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Activity and intracellular location of estrogen receptors α and β in human bronchial epithelial cells

Margarita M. Ivanova^a, Williard Mazhawidza^a, Susan M. Dougherty^a, John D. Minna^b, and Carolyn M. Klinge^{a,*}

^aDepartment of Biochemistry & Molecular Biology, Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, KY 40292, USA

^bHamon Center for Therapeutic Oncology Research NB8.206, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX 75390, USA

Abstract

Gender differences in lung disease and cancer are well-established. We reported estrogenic transcriptional responses in lung adenocarcinoma cells from females but not males despite similar estrogen receptor (ER) expression. Here we tested the hypothesis that normal human bronchial epithelial cells (HBECs) show gender-independent estrogenic responses. We report that a small sample of HBECs express ~twice as much ER β as ER α . ER α and ER β were located in the cytoplasm, nucleus, and mitochondria. In contrast to lung adenocarcinoma cells, estradiol (E₂) induced estrogen response element (ERE)-mediated luciferase reporter activity in transiently transfected HBECs regardless of donor gender. Overexpression of ER α -VP16 increased ERE-mediated transcriptional activity in all HBECs. E₂ increased and 4-hydroxytamoxifen and ICI 182,780 inhibited HBEC proliferation and cyclin D1 expression in a cell line-specific manner. In conclusion, the response of HBECs to ER ligands is gender-independent suggesting that estrogenic sensitivity may be acquired during lung carcinogenesis.

Keywords

Estrogen receptor; Lung adenocarcinoma (human); Normal lung (human); SMRT; Cyclin D1

1. Introduction

Gender differences in lung function and in lung disease indicate roles for estrogens and androgens in lung physiology (Carey et al., 2007^{a,b}; Lim and Kobzik, 2008). Studies in rodents have revealed that estrogen receptors α and β (ER α and ER β) play roles in normal lung function and pathophysiological conditions in lung (Carey et al., 2007^{a,b}; Couse et al., 1997; Kuiper et al., 1997; Massaro et al., 2007; Massaro and DeCarlo Massaro, 2007; Massaro and Massaro, 2004, 2006; Matsubara et al., 2008; Morani et al., 2006; Patrone et al., 2003). Studies in mice in which ER α and/or ER β were deleted (α ERKO and β ERKO) revealed that both ER subtypes are required for the formation of a full complement of alveoli in female mice, that ER α mediates the sexual dimorphism of body mass-specific alveolar number and surface area, and that absence of ER β diminishes lung elastic tissue recoil (Massaro and Massaro, 2004, 2006). In humans, pre-pubescent boys have a

higher incidence of asthma than girls, but after puberty, women have a higher incidence and worse symptoms than men, an observation that is likely related to the effect of estrogens on immune as well as lung functions (Almqvist et al., 2008). Indeed, postmenopausal use of hormone replacement therapy (HRT, estrogen plus progestin) and estrogen replacement therapy (ERT) was associated with a 2.29-fold higher risk for developing asthma, but not with development of chronic obstructive pulmonary disease (COPD) (Barr et al., 2004). Studies indicate that women are at higher risk for developing COPD (Han et al., 2007; Machado, 2008; Machado et al., 2006; Silverman et al., 2000). These studies, and numerous others, illustrate a role for gender, and likely sex steroids, in lung disease (Carey et al., 2007^{a,b}), as well as the continued need for further study (Machado, 2008).

Lung cancer is the most common cancer in both men and women in the USA; however, although the incidence of lung cancer is declining in men, it is only now plateauing in women after rising for many decades (Jemal et al., 2007). Women are more likely than men to have lung adenocarcinoma, a histologic non-small cell lung cancer (NSCLC) subtype that correlates with worsened prognosis, but women have improved survival compared with men (Schwartz et al., 2005). The higher risk of lung adenocarcinoma in women prompted investigation of the possible role of ER in lung cancer (Dougherty et al., 2006; Hersherberger et al., 2005; Marquez-Garban et al., 2007; Pietras et al., 2005; Stabile et al., 2002, 2005; Weinberg et al., 2005). These reports revealed that ER β appears to be more highly expressed in lung cancer cells than ER α and that estrogen treatment activates both genomic (transcriptional) and non-genomic, *i.e.*, membrane-initiated intracellular ERK1/ERK2 (MAPK) signaling, in lung cancer cells. A new clinical case study reported that 73% of 59 NSCLC patient samples examined had higher estradiol (E₂) concentration in the carcinomas compared to the corresponding non-neoplastic lung tissues from the same patient, regardless of gender (Niikawa et al., 2008). Intratumoral E₂ concentrations correlated with aromatase and PCNA mRNA, but not ER α or ER β staining (Ishibashi et al., 2005). Thus, local estrogen production may play a role in NSCLC. We previously reported that both ER α and ER β are expressed in human lung adenocarcinoma cell lines, normal lung fibroblasts, and normal human bronchial epithelial cells (HBECs) (Dougherty et al., 2006). However, only the lung adenocarcinoma cell lines derived from female patients responded proliferatively and transcriptionally to estradiol (E₂) and other ER ligands (Dougherty et al., 2006). The subcellular location and activity of ER α and ER β in HBECs has not been investigated.

