Human Non-Small Cell Lung Tumors and Cells Derived from Normal Lung Express Both Estrogen Receptor α and β and Show Biological Responses to Estrogen

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ABSTRACT

Lung cancer is becoming increasingly common in women and in the United States accounts for more female cancer deaths annually than breast cancer. Many epidemiological studies have provided evidence that women are more susceptible than men to the adverse effects of tobacco smoke. These observations suggest the possible role of estrogens in lung carcinogenesis. We report here the expression of mRNA for estrogen receptor α (ERα) and β (ERβ) in cultured human non-small cell lung cancer cells, cultured lung fibroblasts, and primary cultures of normal bronchial epithelium. Western analysis of ERα suggested that the main protein expressed in lung tumor cells is a variant, probably attributable to alternative splicing. Protein for ERβ was found to be a mixture of full-length as well as alternatively spliced variants. β-Estradiol produced a proliferative response in vitro in both normal lung fibroblasts and cultured non-small cell lung tumor cells. This effect was also observed in vivo. In this regard, β-estradiol stimulated growth of the non-small cell lung tumor line, H23, grown as tumor xenografts in SCID mice. This effect was blocked by fluvestrant (ICI 182,780). In paraffin sections of non-small cell lung tumors, ERβ immunoreactivity was localized to the nucleus, whereas ERα immunoreactivity was mainly localized to the cytoplasm, suggesting that both nuclear and cytoplasmic signaling may be involved in estrogenic responses in the lung. To show that the ERs found in the lung are functional, we demonstrated that β-estradiol stimulated transcription of an estrogen response element-luciferase construct transfected in non-small cell lung tumor cell lines. Antiestrogens blocked this effect. Treatment of lung fibroblasts with β-estradiol also increased secretion of hepatocyte growth factor by 2-fold. These results suggest that estrogen signaling plays a biological role in both the epithelium and the mesenchyme in the lung and that estrogens could potentially promote lung cancer, either through direct actions on preneoplastic or neoplastic cells or through indirect actions on lung fibroblasts. Additionally, it is possible that antiestrogens may have therapeutic value to treat or prevent lung cancer.

INTRODUCTION

Lung cancer is the leading cause of death from neoplasia in the United States. The number of new cases of lung cancer occurring in ex-smokers now equals or surpasses that of active smokers, demonstrating that lung cancer risk declines slowly after smoking cessation (1). Smoking among teenagers has increased in the past decade, especially among girls who use smoking for weight control, and this trend shows no signs of abating (2, 3). There is also increasing evidence that women are more susceptible, dose for dose, to the carcinogenic effects of cigarette smoke (2). Because females now make up almost half the smoking population and young women are beginning to smoke at rates equal to those of young men, we can expect future female lung cancer rates to remain high. The aging ex-smoker population will also be made up of an increasingly larger number of women because women live longer than men.

A number of studies have suggested that women are more susceptible to tobacco carcinogenesis than men (2–5), taking into account baseline exposure, body weight, height, and body mass index (2). The OR2 for adenocarcinoma of the lung in females was found to be 6.8 at 1–19 pack-years of tobacco exposure, compared with 2.4 for men, and 32.7 at 50 pack-years of exposure, compared with 13.8 for men. This same relationship was found for squamous cell carcinoma (2). Not all studies have demonstrated this relationship (6–8). For example, in a recent case-control study, Kruezer et al. (8) did not find increased ORs for female smokers compared with male smokers. However, there was also a large disproportion of female-to-male cases in the never-smoking group (286 versus 88); thus, there may be differences in baseline exposure (such as ETS exposure) that may confound the interpretation of these results.

There also is a difference in the relative distribution of lung cancer histological features between men and women that is not explained by differences in smoking patterns. Women who smoke appear to be at higher risk of developing small cell lung cancer, which has neuroendocrine features, than squamous cell lung cancer, whereas men who smoke have a similar risk for the two histological conditions (4). Furthermore, women smokers are more likely to develop adenocarcinoma of the lung, which is a secretory tumor. Nonsmokers with lung cancer (predominantly diagnosed with adenocarcinoma) are also ~2.5 times more likely to be female than male (9–13). Estrogens may play a causative role in this phenomenon of secretory types of differentiation being more prominent in female lung cancer.

Several genetic factors have been suggested to contribute to the higher ORs in women compared with men. GRPR is a receptor for the hormone gastrin-releasing peptide, which mediates cell proliferation in the lung. Gastrin-releasing peptide plays a role in lung development and possibly in lung wound healing. A sex difference in expression of GRPR, an X-linked gene, has been found, which may be caused by the ability of the GRPR gene to escape X inactivation (5). Women were found to express this gene in their airway cells frequently without smoking, whereas men were found not to express the gene in airway cells unless they smoked (5). Other factors that might increase the carcinogenic effects of cigarette smoke in women include increased expression of cytochrome P450 1A1 isozyme in the lungs of females compared with males (14) and a higher frequency of G-to-T transversions in the p53 gene in females compared with males (15).

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4 The abbreviations used are: OR, odds ratio; ETS, environmental tobacco smoke; GRPR, gastrin-releasing peptide receptor; ERα and ERβ, estrogen receptor α and β; AP-1, activator protein; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBS-T, Tris-buffered saline-Tween; Brefeldin, bromoecdysterine; HGF, hepatocyte growth factor; ERE, estrogen response element.
Additionally, women were found to be three times more likely than men to carry the K-ras gene mutation, which is a marker for aggressive lung cancer found only in smokers or former smokers (16). In a separate study, never-smoking women exposed to ETS who developed lung cancer were two times more likely than never-smoking women without ETS exposure who developed lung cancer to have a defective glutathione-S-transferase M1 gene, which normally deactivates carcinogens in tobacco smoke (17). Reduced DNA repair capacity is also associated with an increased risk of lung cancer, and females were reported to have lower DNA repair capacity than males (18). Whether regulation of cellular processes by estrogens can influence these cancer-related events is unknown.

