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

Susan M Dougherty¹, Williard Mazhawidza¹, Aimee R Bohn¹,
Krista A Robinson¹, Kathleen A Mattingly¹, Kristy A Blankenship¹,
Mary O Huff², William G McGregor³ and Carolyn M Klinge¹

¹Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY 40292, USA

²Department of Biology, Bellarmine University, Louisville, KY 40205, USA

³Department of Pharmacology and Toxicology, Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, KY 40292, USA

(Requests for offprints should be addressed to C M Klinge; Email: carolyn.klinge@louisville.edu)

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 ER α and ER β proteins were expressed in all cell lines with higher ER β than ER α . 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, ER α and ER β 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.

Endocrine-Related Cancer (2006) 13 113–134

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*-acetyltransferase (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.* 1998).

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 14 676 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), ER α and ER β . In the original characterization of ER α and ER β mRNA tissue distribution, in rats, ER β was predominant in lung (Kuiper *et al.* 1997). There was no reported lung phenotype for ER α null (α ERKO) mice (Couse *et al.* 1997, Rubanyi *et al.* 1997), but a comparison of the lungs of wild-type (wt) versus ER β null (β ERKO) mice revealed decreased numbers of alveoli in adult female ER β -/- mice (Patrone *et al.* 2003). Lungs of female β ERKO mice also showed decreased surfactant, platelet-derived growth factor A (PDGF-A), and granulocyte-macrophage colony-stimulating factor (GM-CSF), indicating that ER β plays a role in lung homeostasis in mice (Patrone *et al.* 2003). Immunohistochemical staining revealed ER β expression in human lung in columnar epithelium and in intermediate, basal and smooth muscle cells whereas ER α was expressed in basal and smooth muscle cells (Taylor & Al-Azzawi 2000). ER α and ER β have been proposed to play opposite roles, i.e. 'yin and yang', in cell proliferation with ER α being proliferative and ER β anti-proliferative (Lindberg *et al.* 2003). For example, ER α expression is increased in breast cancer and over-expression of ER β inhibits estradiol (E₂)-stimulated breast cancer cell proliferation (Paruthiyil *et al.* 2004). Whether the ER α :ER β ratio increases in lung cancer is unknown.

There are a limited number of studies with conflicting data regarding ER α and ER β 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 ER α and ER β in females and males (Mollerup *et al.* 2002). In contrast, another group reported higher ER α expression in females than in males whereas ER β expression was similar (Fasco *et al.* 2002). A general conclusion from these reports is that lung tumors from women are more likely to express ER α 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

Table 1 Characteristics of the human non-small cell lung cancer (NSCLC) cell lines used in this report. All cell lines were obtained from ATCC Manassas, VA, USA

Cell line	Sex of patient	Smoker/non-smoker (NS)	Reported ER status (reference)	K-ras mutation (codon GGT-GTT; aa G12C) (http://www.sanger.ac.uk , unless otherwise indicated)
A549	male	unknown	<ul style="list-style-type: none"> no ERα (Croxtall <i>et al.</i>, 1994) Low ERα and high ERβ (Stabile <i>et al.</i>, 2002) 	K-ras point mutation (Okudela <i>et al.</i> , 2004)
NCI-H23	male	smoker	<ul style="list-style-type: none"> No ERα, but ERβ (Hershberger <i>et al.</i>, 2005) Low ERα and high ERβ (Stabile <i>et al.</i>, 2002) ERα and ERβ (Pietras <i>et al.</i>, 2005) 	K-ras point mutation (ATCC information)
NCI-H1299	male	smoker		No K-ras mutation, but mutant N-ras
NCI-H1792	male	smoker		K-ras point mutation
NCI-H1395	female	smoker		No K-ras mutation
NCI-H1435	female	NS	<ul style="list-style-type: none"> ERβ > ERα (Mollerup <i>et al.</i>, 2002) ERα; < 10% of the level of ERα; in MCF-7 cells (Mollerup <i>et al.</i>, 2002) 	No K-ras mutation
NCI-H1793	female	NS		No K-ras mutation
NCI-H1944	female	smoker		K-ras mutation
NCI-H2073	female	smoker		No K-ras mutation

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 ER α and ER β 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, a normal human lung fibroblast cell line (NF1604), and normal human bronchial epithelial (NHBE) cells express ER α and ER β proteins. Although no differences in the levels of ER α and ER β 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-triyl)trisphenol (PPT, an ER α -selective agonist) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, an ER β -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).