In this study, we examined the expression and activity of ER α and ER β in HBECs immortalized with Cdk4 and human telomerase reverse transcriptase (hTERT) (Ramirez et al., 2004). Microarray studies showed that these HBEC cell lines have similar gene expression signatures that were distinct from those of lung cancer cell lines and were comparable to those from primary HBECs (Ramirez et al., 2004). To address whether the differences in estrogenic responses that we detected between lung adenocarcinoma cell lines from female *versus* male lung adenocarcinoma patients (Dougherty et al., 2006) was present in HBECs, we evaluated ER α and ER β expression, intracellular location, and function in HBECs derived from female and male patients. We describe differences in estrogen and antiestrogen responses between HBECs and lung adenocarcinoma cells. As shown in the present report, there were no gender-dependent differences in the ER responses in HBECs, thus differentiating HBECs from lung adenocarcinoma cell lines.

2. Materials and methods

2.1. Chemicals

17 β -estradiol (E₂) and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma–Aldrich (St. Louis, MO). ICI 182,780 was purchased from Tocris (Ellisville, MO).

2.2. Antibodies

Antibodies were purchased as follows: ER α (HC-20) and ER β (H-150) from Santa Cruz Biotechnology (Santa Cruz, CA); ER α (AER320) and α -tubulin from NeoMarkers (Freemont, CA), ER β (PA1-311) from Affinity BioReagents (Gordon, CO), ER β (6A12, MS-ERB11-PX2) from GeneTex (Austin, TX), SMRT monoclonal antibody from BD Biosciences Pharmingen (San Diego, CA), and β -actin from Sigma–Aldrich.

2.3. Cell culture and treatment

The properties of the 5 human bronchial epithelial cells cell lines prepared from 1 male and 2 female patients used in this study are provided in Table 1 and were originally described in (Ramirez et al., 2004). HBEC were maintained in keratinocyte-serum free medium supplemented with 2.5 μ g recombinant human (rh) epidermal growth factor (EGF) and 25 mg bovine pituitary extract from Invitrogen (Carlsbad, CA). MCF-7 cells were purchased from ATCC (Manassas, VA) and maintained as described (Dougherty et al., 2006). Prior to treatment the cells were placed in phenol red-free media supplemented with 5% dextran-coated charcoal stripped FBS (DCCFBS) for 24–48 h. Ethanol (EtOH) was used as the vehicle control for all experiments. Cells were treated with various concentrations of E₂ as indicated in the Figures. For the indicated experiments the cells were pretreated with 100 nM ICI 182,780 for 6 h prior to E₂ treatment.

2.4. Cell proliferation/Bromodeoxyuridine (BrdU) incorporation assays

For measuring cell proliferation, cells were plated in 96 well plates in keratinocyte-serum free medium for 24 h. Treatments (vehicle control, *i.e.*, ethanol (EtOH), E₂, 4-OHT, or ICI 182,780, alone or in combination) were added for 48 h. The BrdU ELISA assay from Roche Diagnostics (Indianapolis, IN) was performed according to the manufacturer's instructions. Within each experiment, each treatment was performed in quadruplicate and values were averaged. Values were compared to those in the wells treated with vehicle (EtOH) control which was set to 100. At least 4 separate experiments were performed for each cell line.

2.5. Transient transfection

For transient transfection, cells were plated in 24-well plates at a density of 2.5×10^4 cells/well in keratinocyte-serum free medium supplemented with 2.5 μ g recombinant human epidermal growth factor and 25 mg bovine pituitary extract (Invitrogen). Transient transfection was performed using FuGene6 (Roche) in phenol red-free OPTI-MEM I reduced serum medium (GIBCO/Invitrogen). Each well received 250 ng of a pGL3-pro-luciferase reporter (Promega, Madison, WI) containing 2 tandem copies of a consensus estrogen response element (*i.e.*, EREc38 (Tyulmenkov et al., 2000)) and 5 ng of a *Renilla* luciferase reporter (pRL-tk) from Promega. For the indicated experiments, HBECs were also transfected with 10 ng of the expression vectors ER α -VP16, ER β -VP16, and ER α -AF-1 (ER α -AF-1 has 3aa mutations in the LBD that preclude ligand binding (Tzukerman et al., 1994)) that were created by Dr. Donald McDonnell (Hall and McDonnell, 1999; McDonnell et al., 1995) and purchased from Addgene (Cambridge, MA). Prior to treatment the cells were placed in keratinocyte-serum free medium for 24 h. Forty-eight hours after transfection, triplicate wells were treated with EtOH (vehicle control), E₂, 4-OHT, and ICI 182,780, 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). 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.