Estrogen status is a recognized factor in lung cancer risk in women, as it is in the development of adenocarcinoma of the breast, endometrium, and ovary (19). Tàuoli and Wynder (20) presented evidence that exogenous and endogenous estrogens may play a role in the development of lung cancer, particularly, adenocarcinoma, among women. Using case-control data, they demonstrated that (a) early age at menopause (40 years or younger) is associated with a reduced risk of adenocarcinoma of the lung (OR, 0.3); (b) the use of estrogen replacement therapy is associated with a higher risk of adenocarcinoma of the lung (OR, 1.7); and (c) there is a positive interaction between estrogen replacement therapy, smoking, and the development of adenocarcinoma of the lung (OR, 3.4). A recent study using a mouse model also confirmed the role of estrogen in lung tumorigenesis. In this regard, diethylstilbestrol was shown to increase the number of lung tumors when administered in conjunction with urethane (21).

Estrogens may act directly as carcinogens through metabolism to catechol estrogens and the formation of adducts (22, 23). Alternatively, estrogens could act as tumor promoters through a receptor-mediated mechanism. There have been conflicting reports of the presence of ERs in lung tumors (24–30), based mainly on immunohistochemical detection of the classical ER, now termed ERα. Lung tumors from females were reported in one study to express ERs more frequently than those from males (26), and the ER-related protein, p29, has been reported to be expressed in lung tumors from females, where it is a negative prognostic indicator (31). However, other studies reported little or no ERα in most lung tumors as evidenced by immunohistochemistry (25, 27–30). There have been few quantitative studies of ERα mRNA expression in the lung, using gene expression, or protein expression, using immunoblotting, and the biological significance of ERα expression in lung tumors has not been determined.

A novel ER referred to as ERβ was cloned and characterized in human tissues and found to be localized to chromosomes 14 (32, 33). The ERβ protein has similarities to the classical ERα in terms of structure and function. The tissue distribution of these two receptors is not identical, but it appears to overlap in some cases (33). ERα and ERβ also differ in their effect on transcription at AP-1 sites (34). Whereas ERα liganded to estradiol activates transcription at AP-1 sites, ERβ liganded to estradiol inhibits transcription at AP-1 sites. In addition, several antiestrogens are transcriptional activators with ERβ at AP-1 sites, whereas the same compounds are inhibitors with ERα at this same site. This evidence suggests that ERα and ERβ may be regulated by distinct mechanisms and play different roles in gene regulation although they share functional characteristics. Although ERβ shows some expression in the mammary gland, relative levels of ERβ mRNA are highest in human granulosa cells, endothelial cells, ovary, and lung (33, 35). Recently, ERβ has been shown to be expressed in both normal lung as well as lung tumors and to have biological function (30). In the present study, we demonstrate that both ERα and ERβ mRNA and protein are expressed in normal bronchial epithelial cultures, cultured lung fibroblasts, and lung tumor cells and that these receptors play a biological role in the lung.

Additionally, we provide evidence that antiestrogens inhibit tumor growth and may be a useful strategy for lung cancer therapy.

MATERIALS AND METHODS

Cell Lines. Cell lines H23 (lung adenocarcinoma), A549 (bronchioloalveolar carcinoma, Calu-6 (lung adenocarcinoma), and MCF-7 (breast adenocarcinoma) were purchased from American Type Culture Collection (Rockville, MD). Cell lines 91T, 784T, 54T, and 128-88T were established in our laboratory from primary lung tumors. The donors of these primary lung tumors were verified to have no history of breast cancer, and by pathological assessment, the tumors were considered to be primaries to the lung. All normal lung fibroblast cell lines (851NLFB, 935NLFB, 948NLFB, 998NLFB, 999NLFB, and 1022NLFB) originated from peripheral lung tissue and should also be of lung origin. We specifically used cell lines derived from both males (91T, 784T, H23, and A549) and females (54T, 128-88T, Calu-6, and MCF-7) to eliminate the possibility that these cancers could be metastases of breast tumors. Primary bronchial epithelial cells were also from both males (941B and 967B) and females (983B), as were normal lung fibroblasts (948NLFB, 999NLFB, and 1022NLFB from males; 851NLFB, 935NLFB, and 998NLFB from females). The primary tissues were derived from biopsies of the upper airway taken at the time of resection (36).

RNA Isolation and RT-PCR. RNA was isolated from cells by the guanidinium thiocyanate method (37). Oligo(dT)12–18 (Life Technologies, Inc., Grand Island, NY) was annealed to 1 μg of total RNA and then reverse transcribed with Superscript II (Life Technologies). The reaction contained 1 μg of total RNA, 500 ng of oligo(dT)12–18, 50 μM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 1 mM each deoxynucleotide triphosphate and 200 units of Superscript. Briefly, total RNA and oligo(dT)12–18 were incubated at 70°C for 10 min and then placed on ice; the remaining components were then added, and the reactions were incubated at 42°C for 50 min and 70°C for 10 min. The cDNA generated was used as a template for PCR with primers specific for ERα and ERβ. PCR amplification was performed in a 20-μl reaction containing 2 μl of the reverse transcription reaction, Taq DNA polymerase (Perkin-Elmer, Foster City, CA), 1× PCR buffer, 1.5 mM MgCl2, 1 mM each deoxynucleotide triphosphate, and 1 μM each primer. PCR was carried out in a Perkin-Elmer 9600 Thermocycler, and the conditions were initial denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55 or 56°C (ERα or ERβ, respectively) for 30 s, and 72°C for 30 s. Final extension was at 72°C for 10 min. Ten μl of each reaction were run on a 1% agarose 1× Tris-borate-EDTA gel.