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 (Manassas, 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 McDaniels (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 McDaniels 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 $\times$ 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.

ER α , ER β 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 ER α , ER β 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 μ l 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 (C_T) method as described in *ABI Technical Bulletin 2* (Bustin 2002, Ginzinger 2002). Fold change was calculated from the $\Delta\Delta C_T$ values with the formula $2^{-\Delta\Delta C_T}$ 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 on to polyvinylidene fluoride (PVDF) membranes (Pall Corporation, Pensacola, FL, USA). As positive and negative controls, known amounts (in fmoles 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

Figure 1 E₂ stimulates and anti-estrogens inhibit the proliferation of lung adenocarcinoma cell lines from females. H1944 (A), H1793 (B), H1395 (C), H1435 (D) and H2073 (E) lung adenocarcinoma cells, all from females, and MCF-7 human breast cancer cells (F) 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 with media (plus treatment) changed on day 2. Cell proliferation was measured by MTT assay. Each bar represents the mean $\pm$ S.E.M. of at least six independent experiments. a, significantly different from control (EtOH) values; b, significantly different from the 10 nM E₂ alone value, $P < 0.05$.

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 β -selective

antagonist/ER α agonist (Sun *et al.* 1999), was similar to that with ICI 182,780, but less than 4-OHT, suggesting the possibility that ER β 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

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 $\pm$ s.e.m. of at least six independent experiments; a, significantly different from control (EtOH) values, $P < 0.05$.

(Stabile *et al.* 2002). 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 3 ER α and ER β are expressed in lung adenocarcinoma cells. (A) Quantitation of mRNA expression of ER α and ER β by real-time RT-PCR was performed as described in the Materials and methods section. Values are the means $\pm$ S.E.M. of quadruplicate determinations and are normalized by ER α and ER β expression in MCF-7 cells. (B and C) Western blots for ER α using ER α antibody AER320. (D and E) Western blots for ER β using ER β antibodies N-19 and H-150 respectively. The blots were stripped and re-probed for β -actin expression for normalization. Known amounts of rhER α (25 and 62 fmol) and rhER β (150, 225 and 300 fmol), determined by HAP assay as described in the Materials and methods, were separated in parallel with the lung sample WCE. Estimation of ER α and ER β 1 concentrations was performed as described in the Materials and methods. (F) The expression of ER α and ER β 1 from different WCE preparations was quantitated by Western blotting. Each bar represents the mean $\pm$ S.E.M. of at least five independent experiments. The inset shows the mean $\pm$ S.E.M. for values from the adenocarcinoma cell lines from males and females; a, significantly different from ER α expression in MCF-7, $P < 0.001$; b, significantly different from ER β 1 expression in any other cell line, $P < 0.001$; as determined by one-way ANOVA followed by Student–Newman–Keuls multiple comparison test. Specific [³H]E₂ binding to WCE (G) and CEs and NEs (H) was measured by HAP assay as described in the Materials and methods. Values are the means $\pm$ S.E.M. of triplicate determinations. *Statistically different from values for lung adenocarcinoma cell lines from male and female, $P < 0.01$; one-way ANOVA followed by Student–Newman–Keuls multiple comparison test.

Expression of ER α and ER β 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 ER α and ER β 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 ER α mRNA than NHBE, NF1604 or any of the lung adenocarcinoma cell lines. ER β mRNA expression was higher than ER α expression in all the lung cell lines. There was no difference in ER α mRNA expression between NHBE, NF1604 and the lung adenocarcinoma cell lines. H23, H1299, H1395, H1792,

H1793, H2073 and NF1604 expressed higher levels of ER β mRNA than MCF-7 cells.