2.6. Protein isolation

Whole cell extracts (WCE) were prepared in modified RIPA buffer as described in Dougherty et al. (2006). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2.7. Western blotting

Western analysis was performed as described (Dougherty et al., 2006). Briefly, WCE were resolved by SDS-PAGE and electroblotted onto PVDF membranes (Pall Corporation, Pensacola, FL). Membranes were blocked in 5% dry milk TRIS-buffered saline (TBS) and probed with anti-ER primary antibodies. Membranes were stripped and re-probed for α -tubulin or β -actin for normalization. For SMRT westerns, 35 μ g of WCE were separated on 3–8% Tris-Acetate NuPAGE gels (Invitrogen) prior to transfer to PVDF. To estimate ER α and ER β protein expression, known concentrations, as assayed by [3 H]E $_2$ binding (Kulakosky et al., 2002), of baculovirus-expressed recombinant human ER α and ER β protein were separated in parallel with the WCE of the HBECs and used as calibrators to estimate ER α and ER β expression levels by Western blot (Figs. 1 and 2) (Dougherty et al., 2006).

2.8. RNA extraction and quantitative real time RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA quality was determined using the RNA 6000 Nano Assay kit on the Agilent 2100 Bioanalyzer (Wilmington, DE) by absorbance at 260 nm. 2 μ gRNA was reverse transcribed using random hexamers and the High Capacity cDNA archive kit (PE Applied Biosystems (ABI), Foster City, CA). The QIAquick PCR purification kit (Qiagen) was used to purify cDNA.

Progesterone receptor (PR, *PGR*), cyclin D1 (*CCND1*), NRF-1 (*NRF1*), pS2 (*TFF1*) and 18S rRNA Taqman primers and probes were purchased as Assays-on-DemandTM Gene Expression Products (ABI). Each sample was normalized using 18S rRNA. Real-time PCR was performed in the ABI PRISM 7700 SDS 2.1 using relative quantification as described previously (Dougherty et al., 2006). Analysis and fold differences were determined using the comparative C_T method as described in the 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 are relative expression compared to vehicle control in each cell line.

2.9. Confocal laser scanning microscopy

Cells were plated on chamber slides (Lab-TekII, Nalge Nunc, Rochester, NY) for 48–72 h prior to incubation with 25 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) for 45–60 min. Mitotracker Red is oxidized in active mitochondria thus providing a functional assay of mitochondrial activity. Cells were then fixed with 1:1 methanol/acetone followed by incubation with ER primary antibodies (ER α : HC-20 or ER β : H150, both from Santa Cruz) at a 1:300 dilution for 1 h. The secondary antibody was labeled with Quantum Dot 525 (Quantum Dot Corporation). Cells were then incubated with ProLong[®] Gold antifade reagent with DAPI (Molecular Probes) before analysis by confocal microscopy (Zeiss Axiovert100) using LSM510 software (Zeiss).

2.10. Statistics

Data are presented as mean $\pm$ SE. 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).

3. Results

3.1. HBECs express ER α and ER β

The expression of ER α and ER β proteins in WCEs from HBECs was investigated by western analysis. Standard amounts of baculovirus-expressed recombinant human ER α and ER β were resolved in parallel and used to estimate ER α and ER β concentrations (Dougherty et al., 2006). WCE from MCF-7 breast cancer cells served as an additional control for ER α and ER β (Dougherty et al., 2006). Full length (67kDa) ER α was expressed in HBECs, H1395 lung adenocarcinoma cells and MCF-7 cells (Fig. 1A). The average expression of ER α in the HBECs ranged from 0.08 to 0.11 fmol/ μ g of protein (Fig. 1B). Concurrent analysis revealed that the expression of ER α in MCF-7 cells was 0.22 fmol/ μ g, *i.e.*, ~twice that of the HBECs (Fig. 1B). Antibodies raised against the N-terminus of ER α also detected ER α in HBECs (Supplemental Fig. 1). Similar analyses revealed that ER β expression ranged from 0.16 to 0.22 fmol/ μ g protein which was similar to the amount of ER β expressed in MCF-7 cells (Fig. 2B). Uncropped images of the ER β westerns are included as Supplemental Fig. 2. Thus, ER β is ~2-fold higher than ER α in HBECs.