The ERα forward and reverse primers were 5′-GAGGACATGAGGAGCT-GCCA-A-3′ and 5′-CACCACCGTCGTGACTACCA-3′, respectively, and were located in exons 4 and exon 4, respectively. The expected size product for ERα was 752 bp. The ERβ forward and reverse primers were 5′-CTGTACTGGTCCAGGTCCA-3′ and 5′-CCAAGCTCATGTGTACCA-3′, respectively. These primers were located in exons 2 and 4 of ERβ and produce a 529-bp fragment. The RNA from each cell line was also analyzed for GAPDH as a control for RNA integrity, using the forward primer 5′-GTCACGGATT-TGGTCGTATT-3′ and reverse primer 5′-AGTCTCCTGGTGCGACT-GAT-3′. These primers produce a 520-bp fragment of GAPDH.

Protein Extraction and Western Analysis. Cells were grown to 90% confluency in three 75-cm2 flasks per cell line. The medium was removed from the cells, which were then rinsed with 1× PBS at room temperature. Protein was extracted by adding 2 ml of ice-cold RIPA buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.045 mg/ml aprotinin (Sigma Chemical Co., St. Louis, MO), and 1 mM sodium orthovanadate) to each flask. Cells were scraped, and the lysate was passed through a 21-gauge needle to shear the DNA. Twenty μl of 10 mg/ml phenylmethylsulfonyl fluoride stock were added to each lysate and incubated on ice for 30–60 min. The cell lysate was centrifuged at 10,000 × g for 10 min at 4°C. The protein concentration was measured in the supernatant using the Pierce BCA-200 Protein Assay Kit.

Protein aliquots (25 μg of all lung cells and 10 μg of MCF-7 breast cancer cells) were separated by size on a 10% SDS-tricine-polyacrylamide gel (Novex/Invitrogen, San Diego, CA) and transferred to nitrocellulose membrane. Non-specific binding sites were blocked by incubation in 1× TBS-T (0.2 μg Tris, 0.14 μg NaCl, 0.1% Tween 20) containing 5% milk for 1 h at room
temperature, followed by incubation overnight at 4°C with either a 1:3000 dilution of rabbit polyclonal anti-ERα antibody (HC-20; Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:1000 dilution of rabbit polyclonal anti-ERβ antibody (PanVera, Madison, WI) in 1× TBS-T containing 1% dry milk. After cells were washed three times in 1× TBS-T (10 min each at room temperature), horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Piscataway, NJ) was added at a 1:2000 dilution and incubated for 2 h at room temperature. After three more washes with 1× TBS-T, the immunoreactive peptide was detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), followed by exposure to autoradiography film. Reombinant human ERα (0.5 ng) and ERβ (2 ng; PanVera) were used as standards. SeeBlue Plus 2 prestained protein standards (Invitrogen, Carlsbad, CA) were also included in each gel.

**Cell Proliferation Assay.** Incorporation of BrdUrd was performed in duplicate wells in chamber slides (Nunc, Naperville, IL) as described previously (38) using the BrdUrd Labeling and Detection Kit II (Boehringer-Mannheim, Indianapolis, IN). Normal lung fibroblasts (935NFLB) and lung tumor cells (H23) were plated at a density of 1×10^4 cells/well in DMEM or RPMI, respectively, plus 10% FBS and were allowed to reach 50% confluency. After the wells were rinsed twice with 1× PBS, they were exposed to phenol red-free medium without serum for 24 h to deplete the cells of estrogen. Fresh phenol red-free medium plus 10% charcoal-stripped serum with or without (0.1–10 nM) β-estradiol was placed on the cells for 48 h. BrdUrd (10 μM) was added for the last 5 h of the incubation. Cells were washed, fixed, and exposed to a 1:250 dilution of the anti-BrdUrd working solution provided in the kit. The binding of the anti-BrdUrd antibody was detected by a 1:200 dilution of the immunoglobulin alkaline phosphatase working solution. A total of 1000 nuclei in each duplicate chamber were scored for BrdUrd labeling by light microscopy. The chambers were coded to prevent bias on the part of the observer. In addition to a saline control, a no-BrdUrd control was used to determine nonspecific background reaction. The no-BrdUrd control value was subtracted from all other values to normalize samples.