To determine ER α and ER β protein expression, known concentrations, as assayed by [³H]E₂ binding (Kulakosky *et al.* 2002), of baculovirus-expressed rhER α and rhER β protein were separated in parallel with the WCEs of the lung adenocarcinoma cell lines and used as calibrators to estimate ER α and ER β expression levels by Western blot (Fig. 3B–E). All cell lines expressed full-length ER α and no shorter ER α products were detected (Fig. 3B and C). These data indicate that intact ER α is expressed in the adenocarcinoma cell lines. Our data differ from a report that MCF-7, H23 and A549 expressed ER α as 80 and 54 kDa bands, but that only MCF-7 expressed 67 kDa ER α (Stabile *et al.* 2002). ER α 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 ER α , we used two different antibodies to examine ER β expression. We have demonstrated the specificity of the AER320 ER α and H150 ER β antibodies for their respective ER subtype (Klinge *et al.* 2005). The N-19 antibody only recognized the

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 E_2 , 100 nM 4-OHT and 1 μ M 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 $\pm$ S.E.M. of three separate transient transfection assays; a, significantly different from EtOH control ($P < 0.05$, Student's *t* test).

ER β 1 60 kDa band (Fig. 3D), i.e. the 'long' isoform, and not the ER β 1s 'short' isoform of ER β (Scobie *et al.* 2002). Western blots using the H150 ER β antibody indicated that most cells express the ER β 1 and ER β 1s isoforms as 61–64 and 50–53 kDa sizes respectively (Fig. 3E). A summary of the estimated ER α and ER β expression in the cells based on Western blotting is presented in Fig. 3F. ER β expression was greater than ER α expression in all lung adenocarcinoma cell lines, and in NHBE and NF1604 cells than

in MCF-7 cells. NHBE cells showed higher ER β expression than NF1604, MCF-7 or any of the lung adenocarcinoma cell lines. Others have reported ER β 1 migrating as 62–64 kDa in human tissues (Choi *et al.* 2001) and ER β from mouse lung was 65 kDa (Patrone *et al.* 2003). As ER β splice variants ER β 2/cxL and ER β 2/cxS are expressed in human tissues as 55–51 kDa proteins, it is possible that the lower ER β band detected in the Western blots shown here may represent more than one isoform of ER β . Further testing will be

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).

required to examine this possibility. There was no correlation between the expression of the shorter ER β 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 ER β 2/cx isoform accounts for differences in estrogen responsiveness between cells.

[³H]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 [³H]E₂ binding.

Figure 6 PR is a marker of estrogen responsiveness in some of the lung adenocarcinoma cell lines from females and E-cadherin is not a marker of estrogen response. Cells were treated with EtOH (vehicle) or 10 nM E₂ for 24 h. WCEs (35 µg) were separated on 10% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted for PR (A). PR-B is 116 kDa and PR-A is 81 kDa. Membranes were stripped and re-probed for β-actin as a loading control. (B) Mean $\pm$ S.E.M. ($n=3-5$) of the total PR (PR-B+PR-A) normalized by β-actin and with EtOH-treated MCF-7 PR expression set to 100%; a, significantly different from EtOH control ($P<0.01$). (C) Indicated cell lines were treated with EtOH (vehicle) or 10 nM E₂ for 24 h. WCEs (35 µg) were separated on 12% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted for E-cadherin. Molecular weight markers run in parallel in each gel (indicated in kDa). Membranes were stripped and re-probed for β-actin. (D) Normalized E-cadherin expression relative to EtOH-treated MCF-7 cells that were set to 100%. Values are the means $\pm$ S.E.M. of three to six separate experiments; a, significantly different from EtOH control ($P<0.05$).

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 CE 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 ERα to the 26S proteasome for degradation (Marsaud *et al.* 2003), these results suggest a possible role for ERα 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 ERα and ERβ protein expression and specific [³H]E₂ binding activity. 4-OHT and ICI

182,780 suppressed basal transcription in H1793 cells indicating that, as reported by others, 4-OHT-occupied ER can actively suppress transcription (Barsalou *et al.* 1998, Zhang *et al.* 1998, Planas-Silva *et al.* 2001, Thomas *et al.* 2003).

