expression in lung adenocarcinoma cell lines from females was significantly higher than that in the cell lines from males.

**Discussion**

The mechanisms accounting for the observed higher incidence of lung adenocarcinoma in females versus males, whether smokers or non-smokers, are unknown, although there are epidemiological as well as biochemical studies indicating that estrogen may play a role in this difference (Stabile & Siegfried 2003). Here, we report that lung adenocarcinoma cell lines from female, but not male patients proliferate in response to E2 and this proliferation is blocked by 4-OHT and ICI 182,780, indicating that the response is ER mediated. These results are similar to E2, 4-OHT and ICI 182,780 responses in MCF-7 breast cancer

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**Figure 7** Coactivator DRIP205 is expressed in lung adenocarcinoma cell lines. Western blots for PBP/TRAP220/DRIP205 were performed using a monoclonal antibody to DRIP205 (Pineda Torra et al. 2004). The blots were stripped and re-probed for α-tubulin for normalization. Values of pixel densities were normalized by MCF-7 cells, which were set to 1, and are the average of two blots ± S.D. The inset shows the mean ± S.E.M. for values from the adenocarcinoma cell lines from males and females; a, significantly different from the average value from cell lines from males (P < 0.05).
lungs. Additionally, non-genomic pathways activated by ER ligands, such as the mitogen-activated protein kinase (MAPK)/epidermal growth factor receptor (EGFR) pathway, recently described as being E2 activated in the 201T (Stabile et al. 2005) and H23 (Pietras et al. 2005) lung adenocarcinoma cell lines from males, may also play a role in the estrogen responses detected in our study. Clearly, this possibility will be another important avenue of further investigation.

We compared the ER expression in lung adenocarcinoma to that measured in other studies of lung cancer and ER expression in breast cancer. To our knowledge, there is only one report that estimated ER concentration in the H23 adenocarcinoma cell line by [3H]E2 binding to be 1.9 fmol/µg cell membrane protein (Pietras et al. 2005) and no reports of ER concentration in extracts from human lung tumors or lung cancer cell lines. In the lung adenocarcinoma cells, ERα expression from Western blot estimation using baculovirus-expressed ERα as a calibrator were an average of 1.54 and 1.61 fmol/µg for males and females respectively. The values for ERα were the 3.98 and 4.02 fmol/µg for males and females respectively. In contrast, [3H]E2 binding assays gave total ER concentration as 0.14 and 0.11 fmol/µg WCE for males and females respectively. Western blot analysis gave an estimate of 2 fmol ERα/µg MCF-7 WCE using baculovirus ERα as a standard. However, our HAP assay data indicate an average [3H]E2 binding value of 0.15 fmol/µg WCE, which closely agrees with the published value of 0.16 fmol ER/µg NE (Chaloupka et al. 1992). This value agrees with those for the NE from lung adenocarcinoma cells (Fig. 3). ER expression, as measured by [3H]E2 binding, in breast tumors ranges between 10–1000 fmol/mg nuclear protein (0.01–1 fmol/µg) and 2–3 pmol/mg (2–3 fmol/µg) cytosolic protein (Forster et al. 2004). The latter values are greater than the lung adenocarcinoma cells and agree with the higher [3H]E2 binding that we detected in the MCF-7 cells.

We suggest that the difference between the Western and [3H]E2 binding estimates of ER concentration indicate that not all of the expressed ER protein is able to bind ligand. From these values, it appears that only 10% of ER binds [3H]E2. Whether this is the result of cell extraction and the assay techniques or a biological property of ER remains to be determined.

Our results in lung adenocarcinoma differ from breast cancer where progression to estrogen independence and anti-estrogen resistance is often associated with decreased ERα expression (Yang et al. 2001); the results also differ from endometrial and ovarian cancer where the ERβ:ERα ratio is decreased compared with the respective non-cancer tissues (Pujol et al. 1998, Fujimoto et al. 2000, Li et al. 2003). Moreover, E2 binding was higher in NEs compared with CEs, indicating that sequestration of ER outside the nucleus does not appear to be the cause of the lack of ER responses in the adenocarcinoma cells from males. Furthermore, gender had no apparent effect on ERα or ERβ expression. ERβ expression was higher than ERα in all adenocarcinoma cell lines. These data agree with previous reports that ERβ expression was higher than ERα in A549 (Stabile et al. 2002, Hershberger et al. 2005) and H1435 (Mollerup et al. 2002) cell lines.