**In Vivo Tumor Xenograft Model.** 17-β-Estradiol pellets (1.7 mg; 60-day release; Innovative Research of America, Sarasota, FL) or placebo pellets were implanted s.c. into 20 female SCID mice (10 estrogen-treated and 10 placebo-treated controls; 5 weeks of age; Harlan Sprague Dawley, Indianapolis, IN). Three days after pellet implantation, H23 lung tumor cells were injected s.c. at two sites per mouse (4 × 10^4 cells/site). The mice were divided into four treatment groups (five animals per group): (a) estrogen only; (b) estrogen + ICI 182,780 (fluvestrant); (c) no estrogen + vehicle control; and (d) no estrogen + ICI 182,780. ICI 182,780 (30 mg/kg of mouse weight; Tocris, Ballwin, MO) or vehicle control (peanut oil) was injected s.c. twice a week for 5 weeks. Tumor size was measured before each weekly injection and reported as an average relative tumor volume, calculated as: (l × w × h) / 2 (mm^3), where l is the length, w is the width, and h is the height of the tumor measured with calipers. At the end of the 5-week period, the animals were sacrificed, and the tumors were removed. One-third of the tumor was harvested for protein analysis, one-third for RNA analysis, and the other third was fixed in 10% formalin for immunohistochemical analysis. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

**Immunohistochemical Staining.** Tissue samples were fixed in 10% formalin for 15–30 min at room temperature and stored in 100% ethanol. Tissues were then paraffin embedded, sliced, and mounted on slides. Paraffin was removed from the slides with xylene, and slides were stained according to standard procedures. Primary antibodies used were rabbit polyclonal anti-ERα antibody (HC-20) or rabbit polyclonal anti-ERβ antibody. The secondary antibody was a biotinylated IgG specific for the primary antibody. Brown staining was considered positive. The positive control for both ERα and ERβ was breast adenocarcinoma tissue. The negative control staining was done without the addition of primary antibody or with neutralization of the antibody with a blocking peptide.

**Transient Transfection and Luciferase Assay.** Cells were plated in phenol red-free medium containing 10% supplemented calf serum at 1×10^4 cells/well in 6-well plates. The next day, the medium was changed to one containing 10% charcoal-stripped serum to deplete the cells of estrogen and then transfected the following day. Transfections were performed using Lipofectamine (Life Technologies) or DC-Chol (synthesized by L. Huang, University of Pittsburgh, Pittsburgh, PA) for MCF-7 and H23 cells, respectively, according to the manufacturers’ instructions. The conditions for transfection in these different cell lines had been optimized previously. All plasmids used for transfection were purified using the Qiagen EndoFree kit to remove harmful endotoxins. Cells were washed twice with 1× PBS and transiently cotransfected with reporter plasmid (pERE-TK-LUC; a gift from M. Nichols, University of Pittsburgh, Pittsburgh, PA) and the pRL-CMV (Promega, Madison, WI) control to correct for transfection efficiency. Cells were incubated with 0–10 nM estrogen with or without 1 μM ICI 182,780 or tamoxifen for 24 h and then harvested using passive lysis buffer (Promega). Luciferase activity was measured using the Dual-Luciferase System (Promega) and a TD 20/20 luminometer. Values were corrected for protein concentration and are presented as the mean ± SE of three independent experiments, each of which had two samples per treatment.

**Isolation of Conditioned Medium and Detection of Secreted HGF.** A total of 6 × 10^3 normal lung fibroblasts (999NFLB) were plated on 100-mm plates in phenol red-free DMEM containing 10% supplemented calf serum and allowed to attach to the plate overnight. The medium was then replaced with one containing 10% charcoal-stripped serum to deprive cells of estrogen. Twenty-four h later, estrogen (0.1–10 nM) or vehicle control (ethanol) was added. After 48 h in the presence of estrogen, conditioned medium was isolated to measure secreted HGF protein. Briefly, the medium was removed from the plates, centrifuged for 10 min at 3000 × g, acidified with 0.1% acetic acid, and concentrated using a Sep-Pak C18 cartridge (Waters, Milford, MA). The concentrate was eluted with 2 ml of elution buffer (60% acetonitrile, 0.1% acetic acid, 2 mM sodium phosphate, pH 6.8). Five μg of protein from each sample were separated on a 10–20% SDS-tricine-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was immunoblotted with a 1:1000 dilution of monoclonal antibody HGF antibody (R&D Systems, Minneapolis, MN), followed by a 1:1000 dilution of a horseradish peroxidase-conjugated secondary antibody (Amersham). Immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate (Pierce), followed by exposure to autoradiography film. Signals were quantified by ImageQuant analysis.

**RESULTS**

**ERα and ERβ mRNA Expression in Normal and Tumor Lung Cells.** Previous reports have been conflicting as to the presence of ERs in lung cells (24–30, 39–41). These reports have been based mainly on immunohistochemistry of ERs. We examined both cultured normal lung fibroblast cell lines and normal bronchial epithelial cells for the presence of ERα and ERβ, using RT-PCR (Fig. 1A). A RT-PCR product for ERα was present in all of the normal lung fibroblast cell lines and cultured normal bronchial epithelial cells that we examined. Two major bands for ERα were amplified from most cell lines. The upper band was the expected 752-bp fragment that represents the full-length sequence between exons 4 and 8. The DNA fragment from this band was isolated and sequenced to confirm the product and was found to be the predicted ERα sequence. The lower band was 568 bp and corresponded to an exon 7 deletion (ERαΔ7), which was also confirmed by sequencing. This deletion is the most widespread deletion present in most human breast, uterus, and endometrial tumors (42–44). In some samples (i.e., 851NFLB) a small band slightly above ERαΔ7 was observed, which might correspond to ERαΔ5 or ERαΔ6, which should be 613 and 618 bp, respectively, with this primer set. We could also observe any double or triple exon deletions in exons 5–7 with this assay, which may account for some of the smaller bands that we observed. However, this RT-PCR assay for ERα would not detect deletions in exons 2, 3, or 4.

One main product was observed for ERβ in all of the normal lung cells that we examined. This fragment was sequenced and found to represent the full-length sequence between exons 2 and 4. The only ERβ deletion that we should have observed with these primers would be ERβΔ3, which is 413 bp and may be present in sample 1022. All of the normal bronchial epithelial cells that we examined also ex-
pressed ERβ. To verify the integrity of the RNA in the samples, we also analyzed GAPDH expression in all of the cell lines. All samples expressed GAPDH at similar levels.