PR may be a marker of estrogen responsiveness in some lung adenocarcinoma cells from females. PR is a well-established marker of estrogen responsiveness in breast cancer (McGuire *et al.* 1986). Reports on PR expression in NSCLC are mixed. Some paraffin-embedded tumors showed PR expression by immunohistochemical staining (Kaiser *et al.* 1996, Su *et al.* 1996), but other studies reported no PR expression (Di Nunno *et al.* 2000, Radzikowska *et al.* 2002). Although some studies found no correlation between ER α and PR immunoreactivity in NSCLC (Kaiser *et al.* 1996, Su *et al.* 1996), one study reported higher PR in lung adenocarcinoma tumors from female patients compared with SCLC and adenocarcinomas from male patients (Kaiser *et al.* 1996). To determine if E₂ stimulated PR expression in lung adenocarcinoma cell lines, cells were grown in DCC-FBS for 24 h and then treated for 24 h with 10 nM E₂. All the lung cell lines expressed PR-B (Fig. 6A). The positive control MCF-7 cells expressed predominately PR-B and E₂ increased PR protein expression by ~50% (Fig. 6B). Interestingly, E₂ increased PR expression in H1395 and H1435 cells from females. Thus, two out of five of the cell lines from females showed E₂-stimulated PR expression. E₂ decreased PR expression in A549 and H1299 cells from males. We conclude that PR may be an endogenous marker for E₂ responsiveness in some lung adenocarcinomas from females.

E-cadherin expression is not stimulated by E₂ 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 E₂ in 201T adenocarcinoma and in 273T squamous cell carcinoma lung cancer cell lines (Hershberger *et al.* 2005). Thus, we investigated whether E₂ stimulates E-cadherin expression in the cell lines used in this study (Fig. 6C). As anticipated, E-cadherin was not expressed in NF1604 cells. E₂ decreased E-cadherin expression in MCF-7 cells (Fig. 6D). This agrees with previous reports on E₂–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 E₂. 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 — E₂ either had no effect or decreased E-cadherin expression (Fig. 6D). These results contradict the conclusion that E-cadherin is an E₂-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 E₂ 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 α 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 (MED1) and that DRIP205 is recruited to estrogen target genes in an E₂-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

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 $\pm$ S.D. The inset shows the mean $\pm$ 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 ($P < 0.05$).

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 E_2 and this proliferation is blocked by 4-OHT and ICI 182,780, indicating that the response is ER mediated. These results are similar to E_2 , 4-OHT and ICI 182,780 responses in MCF-7 breast cancer

cells (Bowers *et al.* 2000). In addition to E_2 -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 E_2 .

Surprisingly, despite the proliferative and transcriptional response to E_2 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 ER β in all the lung adenocarcinoma cell lines was expressed at a level comparable with that of ER β in MCF-7 cells and at a higher level than ER α in MCF-7 or any of the normal or neoplastic lung cells. There was no gender-dependent difference in ER α or ER β mRNA expression levels between adenocarcinoma cells. Specific [3 H] E_2 binding and the estimated expression levels of full-length ER α and ER β , based on immunoblotting with known concentrations of rhER α or rhER β 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 E_2 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 ER α and components of the RNA polymerase II holoenzyme/mediator complex (Burakov *et al.* 2000, 2002, 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 knowledge, 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

lung adenocarcinomas from male and female patients. 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 E_2 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]E $_2$ binding to be 1.9 fmol/ μ g 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, ER α expression from Western blot estimation using baculovirus-expressed ER α as a calibrator were an average of 1.54 and 1.61 fmol/ μ g for males and females respectively. The values for ER β were the 3.98 and 4.02 fmol/ μ g for males and females respectively. In contrast, [3H]E $_2$ binding assays gave total ER concentration as 0.14 and 0.11 fmol/ μ g WCE for males and females respectively. Western blot analysis gave an estimate of 2 fmol ER α / μ g MCF-7 WCE using baculovirus ER α as a standard. However, our HAP assay data indicate an average [3H]E $_2$ binding value of 0.15 fmol/ μ g WCE, which closely agrees with the published value of 0.16 fmol ER/ μ g 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]E $_2$ binding, in breast tumors ranges between 10–1000 fmol/mg nuclear protein (0.01–1 fmol/ μ g) and 2–3 pmol/mg (2–3 fmol/ μ g) cytosolic protein (Forster *et al.* 2004). The latter values are greater than the lung adenocarcinoma cells and agree with the higher [3H]E $_2$ binding that we detected in the MCF-7 cells.