To our knowledge, the only report of ERα and ERβ protein expression in normal human lung, by immunostaining, indicated overlapping as well as cell-type-distinct bronchiolar expression of each subtype (Taylor & Al-Azzawi 2000). There are only three reports evaluating the expression of ERα and ERβ proteins in lung adenocarcinoma tumors or cell lines, and these reports reached different conclusions, especially regarding ERα expression (Omoto et al. 2001, Fasco et al. 2002, Hershberger et al. 2005). There are many more reports on ERα and ERβ mRNA expression in normal human lung and NSCLC by RT-PCR (Fasco et al. 2002, Mollerup et al. 2002, Radzikowska et al. 2002, Stabile et al. 2002). Notably, ER expression is highly variable between samples and laboratories, differences that are probably due to the technique employed and the method of sample preparation.

Although adenocarcinoma cells from females and males had comparable expression of ERβ, the ERβ antagonist R.R-THC inhibited E2-induced cell proliferation selectively in the female cell lines. These cell lines did not have a lower ERα:ERβ ratio versus cells from males. Thus, the mechanism accounting for this observation is unknown. Clearly further experiments to ascertain the roles of ERα and ERβ in adenocarcinomas from female and male patients is warranted. The inhibition of E2-induced proliferation by R.R-THC was similar to that detected with ICI 182,780, but less than that detected for 4-OHT, suggesting the possibility that ERβ as well as ERα may be involved in E2-induced cell replication in these cell lines.

4-OHT alone inhibited adenocarcinoma cell proliferation in cells from females, but the pure steroidal anti-estrogen ICI 182,780 alone inhibited only H1395 (female) proliferation. Both 4-OHT and ICI 182,780 inhibited E2-induced cell replication. In the two
previous reports showing that ICI 182,780 inhibits E2-induced responses in H23 and 201T cell lines, ICI 182,780 was not tested alone (Pietras et al. 2005, Stabile et al. 2005). Previous investigators have reported that, in addition to inhibiting E2–ER binding competitively, 4-OHT also inhibits protein kinase C (Gundimeda et al. 1996) and calmodulin-dependent cAMP phosphodiesterase (Colletta et al. 1994), but the mechanism for the inhibition of proliferation of the lung cancer cells seen with 4-OHT alone has not yet been determined.

Overall, we conclude that adenocarcinoma cell lines from males are not estrogen responsive, as measured by cell proliferation. Although 4-OHT inhibited the proliferation of H1299 and H1792 cell lines by ~25%, this inhibition is significantly less than that observed in the adenocarcinoma cell lines from females. Both H1299 and H1792 cell lines were from male smokers, whereas the H23 and A549 cell lines that were not inhibited by 4-OHT were from non-smokers. However, whether smoking increases sensitivity to 4-OHT and the mechanism for this response in these cells is unknown. The only common published markers for H23 and A549 are that both lines show low activation of the PI3K/Akt pathway (Kandasamy & Srivastava 2002) and neither has EGFR mutations (Tracy et al. 2004). 4-OHT inhibits the mitogenic activity of EGF in breast cancer cells (Chalbos et al. 1993) but whether 4-OHT impacts EGFR activity in H23 and A549 or other lung adenocarcinoma cell lines is unknown. ER–EGFR cross-talk and ‘non-genomic’ or membrane-initiated E2 action, characterized most extensively in endothelial cells (Klinge et al. 2005), has recently been reported to occur in NSCLC cell lines (Stabile et al. 2005). Further experiments will be required to examine the role of membrane-initiated (non-genomic) E2 signaling in lung adenocarcinoma and whether this contributes to gender-specific differences or the ability of 4-OHT to inhibit proliferation of the H1299 and H1792 cell lines from males.