We next looked at mRNA levels in lung tumor cell lines from both males and females (Fig. 1B). We examined samples from both males and females to diminish the possibility that a female’s lung tumor had been mistakenly diagnosed and was actually a breast tumor that had metastasized to the lung. These samples represented adenocarcinomas (91T, 784T, H23, and Calu-6), squamous cell lung tumors (54T and 851NLFB), and bronchioloalveolar (A549) tumor cells. MCF-7 breast cancer cells were used as a positive control. ERα mRNA was found in high levels in the 91T, 784T, and 54T cell lines and low levels in the 128-88T, H23, A549, and Calu-6 cell lines. Thus, ER is not confined to adenocarcinomas, but can also be found in squamous cell tumors. In H23 cells, the predominant band was ERαΔ7 and what could possibly be a double exon deletion of ~450 bp. The expected sizes of Δ5 + 6, Δ6 + 7, and Δ5 + 7 are 479, 434, and 429 bp, respectively. Additionally, a larger product was present in MCF-7 and 54T cells. This could possibly represent a variant of ERα that contained an in-frame duplication of certain exons. This idea is not without precedence. For example, a duplication of exons 6 and 7 has been described in breast cancer cells (45). The expected size of this variant would be 1025 bp with this primer set. ERβ mRNA was present in all tumor cell lines examined. A possible ERβΔ3 band was observed in H23 and MCF-7 cells.

The MCF-7 positive control showed ~2–40-fold higher levels of ERα mRNA than in lung tumors. On the other hand, similar levels of ERβ were observed in both MCF-7 cells and lung cancer cell lines. GAPDH mRNA levels were similar in all samples. There appeared to be no correlation between ER levels and whether the cells were derived from males or females. Additionally, the same bands were present in both normal lung cells (Fig. 1A) and tumor cells (Fig. 1B) and at similar ratios. One trend that was noticeable was that the lung tumor lines that expressed high ERα mRNA (784T and 54T) also expressed low ERβ. On the other hand, those that expressed low ERα mRNA (128-88T, H23, A549, and 91T) expressed high levels of ERβ.

**ERα and ERβ Protein Levels in Both Normal and Tumor Lung Cells.** We next sought to examine protein levels of the receptor in the lung cells. We analyzed the same cell lines that were used for the RNA analysis. Using an antibody to the COOH terminus (last 20 amino acids, encoded by exon 8) of ERα, we would not detect any alternatively spliced variants that produced a frameshift and truncated protein attributable to a premature stop codon. Deletions in ERα exons 2, 5, 6, and 7 resulted in frameshifting that produced premature stop codons at nucleotides 1008, 1613, 1941, and 1943, respectively. The normal ERα stop codon is located in exon 8 at nucleotide 2146. We could detect variants that contained deletions in exons 3 or 4 with this antibody. These alternatively spliced variants use the normal stop codon and thus would be recognized by the antibody used in this assay.

The full-length ERα 66-kDa protein was observed in MCF-7 cells and the recombinant human ERα standard control, but little or no full-length ERα protein was present in the lung cancer cell lines that we examined (Fig. 2A). Cell lines H23 and 91T showed a small amount of full-length protein. We observed a band that was smaller than the full-length ERα band and could possibly represent an exon 4 deletion, which should be 53.6 kDa. This band was present in all of the lung tumor lines as well as the breast cancer cell line. We did not observe the exon 3 deletion protein, which would be 61.7 kDa. We also observed a larger band in all of the cell extracts, which could possibly represent an ERα variant that contains an in-frame duplication of certain exons. The expected size of the previously reported exon 6 and 7 duplication form of the protein is 80 kDa. Alternatively, this larger band may represent a novel form of ERα that may use a unique translation initiation start site further upstream. The less intense bands observed may represent other variants or degradation products. There was no difference in pattern of expression between cell lines that were derived from males versus females. In addition, the same bands were observed in adenocarcinomas and squamous and bronchioloalveolar tumor cells. We obtained the same data using a polyclonal antibody to the same epitope from a different source (data not shown).

When the antibody was neutralized with a 5-fold excess of blocking peptide containing the same region that the antibody epitope corresponded to, the full-length ERα 66-kDa band, the 53.6-kDa putative exon 4 variant, and the larger 80-kDa band were completely blocked in all of the cell extracts (data not shown). The band that is slightly smaller than the 66-kDa band was not blocked, suggesting that all three of the intense bands are indeed specific for ERα. Thus, the full-length ERα is not present at all or is present in a very small amount in the various lung tumor cell lines that we examined; how-
ever, it is the most abundant form in breast cancer cells. This suggests that there may be tissue-specific differences in the expression of ERα full-length versus alternatively spliced variants.

We also examined ERα in normal lung fibroblasts. The full-length ERα was also not observed in these normal cells (data not shown). Thus, there is not a difference in ERα variants between normal lung fibroblasts and tumor cells. The fact that we do not observe a full-length ERα protein but do see the full-length ERα mRNA encompassing exons 4–8 suggests that there must be other deletions in exons 2 or 3 that the RT-PCR assay does not recognize.