We suggest that the difference between the western and [3H]E $_2$ 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]E $_2$. 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 ER α expression (Yang *et al.* 2001); the results also differ from endometrial and ovarian cancer where the ER β :ER α ratio is decreased compared with the respective non-cancer tissues (Pujol *et al.* 1998, Fujimoto *et al.* 2000, Li *et al.* 2003). Moreover, E_2 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 ER α or ER β expression. ER β expression was higher than ER α in all adenocarcinoma cell lines. These data agree with previous reports that ER β expression was higher than ER α 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 ER α and ER β 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 ER α and ER β proteins in lung adenocarcinoma tumors or cell lines, and these reports reached different conclusions, especially regarding ER α expression (Omoto *et al.* 2001, Fasco *et al.* 2002, Hershberger *et al.* 2005). There are many more reports on ER α and ER β 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 ER β , the ER β antagonist R,R-THC inhibited E_2 -induced cell proliferation selectively in the female cell lines. These cell lines did not have a lower ER α :ER β ratio versus cells from males. Thus, the mechanism accounting for this observation is unknown. Clearly further experiments to ascertain the roles of ER α and ER β in adenocarcinomas from female and male patients is warranted. The inhibition of E_2 -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 ER β as well as ER α may be involved in E_2 -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 E_2 -induced cell replication. In the two

previous reports showing that ICI 182,780 inhibits E_2 -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 E_2 -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 E_2 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) E_2 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 $ER\alpha$ and $ER\beta$ expression and the effects of E_2 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 E_2 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 E_2 -induced

ERE-driven luciferase activity whereas E_2 induced, and concomitant 4-OHT or ICI 182,780 inhibited, the E_2 -induced luciferase activity in cells from females. Thus, despite expressing comparable $ER\alpha$ and $ER\beta$, 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 $ER\alpha$ or $ER\beta$ expression does not account for the differences in E_2 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, E_2 induced PR only in two of the five cell lines from females, indicating a lack of full correlation between gender and E_2 -induced PR response. Similarly, although E-cadherin was recently reported to be a marker for E_2 in NSCLC (Hershberger *et al.* 2005), we found that E_2 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 E_2 -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 [3 H]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 E_2 -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 ER β -selective antagonist R,R-THC, to inhibit E₂-induced proliferation of lung adenocarcinoma cells from females, but not males, despite similar expression of ER α and ER β , 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 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 ER α , ER β 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.

Acknowledgements

We thank Dr John A Katzenellenbogen for providing the R,R-THC for this study. We thank Dr Michael Garabedian for providing the DRIP205 antibody and Dr Nalinie S Wickramasinghe for performing some of the HAP assays on MCF-7 cells. We thank Drs Barbara J. Clark and Richard E. Goldstein for their insightful comments on our manuscript.

Funding

This work was supported by grants from the Commonwealth of Kentucky Lung Cancer Research Program to WGM and CMK and by NIH RO1 DK 53220 and an Intramural Research Incentive Grant from the Office of the Senior Vice President for Research to CMK. ARB was supported by a

summer medical student fellowship from the Summer Research Scholars Program, School of Medicine; K AR is supported by a pre-doctoral fellowship from NIH/NIEHS T32 ES011564; K AM is supported by pre-doctoral fellowship 315087B from the American Heart Association Ohio Valley Affiliate. MOH is supported by a grant from the Kentucky Biomedical Research Infrastructure Network (KBRIN) funded by NIH P20 RR16481. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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