This study is unique in not only examining ERα and ERβ expression and the effects of E2 and anti-estrogens on lung adenocarcinoma cell proliferation, but because we also characterized the transcriptional response of endogenous ER using an ERE-reporter assay, examined the effect of E2 on the expression of two endogenous estrogen-responsive genes (PR and E-cadherin) and examined a critical coactivator of ER (i.e. PBP/TRAP220/DRIP205). The results of the transient transfection assays reflected results of the cell proliferation assays: NF1604 cells and the adenocarcinoma cell lines from males showed no E2-induced ERE-driven luciferase activity whereas E2 induced, and concomitant 4-OHT or ICI 182,780 inhibited, the E2-induced luciferase activity in cells from females. Thus, despite expressing comparable ERα and ERβ, cells from males did not show induction of ERE-driven luciferase activity in these transient transfection studies, indicating a lack of ER genomic function, i.e. transcriptional activation from an ERE, in these cells. We conclude that ERα or ERβ expression does not account for the differences in E2 proliferative or transcriptional responses in adenocarcinoma cells from male versus female patients. Although the average DRIP205 expression is higher in cell lines from females than males, we do not believe that DRIP205 is the only coregulator whose expression may differ between the cell lines or between genders. Further examination of ER coregulator expression in lung adenocarcinoma cell lines and tumor samples is a next logical step in our investigation of the mechanisms involved in gender differences in NSCLC.

In contrast, endogenous estrogen target gene expression did fully follow this pattern of female responsiveness. Although PR was expressed in all the adenocarcinoma cell lines, E2 induced PR only in two of the five cell lines from females, indicating a lack of full correlation between gender and E2-induced PR response. Similarly, although E-cadherin was recently reported to be a marker for E2 in NSCLC (Hershberger et al. 2005), we found that E2 did not stimulate E-cadherin expression in any of the adenocarcinoma cell lines. Clearly, a reasonable next step in our study will be to identify E2-regulated genes in adenocarcinoma cell lines from females versus males.

There are a few reports demonstrating that androgen receptor (AR) is expressed in normal human lung and in SCC and NSCLC (Beattie et al. 1985, Kaiser et al. 1996, Wilson & McPhaul 1996, Lin et al. 2004). Based on specific [3H]ligand binding assays, lung adenocarcinomas had twice as much ER as AR, i.e. 4.8 and 2.6 fmol/mg protein respectively (Beattie et al. 1985). However, to our knowledge, no one has compared AR expression or activity in lung tumors from men versus women. Activation of AR by dihydrotestosterone (DHT) was reported to inhibit E2-stimulated proliferation by ZR-75-1 breast cancer cells (Poulin et al. 1988), but the effect of DHT on lung adenocarcinoma cell lines is not well characterized. One recent study showed that DHT stimulated the proliferation of NCI-H1355 adenocarcinoma cells and that the cigarette smoke carcinogen B[a]P inhibited DHT-stimulated proliferation and AR expression in vitro (Lin et al. 2004). Interestingly, this cell line was derived from a male patient. Another logical set of
experiments would be to examine AR expression and function in adenocarcinoma cell lines from male and female patients to determine if AR ‘squelches’ (Meyer et al. 1989), e.g. competes for limited coregulators, the activity of ER in the adenocarcinoma cells from male patients.

In summary, our studies suggest that the gender of a patient with lung adenocarcinoma may have an impact on the estrogen/anti-estrogen response of the tumor. The ability of ER antagonists 4-OHT and ICI 182,780, and the ERβ-selective antagonist R,R-THC, to inhibit E2-induced proliferation of lung adenocarcinoma cells from females, but not males, despite similar expression of ERα and ERβ, indicates the possible use of these agents to inhibit selectively tumor growth in female patients. There is currently a clinical trial at the University of Pittsburgh addressing tumor growth in female patients. There is currently a clinical trial at the University of Pittsburgh addressing whether a combination of ER and EGFR inhibitors (Faslodex and Iressa) may be effective in treating lung cancer, regardless of gender (Hershberger et al. 2005). Although ERα, ERβ and E-cadherin do not appear to be useful biomarkers of estrogen responsiveness in lung adenocarcinoma cell lines, whether PR may be a biomarker in a subset of E2/anti-estrogen-responsive tumors, and whether it is female specific, remains to be determined. The higher DRIP205 expression detected in some of the lung adenocarcinoma cell lines from females may play a role in the greater estrogenic responses seen, but is unlikely to be the sole determinant of gender-specific differences in response. Identification of the precise molecular mechanisms involved in the gender-specific response of the adenocarcinoma cells to E2 and anti-estrogens warrants further study.

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