ERβ immunoblots of the same cell lines showed the full-length ERβ 59-kDa protein as well as several smaller bands (Fig. 2B). The ERβ antibody recognizes the COOH terminus of the protein from amino acids 512–530. As was the case with the ERα antibody, we would not observe any variants that produced a frameshift that resulted in a premature stop codon and truncation of the protein. The variants that we could detect with this antibody were ERβΔ3 and ERβΔ4, which would result in proteins of 54.9 and 48.1 kDa, respectively. We have not further elucidated which variants the observed bands represent. The same bands that we observed in the lung tumor extracts were present in extracts from MCF-7 cells. When the antibody was neutralized with a peptide specific to the region that the antibody recognized, the full-length protein band and the recombinant ERβ protein were completely blocked (data not shown). The smaller bands were only partially blocked and thus may not be specific for ERβ. As an additional control, an antactin antibody was used to show that equal amounts of protein from each sample were loaded on these gels and that the protein was intact (data not shown).

**β-Estradiol Increases Cellular Proliferation of Normal Lung Fibroblasts and Lung Tumor Cell Lines In Vitro**

If estrogen influences lung tumor development, a stimulatory effect attributable to estrogen on the growth of lung tumor cells would be expected. We investigated this in several lung tumor cell lines. Cell proliferation was assessed by measurement of BrdUrd incorporation during DNA synthesis in proliferating cells. Fig. 3 shows the effect of β-estradiol on the growth of normal lung fibroblasts (935NLFB; Fig. 3A) and lung tumor cells (H23 cells; Fig. 3B).

In normal lung fibroblasts, a maximum 3.8-fold increase was observed in the presence of 1.0 nM estrogen (P < 0.05). A higher 17-fold increase in BrdUrd incorporation was observed in the tumor cells exposed to 1.0 nM estrogen (P < 0.01). This suggests that estrogens may stimulate cell growth in tumor cells to a greater extent than in normal lung fibroblasts and that estrogens are indeed involved in lung tumorigenesis. The results were calculated based on the growth of cells in the absence of estrogens by use of phenol red-free medium and charcoal-stripped serum. The results for H23 cells have been repeated three separate times, and the experiments using normal lung fibroblasts (935NLFB) were repeated two separate times. Similar results were observed with other tumor (A549) and normal fibroblast (999NLFB) cell lines. MCF-7 breast cancer cells were included in the assay as a positive control in each experiment. Estrogen increased proliferation of MCF-7 cell 3-fold (data not shown).

**In Vivo Tumor Growth in Immunocompromised Mice.** Growth experiments were also conducted in vivo to assess whether estrogen could stimulate tumor growth and also whether antiestrogens could inhibit growth in immunocompromised mice. The H23 cell line that was used in this experiment showed expression of both ERα and ERβ protein. In addition, because the cells were derived from a male, there is very little possibility that H23 could be derived from a breast tumor that metastasized to the lung rather than a primary lung tumor. Fig. 4 shows a representative experiment with five animals per treatment group. As shown in Fig. 4, estrogen stimulated tumor growth by 1.2-fold compared with placebo-treated controls. This suggests that estrogens do indeed affect lung tumorigenesis, although the increase is not statistically significant. The mice used in this experiment were female and were not ovariectomized, so there were background circulating levels of estrogen present in the placebo-treated controls. In addition, there may be differences in these background estrogen levels that may account for the large variation of tumor size that we observed in this particular group (range of tumor volumes, 42–1304 mm³).
verification of this model, ICI 182,780 treatment in combination with estrogen treatment (β-estradiol + ICI versus β-estradiol) inhibited tumor growth by 41% (ICI alone versus vehicle control, P < 0.05). This experiment was repeated two additional times with similar results. Thus, the use of antiestrogens may be a valuable treatment for lung cancer. Although we achieved <50% tumor growth inhibition, antiestrogens may be useful in combination with other available treatments to achieve further inhibition of tumor growth.

Frozen tumor sections from this experiment were harvested for RNA and protein and used in RT-PCR and Western analysis, respectively. Results showed that both ERα and ERβ were present in these tumors (data not shown). Immunohistochemical staining of these tumors also demonstrated expression of ERα (Fig. 5) and ERβ (data not shown). Curiously, in these tumors, ERα was localized mainly in the cytoplasm with very few nuclei stained (Fig. 5C). On the other hand, ERβ was located primarily in the nucleus (data not shown). The tumors that were treated with estrogen pellets did not show any more ERα nuclear staining than those that received the placebo pellet (Fig. 5, C versus D), suggesting that the presence of estrogen does not noticeably change the subcellular distribution of ERα. The staining was somewhat more intense in the estrogen-treated tumor versus the placebo-treated tumor. There may be physiological significance for this subcellular distribution in the cytoplasm. Because we observed little or no full-length ERα in Western blots (Fig. 2A), it is possible that the major ERα protein in lung cells is a variant protein that functions in the cytoplasm. Control staining of breast carcinoma tissue showed both ERα (Fig. 5A) and ERβ (data not shown) in the nucleus. Without addition of the ERα primary antibody, there was very little background staining (Fig. 5B).

**Immunohistochemical Analysis of Primary Lung Tumor Specimens.** We next examined the subcellular locations of ERα and ERβ in paraffin sections of lung tumor sections from patients. Once again, we observed ERα in the cytoplasm and ERβ in the nucleus (Fig. 6). Fig. 6A shows nuclear ERα staining in a breast adenocarcinoma. Lymphocytes are negative in this section, indicating that the staining is specific. Conversely, in a lung squamous cell dysplasia, very few nuclei stained positive for ERα and most of the staining was cytoplasmic (Fig. 6B). ERβ, on the other hand, was found mainly in the nucleus of both tissue sections from a lung squamous cell dysplasia (Fig. 6C) as well as from a lung squamous cell carcinoma (Fig. 6D).