3.2. Subcellular localization of ER α and ER β in HBECs

We examined the effect of E₂ on the intracellular localization of ER α and ER β in HBECs using immunohistochemistry (IHC) with antibodies unique for each ER subtype and confocal microscopy (Fig. 3, Supplemental Fig 3, and summarized in Table 2). DNA was stained by DAPI. Respiring mitochondria were specifically imaged by MitoTracker Red CMXRos. As a negative control, no green immunofluorescence was detected when the primary ER α or ER β antibodies were excluded from the incubation, although MitoTracker and DAPI staining was appropriately detected (Supplemental Fig 4). A Western blot for ER α using the HC-20 ER α antibody is shown in Supplemental Fig 5. In all cells examined, MitoTracker signaling consistently indicated localization of the active mitochondria in the perinuclear region. In all HBECs, ER α and ER β were predominantly cytoplasmic but were also present in the nucleus in both untreated and E₂-treated cells. ER signal intensity varied between cells. Fig. 3 demonstrates an overlap (yellow) between ER α and ER β with MitoTracker Red, indicating colocalization of each ER subtype within mitochondria of HBEC2-KT cells. Similar results were detected in HBEC2-E, HBEC3-ET, and HBEC4-KT cells (Supplemental Fig. 3). In contrast, ER α did not overlap with MitoTracker Red in HBEC3-KT (Supplemental Fig 3). Treatment of HBEC2-KT with 10 nM E₂ for 45 min. did not alter the subcellular distribution of ER α and ER β (Fig. 3) and similar results were detected with most HBECs (Supplemental Fig 3 and Table 2) with the exception that E₂ appeared to decrease the nuclear and increase the cytoplasmic localization of ER α in HBEC3-ET (Supplemental Fig 3 and Table 2). This is the opposite of the effect of E₂ in MCF-7 human breast cancer cells (Schlegel et al., 1999).

3.3. E₂ and 4-OHT stimulate ERE-reporter gene activity in an HBEC cell line-specific manner

To determine the transcriptional activity of endogenous ER in the HBEC cell lines, transient transfection assays were performed using a luciferase reporter driven by 2 tandem copies of an ERE (Klinge et al., 2004). E₂ increased ERE-driven luciferase activity in all HBECs (Fig. 4). 4-OHT and ICI 182,780 suppressed E₂-induced transcription in HBEC2-E and HBEC3-ET, indicating that the increase in luciferase activity in response to E₂ is ER-mediated. In HBEC2-E, the combination of E₂ and 4-OHT reduced transcription to below basal, lower than either E₂ or 4-OHT alone. 4-OHT did not inhibit E₂-induced luciferase activity in HBEC2-KT, HBEC3-KT, or HBEC4-KT, although ICI inhibited E₂-luciferase activity in these 3 HBEC cell lines, indicating that ER was responsible for the E₂-induced activity. ICI inhibited basal luciferase activity in HBEC4-KT. 4-OHT showed ER agonist activity HBEC2-E and HBEC3-KT. It is well-established that 4-OHT is a selective ER modulator (SERM) that has ER agonist

or agonist activity in a cell line-dependent manner (MacGregor and Jordan, 1998). In sum, these data indicate that endogenous ER is transcriptionally active in HBECs.

3.4. Transfection of HBECs with ER α -VP16 or ER β -VP16 stimulated luciferase transcription

There are many individual steps in the ER signal transduction cascade at which defect(s) may reside. Because the ER α -VP16 and ER β -VP16 chimeric proteins have the VP16 acid transcriptional activation domain (AD) cloned onto the N-terminus of the ER proteins, these constructs provide an assay of transcriptional activity that depends exclusively on DNA binding (McDonnell et al., 1995). Transfection with ER α -VP16 generated significantly higher ERE-driven luciferase activity in each HBEC cell line (Fig. 5). Most cell lines transfected with ER α -AF-1 showed only a modest increase in luciferase activity over basal, indicating that AF-1 is not predominant over AF-2 in HBECs, unlike HepG2, COS-7, or PC-3 cells (Flouriot et al., 2000; Merot et al., 2004; Metivier et al., 2002a,b; Penot et al., 2005). E₂ did not result in a further increase reporter activity with VP16-ER α , possibly indicating that the transcriptional response is saturated, and actually inhibited VP16-ER α activity in HBEC4-KT. Likewise, E₂ inhibited VP16-ER β activity in HBEC3-ET and HBEC3-KT.