Several other tumor specimens were positive for ERs by immunohistochemistry, including the primary tumor tissue used to generate cell lines 128-88T and 784T (data not shown). Several controls were also used in the staining, which included eliminating the primary antibody from the procedure to ensure that the secondary antibody did not give any background staining and neutralizing the primary antibody with a blocking peptide before use in the assay. Both of these control slides showed no background staining, suggesting that the staining is specific for ERα or ERβ.

![Image](image-url)
β-Estradiol Increases Transcription from an ERE in Lung Cancer Cells. To show that the ER is functional in lung cells, we used a gene reporter assay with a single vitellogenin ERE upstream of a minimal thymidine kinase promoter and the firefly luciferase gene (pERE-TK-LUC). In addition, we cotransfected a control plasmid, pRL-CMV, to control for transfection efficiency from plate to plate. The control plasmid contains the CMV immediate-early enhancer/promoter region, which provides strong expression of the Renilla luciferase cDNA. Thus, we were able to measure both firefly and Renilla luciferase levels in one assay based on dissimilar enzyme structures and substrate requirements of the two luciferases.

The results of three independent experiments in a lung cancer cell line, H23, are shown in Fig. 7A. MCF-7 cells were used as a positive control (Fig. 7B). Estrogen increased transcription in a dose-dependent manner in both MCF-7 and H23 cell lines. The effect of estrogen was greater in MCF-7 cells than it was in the lung cancer cell line that we examined. The addition of 10 nM β-estradiol resulted in an ∼4-fold increase in transcription in MCF-7 cells (P < 0.05; Fig. 7B).

Fig. 6. ERα and ERβ immunohistochemical analysis in patient tissue samples. A, ERα staining in a breast adenocarcinoma. B, ERα staining in a lung dysplasia. C, ERβ staining in a lung dysplasia. D, ERβ staining in a lung squamous cell carcinoma.
The presence of either ICI 182,780 or tamoxifen inhibited the stimulation of transcription attributable to estrogen back to basal levels. In H23 cells (Fig. 7A), transcription was stimulated 1.5–2-fold in the presence of estrogen (P < 0.01). ICI 182,780 and tamoxifen blocked this stimulation. These results suggest that at least some forms of the ERs present in the lung cancer cell line that we examined are functioning in the expected manner, i.e., they can bind to an ERE and activate gene transcription. We observed similar results in 784T lung cancer cells (data not shown). The cell lines that we examined expressed both ERα and ERβ. The fact that estrogen does not stimulate transcription to the same extent in lung cancer cells compared with breast cancer cells may reflect the fact that the lung cells do not contain full-length ERα and that the ERα variants present are mainly cytoplasmic. Thus most, if not all, of the stimulation may be attributed to ERβ, which is found in the nucleus. These results also suggest that the stimulation of transcription by estrogen is ER dependent.

Exposure of Lung Fibroblasts to Estrogen Increases HGF Secretion. HGF is a paracrine growth factor in the lung that is produced largely by mesenchymal cells in the submucosa of the airway. HGF acts on normal and neoplastic epithelial cells to produce mitogenesis and motility. It has been reported that the HGF gene contains two putative EREs: one in its promoter region and one in the first intron (46). The sequences of these EREs contain one nucleotide difference from the consensus ERE. We therefore determined whether HGF secretion by lung fibroblasts could be enhanced by treatment with estrogen. Treatment with β-estradiol in the absence of phenol red or serum-derived estrogens resulted in a 2-fold increase in the amount of HGF recovered from conditioned medium of fibroblasts from subject 999, a male who expressed both ERα and ERβ (Fig. 8). These results demonstrate that estrogens can alter the biology of lung fibroblasts to produce more of a growth factor known to promote lung cancer growth, invasion, and progression.

DISCUSSION

The presence of ERs in human lung tumors has been controversial for many years. We report in this study that some forms of both ERα and ERβ are indeed present in normal lung cells as well as in lung tumors. Additionally, we believe that little or no full-length ERα is expressed in these cells, but rather that the lung expresses several variants of ERα, including those lacking the nuclear localization signal in exon 4 and possibly a novel protein with a longer NH2 terminus because of a unique translation start site. This is the opposite of what is observed in breast tissue, which is that the ERα variants in total generally represent a minority of the ER mRNA compared with the full-length form. The expression of these variants in lung cells at levels detectable by Western analysis suggests a possible physiological role for the variants. We also present several lines of evidence that the ERs are functional in the lung: (a) Estrogen induces cell proliferation in the lung in vitro as well as in vivo. Antiestrogens can block this effect. (b) Estrogen can activate transcription from an ERE in lung cancer cells. (c) Estrogen can stimulate secretion of a growth factor thought to be involved in lung tumorigenesis. Collectively, these data demonstrate that ERs play a biological role in the lung.

Past studies of ER expression in lung tissues used reagents optimized to identify the 66-kDa full-length ERα protein. Antibodies raised against epitopes in the deleted exons of ERα or recognizing a conformation of the protein specific for breast tissue might not detect the cytoplasmic form of ERα found in the lung. ERβ protein might also not be recognized. The conclusion might have been that lung tumors are ER negative. We have performed a direct comparison between monoclonal versus polyclonal antibodies to the COOH terminus of ERα. Our results show that only the full-length 66-kDa ERα protein was detected with the monoclonal antibody, whereas two additional ERα-specific bands were detected with the polyclonal antibody. Previous studies have used both biochemical binding assays (39–41) and more specific immunohistochemical assays (24–30). The immunohistochemical reports that showed no or little reactivity to ER used an antibody to the NH2 terminus. The study that reported 96.8% positivity did not identify the source of antibody used (24).

Human ER mRNA is very heterogeneous. Both ERα and ERβ undergo alternative splicing to generate transcripts that have deletions in various combinations of the eight exons in ER. There have been at least 20 ERα alternatively spliced mRNA transcripts created by single, double, or multiple deletions reported using RNase protection assays and RT-PCR (42, 47–50). ERβ splice variants have been identified in human breast tumors and in ovarian, uterine, and mammary tissues (51). Deletions in ERβ exons 3, 5, 6, and 5 + 6 have been characterized. It has not been until recently that a variant protein was actually shown to be produced from an alternatively spliced transcript. In this regard, it has been reported that a 52-kDa truncated ERα protein, ERαΔ7P, is the predominant variant of ERα in MCF-7 breast cancer cells (52). At this time it is not clear whether these ER variants are involved in tumorigenesis or whether they have any physiological role at all. For example, the use of ERαΔ7P in a yeast transfection assay resulted in dominant-negative activity (53). This result, however, was not observed in a transfection assay in mammalian cells (54). Conflicting results of functionality have also been reported for ERαΔ5P (55, 56).

The classical model for the mechanism of action of steroid hormone receptors is that they undergo translocation to the nucleus when bound to steroids. Some evidence suggests that unbound receptors are in equilibrium between the nucleus and the cytoplasm (57). On the basis of immunohistochemistry results, the forms of ERα expressed in the lung were located primarily in the cytoplasm and ERβ in the nucleus in several of the lung cancer samples and normal lung cells that we have examined. There are two possible reasons that this may occur. One reason is that there may be enough ERα in the nucleus in lung cells to act as a transcription factor and any excess ERα in the cytoplasm has no physiological function. An alternative possibility is that there is another mechanism for ERα in the lung that is distinct from that of ERα in breast and other tissues. This may involve a variant protein of ERα that has a physiological function different from that of wild-type ERα. This idea is not without precedence. For example, Pasqualini et al. (58) found a differential subcellular distribution of the different ERα variants. In this regard, full-length ERα was predominantly nuclear and ERαΔ3 and ERαΔ4 were present in both the cytoplasm and nucleus. ERαΔ3 + 4 was predominantly cytoplasmic. Upon hormone treatment, the locations of ERαΔ3 and ERαΔ3 + 4 were unaffected. If indeed lung tumors contain mainly
variant ERα proteins, it is possible that these variants play a unique role in the cytoplasm. The cellular location of ERαs in the lung tumors may be attributable to lack of ligand because the tissue samples studied were from postmenopausal women. Alternatively, this may be a novel mechanism for ERα regulation in lung cancer cells. The fact that ERβ was found in the nucleus when the same specimens were used suggests that lack of ligand may not be a factor. We suggest that lung cancer cells have little or no full-length ERα and that the variant that is present is mainly cytoplasmic. This would be the case if an exon 4 deletion were the predominant protein because the nuclear localization signal is located in exon 4.

We were also interested in determining whether the ERs in the lung played any biological role. We observed an increase in transcriptional activation from an ERE in response to β-estradiol. These increases were not as large as those observed with MCF-7 cells. This could be because all of the effects are attributable to nuclear ERβ only and not to ERα, which is found only in the cytoplasm. In addition, it is possible that the ERα variants have an altered estrogen-binding pocket and thus may not be responsive to estrogen.

Estrogens are known to cause transcriptional activation of several growth factor genes, among them transforming growth factor α, epidermal growth factor, and insulin-like growth factor-1 (59, 60). All of these growth factors are known to mediate mitogenesis in lung tumors. We have found that lung fibroblasts can be induced to secrete more HGF, a paracrine growth factor in the lung, after estrogen exposure. This result also suggests that the stromal cells are affected by estrogen, not just the epithelium. Recently, the stromal cell component in reproductive tissues has been shown to be the site of the majority of ER expression, compared with the epithelial component (61). In addition, activation of the stromal ER is responsible for estrogen-induced epithelial cell proliferation; it is believed that estrogen binding to its receptor in stromal cells causes release of paracrine growth factors that induce epithelial cell growth, whereas the proliferation induced by estrogen binding to the epithelial cell receptor itself is not significant (61). This suggests that in normal lung tissue, lung fibroblasts may be the source of much of the receptor expression and response to estrogens. Our results show expression of ER mRNA in lung fibroblasts from most individuals. Proliferation of lung fibroblasts can lead to thickening of the airways and lung obstructions.

Among smokers, those with obstructive lung disease have the greatest risk for developing lung cancer (62, 63), and there is a growing body of literature showing that women are more susceptible to obstruction induced by smoking than men (64–66).

ER-mediated events may be responsible for at least some of the increased risk of women to lung cancer compared with men because women have higher circulating levels of estrogen. However, in men, tissue levels of estrogen may be high enough to show biological effects because testosterone can be converted to estrone locally in tissues through the action of aromatase. If ER is indeed involved in lung tumorigenesis, inhibiting lung tumor growth may be possible with antiestrogens, and antiestrogen therapy could be used in combination with other available therapies. At present, lung cancer patients have few therapeutic options. Potentially, these studies provide the basis for a new type of adjuvant therapy in selected lung cancer patients. Antiestrogens used at present for prevention of breast cancer may also have utility to prevent lung cancer.

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Human Non-Small Cell Lung Tumors and Cells Derived from Normal Lung Express Both Estrogen Receptor $\alpha$ and $\beta$ and Show Biological Responses to Estrogen

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