3.5. E₂ stimulates transcription of select endogenous estrogen-target genes in HBECs

Quantitative real time PCR (Q-PCR) was performed to examine the effect of E₂ on the transcription of classical endogenous estrogen target gene cyclin D1 (*CCND1*) (1) and on the transcription of nuclear respiratory factor-1 (NRF-1, *NRF1*) which we recently identified as an estrogen-regulated gene (Mattingly et al., 2008). As controls, MCF-7 (ER α positive, estrogen-responsive), H1395 and H1792 (ER α and ER β positive, E₂-responsive (female) and non-responsive (male) lung adenocarcinoma cell lines, respectively (Dougherty et al., 2006)) were treated in parallel with HBECs. E₂ increased *CCND1* in MCF-7 and H1395 (Fig. 6), in agreement with the E₂-induced proliferation in these cell lines (Dougherty et al., 2006). E₂ increased *CCND1* transcription in HBEC2-KT, HBEC3-ET, HBEC3-KT, and HBEC4-KT cells, and statistically reduced *CCND1* expression in H1792 cells. Similarly, E₂ increased *NRF1* transcription in MCF-7, H1395, HBEC2-KT, HBEC3-ET, HBEC3-KT, and HBEC4-KT cells and reduced *NRF1* in H1792 cells. In each cell line in which E₂ increased *CCND1* or *NRF1* transcription, ICI inhibited the E₂-induced increase, indicating that the transcriptional response was ER-mediated. Interestingly, ICI also reduced basal expression of *CCND1* or *NRF1* in each cell line, indicating that ER regulates the basal expression of these genes in these cell lines. In contrast, HBECs did not express two other classical E₂-regulated target genes, *i.e.*, PR and pS2, either in control or E₂-treated cells (data not shown). Likewise, pS2 was not expressed in H1395 or H1792, but PR was E₂-induced in H1395 (Supplemental Fig 6). These data indicate that E₂ activates the transcription of some established estrogen target genes in HBECs.

3.6. SMRT β is the predominant SMRT isoform in HBECs

Although E₂-induced ERE-luciferase activity in each of the transiently transfected HBECs (Fig. 4), E₂ failed to increase cyclin D1 or NRF-1 in HBEC2-E. Coactivators are required for E₂-ER induced gene transcription (Klinge, 2000). To determine whether differential expression of the coregulator SMRT corresponds with E₂-induced *CCND1* transcription in all HBECs except HBEC2-E (Fig. 6), we examined SMRT protein by Western blot using an antibody that recognizes both SMRT β and SMRT α (Fig. 7). We observed 2 bands corresponding to SMRT splice variants SMRT β (full length) and SMRT β which is missing repressor domain 1 (RD-1) at the N-terminus (Ordentlich et al., 1999). SMRT β acts as an ER α -selective coactivator in a cell-type and gene-specific manner and is required for induction of *CCND1* transcription and cell proliferation in MCF-7 cells (Peterson et al., 2007). SMRT β expression was higher than SMRT α in HBECs and total SMRT expression was lower in HBECs compared to H1395, A549, and H1792 lung adenocarcinoma and MCF-7 breast cancer cells (Fig. 7). However, the inability

of E₂ to induce *CCND1* transcription in HBEC2-E and H1792 and the lower induction of *CCND1* transcription in HBEC3-ET and HBEC3-KT is not due to an absence or reduction of SMRT β protein expression.

3.7. E₂ stimulates and selective estrogen receptor modulators (SERMs) inhibit HBEC proliferation in a cell line-specific manner

E₂ stimulated the proliferation of lung adenocarcinoma cell lines from female but not male patients (Dougherty et al., 2006). To determine if E₂ affects HBEC proliferation in a gender-dependent manner, HBECs were treated with E₂, 4-OHT, and ICI 182,780, alone or in combination with E₂ and cell proliferation was measured by BrdU incorporation assays (Fig. 8). E₂ increased proliferation of HBEC2-KT, HBEC3-KT, and HBEC4-KT, but not HBEC2-E or HBEC3-ET. The E₂-dependent increase in HBEC3-KT and HBEC4-KT proliferation was inhibited by 4-OHT and ICI, indicating an ER-mediated effect. In contrast, 4-OHT and ICI did not inhibit the E₂-induced proliferation in HBEC2-KT. Notably, the E₂-induced proliferation of HBEC2-KT, HBEC3-KT, and HBEC4-KT correlates with the induction of Cyclin D1 transcription in these cells (Fig. 6). On the other hand, although E₂ increased Cyclin D1 transcription in HBEC3-ET (Fig. 6A), it did not increase BrdU incorporation (Fig. 8).

4. Discussion

Although post-pubertal, premenopausal women have higher incidences of certain lung diseases, *i.e.*, asthma (Carey et al., 2007^{a,b}), and pre- and peri-menopausal are at increased risk for NSCLC, particularly adenocarcinoma (Schwartz et al., 2007), compared to men, few investigators have examined the effect of estrogens on normal bronchial epithelial cells. Only recently has estrogen been reported to act as a promoter for lung adenocarcinoma development in a genetically defined murine model (Hammoud et al., 2008). To our knowledge, the studies reported here are the first to systematically evaluate the expression, subcellular localization, and genomic function of ER α and ER β in HBECs. Our results showing that all 5 HBEC cell lines express ~twice as much ER β protein compared to ER α support results from IHC studies of human lung tissues (Pietras et al., 2005; Shen et al., 2007) and our previous report of higher ER β than ER α in primary HBECs (Dougherty et al., 2006). Further, both ER subtypes were predominantly cytoplasmic and showed mitochondrial and nuclear staining. These data agree with other reports of mitochondrial localization of ER α and ER β other cell types (Cammarata et al., 2004; Chen et al., 2007, 2004; Jonsson et al., 2007; Monje and Boland, 2001; Pedram et al., 2006; Solakidi et al., 2005; Yang et al., 2004).

Each of the HBEC lines used in our study have microarray gene expression profiles similar to primary HBECs and distinct from lung cancer cell lines (Ramirez et al., 2004). Here E₂-induced ERE-driven luciferase activity in all HBECs, indicating that the endogenous ERs are transcriptionally active. In contrast, E₂ did not induce reporter activity in transiently transfected lung adenocarcinoma cell lines derived from male patients (Dougherty et al., 2006).

Here we observed that the E₂-induced increase in cyclin D1 and NRF-1 transcription was correlated with E₂-induced cell proliferation in HBEC2-KT, HBEC3-KT, and HBEC4-KT cells, but not in HBEC3-ET. *CCND1* is amplified in NSCLC and frequently overexpressed in lung tumors and pre-invasive bronchial lesions (Gautschi et al., 2007). However, to our knowledge, there is nothing published about ER α and cyclin D1 in normal lung. Increased cyclin D1 is associated with ER α expression in breast tumors (Cho et al., 2008; Stendahl et al., 2004) and *CCND1* transcription is induced by E₂ in MCF-7 and other ER α -expressing breast cancer cell lines (Altucci et al., 1996; Eeckhoutte et al., 2006; Hong et al., 1998; Watts et al., 1994). Likewise, *NRF1* transcription is induced by E₂ in MCF-7 cells (Mattingly et al., 2008). NRF-1 is a transcription factor that regulates the transcription of mitochondrial transcription factors and genes involved in cell proliferation (Scarpulla, 2006). Although the NRF-1 transcript was highly

expressed in rat lung (Gopalakrishnan and Scarpulla, 1995), this is the first study examining NRF-1 in normal human lung cells.

Although SMRT was originally characterized as a corepressor of ER and other nuclear receptors (Chen and Evans, 1995; Nagy et al., 1997), E₂-ER α -induced cyclin D1 transcription in MCF-7 cells requires SMRT β (Peterson et al., 2007). A recent editorial highlighted the importance of rethinking about coactivators and corepressors as context-specific coregulators having either stimulatory or inhibitory activities (O'Malley and McKenna, 2008). We observed no correlation between SMRT β expression and E₂-stimulated *CCND1* transcription in the HBECs or in H1792 and H1395 lung adenocarcinoma cell lines. However, SMRT β was higher than SMRT α in HBECs. Since SMRT β is missing RD-1, a hypothesis stemming from these data is that SMRT may play more of a coactivator than corepressor role in HBECs. Interestingly, only HBEC4-KT cells were growth inhibited by 4-OHT alone and HBEC4-KT showed higher SMRT α than the other HBECs, corresponding with the greater repressor role for SMRT α than SMRT β (Peterson et al., 2007). Further experiments are needed to examine this suggestion. To our knowledge, this is the first examination of SMRT protein expression in HBECs or lung adenocarcinoma cells. Lung homogenates from male Sprague–Dawley rats had lower SMRT than those from females, but because the MW of SMRT was given as 160 kD (González-Arenas et al., 2004), the identity of this SMRT iso-form is unclear since SMRT α is 270 and SMRT β is 249 kDa (Peterson et al., 2007). We did not detect a significant difference in SMRT β expression in HBECs from females *versus* a male.

SMRT's ER α -specific coactivator activity is cell-specific (Peterson et al., 2007). The coactivator activity of SMRT correlates with greater ER α AF-2 than AF-1 activity in MCF-7 and HeLa cells compared to HepG2 (Penot et al., 2005). Because E₂ induced ERE-luciferase reporter activity in all HBECs (Fig. 5), and since we observed no increase in ERE reporter activity when HBECs were transfected with ER α -AF-1, a construct in which AF-2 is inactivated by 3 aa substitutions (Tzukerman et al., 1994), we conclude that ER α AF-2 > AF-1 in HBECs. Although 4-OHT was an agonist in HBEC2-E and HBEC3-KT, there is no apparent correlation of these responses to SMRT α or β expression.

There are few studies of ER activity in HBECs. A previous study reported no endogenous ER α at either the mRNA or protein levels in primary HBECs, but demonstrated that adenoviral ER α infection increased cigarette smoke extract-induced CYP1B1 transcription, a result that the authors interpreted as indicating that ER α contributes to gender differences in carcinogen metabolism and mutation (Han et al., 2005). The human bronchial epithelial cell line BEAS-2B, immortalized by SV40, expresses ER β (Møllerup et al., 2002). E₂ stimulates BEAS-2B cell proliferation by ~5% and E₂ synergized with BaP to increase COX-2 transcription and PGE2 production in an ER-dependent manner (Chang et al., 2007). The authors speculated that BaP activated CYP1A1/CYP1B1 and increased catechol estrogen production thereby activating the NF- κ B pathway in BEAS-2B cells (Chang et al., 2007). We have not examined E₂ metabolism in the HBECs used in the present study.

In summary, although E₂ stimulated transcriptional and proliferative activities in lung adenocarcinoma cell lines isolated from female but not male patients (Dougherty et al., 2006), in the present small study no gender-specific differences in the estrogenic phenotype of HBECs was detected. Thus, there appears to be no innate gender-dependent difference in E₂ responses in normal HBECs that correlate with those detected in the lung adenocarcinoma cell lines. Further, the estrogenic responses in lung adenocarcinoma cell lines appear to be greater than those in HBECs, implying a gain-of-function in the transformed cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. HBECs express ERα. WCE (40 μg) from the indicated cell lines and the indicated amounts of baculovirus-expressed ERα were separated by 10% SDS PAGE, transferred to PVDF membranes, and probed with AER320 monoclonal antibody for ERα (A). The membrane was stripped and re-probed for α-tubulin as a loading control. This is a representative blot. (B) Summary of ERα expression from 5 separate Western blots. Values are the mean ± SEM of at least 5 experiments and are plotted as fmol ERα/μg WCE protein and were calculated using the rhERα as a standard as described in Section 2. *Significantly different from MCF-7 ($p < 0.05$).

Fig. 2. HBECs express ER β . WCE (40 μ g for the PA1-311 and 6A12 blots and 30 μ g for the H150 blot) from the indicated cell lines and the indicated amounts of baculovirus-expressed ER β were separated by 10% SDS PAGE, transferred to PVDF membranes, and probed with the indicated antibodies for ER β . The membranes were stripped and re-probed for α -tubulin as a loading control for normalization as described in Section 2. Uncropped images of these blots are in Supplemental Fig. 2. (B) Summary of ER β expression using the ER β antibodies listed in Section 2. Values are the mean $\pm$ SEM of at least 5 experiments and are plotted as fmol ER β / μ gWCE protein and were calculated using the rhER β as a standard as described in Section 2.

Fig. 3.

ER α and ER β localize to the cytoplasm, nucleus, and mitochondria in HBEC2-KT. Confocal microscopic imaging of HBEC2-KT treated with vehicle (EtOH) or 10 nM E₂ for 45 min. Cells were incubated with 25 nM MitoTracker Red CMXRos (red color) for 45 min prior to fixation. After fixation, the cells were incubated with ER α antibody HC-20 or ER β antibody H-150 and then the secondary antibody labeled with Quantum Dot 525 (green color) as described in Section 2. Cells were then incubated with ProLong® Gold anti-fade reagent with DAPI to stain the nucleus (blue color, upper left quadrant and in the merged image). Bars (gold) are 20 μ m. The merged image is in the lower right quadrant. (B) 25% magnification of the same cells seen in (A) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

Fig. 4.

Estradiol increases ERE-reporter gene expression in HBECs. HBECs were transiently transfected with an ERE-luciferase reporter as described in Section 2. The cells were treated with 10 or 100 nM E₂, 100 nM 4-OHT, 1 μ M ICI 182,780, alone or in combination as indicated for 24 h. Luciferase and *Renilla* luciferase were assayed as described in Section 2. Luciferase values were normalized by *Renilla* luciferase values and the RLU value for EtOH (vehicle control) was set to 1. Values are the mean $\pm$ SEM of 3 separate transient transfection assays. *Significantly different from EtOH control; **significantly different from the 10 nM E₂ value ($p < 0.01$, one-way ANOVA, Student–Newman–Keuls post hoc).

Fig. 5.

Transfection of HBECs with ERα-VP16 or ERβ-VP16 increases ERE-driven luciferase activity. HBECs were transiently transfected with an ERE-luciferase reporter and 10ng VP16-ERα, VP16-ERβ, or ERα-AF-1 as described in Section 2. The cells were treated with EtOH or 10 nM E₂, as indicated for 24 h. Luciferase values are the mean ± SEM of 3 determinations.

*Significantly different from EtOH control in the same transfection group ($p < 0.01$, one-way ANOVA, Student-Newman-Keuls post hoc).

Fig. 6.

Estradiol increases endogenous estrogen target gene expression in HBECs. The indicated cell lines were treated with vehicle (EtOH, open bars), 10 nM E₂ (closed bars), or 10 nM E₂ + 100 nM ICI 182,780 (hatched bars) for 6 h. Q-RT-PCR analysis of cyclin D1 (*CCND1*, A) and NRF-1 (*NRF1*, B) were normalized to 18S and values are compared to EtOH within each cell line as described in Section 2. Values are the mean $\pm$ SEM of 3 independent experiments. * And ** indicate values that are significantly different compared to vehicle and 10 nM E₂, respectively ($p < 0.01$, one-way ANOVA, Student–Newman–Keuls post hoc test)

Fig. 7.

SMRT β expression is higher than SMRT α in HBECs. WCE (35 μ g protein) from the indicated cell lines were separated by 3-8% Tris-Acetate gels, transferred to PVDF membranes, and probed with SMRT antibody as described in Section 2 (A). The membrane was stripped and re-probed for α -tubulin as a loading control for normalization as described in Section 2. Summary of relative SMRT expression from 3 separate Western blots in which SMRT α and SMRT β were normalized to α -tubulin and then expressed relative to SMRT α and SMRT β in MCF-7 cells in each separate gel (B). Values are the mean $\pm$ SEM.

Fig. 8.

Estradiol increases the proliferation of HBEC2-KT and HBEC4-KT cells. The effect of the indicated concentrations of E₂, 4-OHT, and ICI 182,780, alone or in combination with 10 nM E₂ on the proliferation of HBEC cell lines was determined after 48 h of treatment by assaying BrdU incorporation as described in Section 2. Data were normalized to the vehicle (EtOH) within each experiment. Values are % of EtOH control and are the mean $\pm$ SEM of at least 3–4 experiments, * and ** indicate values that are significantly different compared to vehicle and 10 nM E₂, respectively ($p < 0.05$).

Table 1

Cell lines used in this study

Cell line	Cell type	Immortalized by	Sex	Age	Cancer history
HBEC2-E	Bronchial epithelial	HPV-16/E6/E7	Male	67	NSCLC
HBEC2-KT	Bronchial epithelial	Cdk4 + hTERT	Male	67	NSCLC
HBEC3-ET	Bronchial epithelial	hTERT	Female	65	No cancer
HBEC3-KT	Bronchial epithelial	Cdk4 + hTERT	Female	65	No cancer
HBEC4-KT	Bronchial epithelial	Cdk4 + hTERT	Female	71	Lung cancer
AS49	Lung adenocarcinoma		Male	58	
NCI-H23	Lung adenocarcinoma		Male	51	Smoker
NCI-H1299	Lung adenocarcinoma		Male	43	Smoker
NCI-H1792	Lung adenocarcinoma		Male	50	Smoker
NCI-H1395	Lung adenocarcinoma		Female	55	Smoker
NCI-H1435	Lung adenocarcinoma		Female	35	Non-smoker
NCI-H1793	Lung adenocarcinoma		Female	52	Non-smoker
NCI-H1944	Lung adenocarcinoma		Female	62	Smoker
NCI-H2073	Lung adenocarcinoma		Female	47	Smoker
MCF-7	Breast adenocarcinoma		Female	69	Pleural effusion of a patient with metastatic breast cancer

Human bronchial epithelial cell (HBEC) cell lines were derived from patients who were smokers and immortalized as described previously (Ramirez et al., 2004). Characteristics of the other cell lines are taken from (Dougherty et al., 2006) and the ATCC website (<http://www.atcc.org/>).

Table 2Subcellular localization of ER α and ER β in HBECs.

Cell line	ER α	ER α +E ₂	ER β	ER β +E ₂
HBEC2-E	C >N, M	C >N, M	C >N, M	C, M
HBEC2-KT	C >N, M	C, M	C >N, M	C >N, M
HBEC3-ET	C >N	C >N, M	C >N, M	C >N, M
HBEC3-KT	C >N, M	C, M	C >N, M	C >N, M
HBEC4-KT	C >N	C, M	C >N, M	C >N, M

HBECs were incubated with 25 nM Meitotracker Red CMXRos for 45 min prior to fixation as described in Section 2. Where indicated as +E₂, cells were treated with 10 nM E₂ for 45 min. Representative images of HBECs 2-E, 3-ET, 3-KT, and 4-KT are shown in Supplemental Fig. 3. C = cytoplasmic; N= nuclear; Co-M = colocalization with MitoTracker (mitochondria).