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Estrogen receptor beta (ERβ) subtype-specific ligands increase transcription, p44/p42 mitogen activated protein kinase (MAPK) activation and growth in human non-small cell lung cancer cells☆

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ABSTRACT

In non-small cell lung cancer (NSCLC) cells, 17β-estradiol increases transcription, activates MAPK, and stimulates proliferation. We hypothesize that estrogen receptor β (ERβ) mediates these responses because it, but not ERα, is detected in our NSCLC cell lines. To test this, we determined the effects of the ERβ-selective agonists genistein (GEN) and 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) and the ERα-selective agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) in 201T cells. The cells were transfected with either an ERα or an ERβ expression vector and an estrogen response element (ERE)-tk-luciferase reporter construct. PPT increased luciferase activity in cells expressing ERα but not ERβ. GEN and DPN selectively increased luciferase activity in ERβ-transfected cells at concentrations ≤10 nM. Fulvestrant blocked the GEN- and DPN-mediated increases, indicating that transcription was ER-dependent. GEN but not PPT mediated a significant 1.5-fold increase in reporter activity upon transfection with ERE-tk-luciferase alone, demonstrating that endogenous ERβ activates transcription. PPT and DPN increased MAPK phosphorylation (2.5-fold and 3.7-fold, respectively). However, only DPN stimulated 201T growth *in vitro* ($p = 0.008$) and *in vivo* ($p = 0.05$). We conclude that ERβ mediates genomic and non-genomic responses to estrogen in 201T cells and that activation of both pathways may be necessary for increased proliferation of these cells.

1. Introduction

Lung cancer is the leading cause of cancer mortality worldwide, with greater than 160,000 deaths expected in 2007 [1]. Although smoking remains the primary cause of this disease, approximately 25% of patients who develop lung cancer are lifetime never smokers [2]. A disproportionate number of these patients are females, which is suggestive of a potential role for estrogens in supporting the development or growth of lung cancer. Although epidemiologic studies have not shown a consistent association between use of exogenous estrogens and lung cancer risk (reviewed in [3]), recent reports point to a detrimental effect of estrogen on lung cancer outcome. In an analysis of the SEER database, we found that women diagnosed with either squamous cell carcinoma or bronchiolalveolar carcinoma who were between 55 and 59 years old (and were presumably post-menopausal) had significantly better survival than women who were 40–49 years old (and were presumably pre-menopausal) [4]. In a retrospective analysis of SWOG clinical trials, Albain et al. identified a survival benefit for women diagnosed with advanced non-small cell lung cancer (NSCLC) compared to men [5]. However, the survival advantage was observed only in women who were age 60 or older. In a third study, Ross et al. quantified free estradiol levels in serum samples collected from males enrolled in phase III clinical trials in advanced NSCLC [6]. These investigators observed that men with high free estradiol levels had significantly poorer survival than men with lower estradiol levels.

To elucidate the role of estrogen in lung cancer, the effects of 17β-estradiol (E2) have been directly evaluated using pre-clinical

Abbreviations: CSS, charcoal-stripped serum; DPN, 2,3-bis(4-hydroxyphenyl)propionitrile; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; FBS, fetal bovine serum; GEN, genistein; ICI, ICI 182,780; NSCLC, non-small cell lung cancer; MAPK, p44/p42-mitogen activated protein kinase; PARP, poly (ADP-ribose) polymerase; PPT, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol.

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models. In an animal model in which lung adenocarcinomas were induced by *K-ras* activation and *p53* deletion, E2 promoted tumor progression: both tumor burden and differentiation were affected by estrogen [7]. In established NSCLC cell lines, E2 significantly increased cell proliferation *in vitro* and *in vivo*, increased endogenous gene expression and promoted VEGF secretion; and induced rapid phosphorylation/activation of p44/p42 mitogen activated protein kinase (MAPK) [8–12]. Conversely, agents that either antagonized ER function (fulvestrant) or blocked endogenous E2 synthesis (exemestane) significantly inhibited the growth of lung tumor xenografts [10,11,13]. The pro-proliferative/pro-survival responses elicited by E2 in these experiments provide an explanation for the poorer clinical outcomes observed in NSCLC patients who are either predicted or documented to have high estrogen levels.

The cellular response to estrogens is mediated by estrogen receptors ERα and ERβ, which belong to the nuclear steroid hormone receptor superfamily. Classically, these proteins function as sequence specific, ligand-dependent transcription factors: upon E2 binding, ERα and ERβ increase the transcription of genes whose promoters contain an estrogen response element (ERE). The two receptors are encoded by distinct genes and display differential tissue distributions. Whereas ERα is expressed primarily in the uterus, liver, kidney and heart, ERβ expression occurs primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, hematopoietic and central nervous systems [14]. Analysis of murine knock-out models uncovered an important role for ERβ in normal lung biology. Targeted inactivation of ERβ results in lung abnormalities in female mice (at 3 months of age) including a decrease in the number of alveoli and altered surfactant homeostasis [15]. By 5 months of age, both male and female ERβ-deficient mice exhibit signs of significant lung dysfunction [16].

A central question to be answered with regard to the estrogen signaling pathways that are operative in lung cancer cells is which of the ER proteins mediates the response. To address this issue, immunohistochemistry studies have been conducted in which the relationship between ER expression in tumor tissue and disease outcome was analyzed. Nuclear localization of ERβ was observed in 45–69% of NSCLC cases [17–20]. In each of four recent studies, nuclear ERβ expression was found to be a favorable prognostic indicator, although this was observed only in males in two of the studies [18,20]. Although ERβ immunoreactivity was also detected in the cytoplasm, it was observed primarily in tumor specimens that also express a nuclear pool of the receptor. The analysis and interpretation of ERα staining patterns in NSCLC are considerably more complex. Nuclear ERα immunostaining is either never, or only rarely, detected in primary lung tumors [17–20]. ERα is detected in the cytoplasm of NSCLC cases, but the proportion of positive cases varies widely from approximately 3% [17] to 73% [19]. In two studies where the prognostic significance of ERα expression was reported, it was found to have either no correlation with survival [20] or to correlate with poor prognosis [19].

Because ERβ is expressed and functional in normal lung tissue and is consistently detected in primary lung cancers, we hypothesized that that this receptor mediates the response of NSCLC cells to estrogen. To ascertain whether activation of endogenous ERβ is sufficient to induce the full range of estrogenic responses described in lung cancer cells, we determined the effects of the ERβ subtype selective agonists genistein (GEN) and 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) on gene transcription, MAPK activation and cell growth in 201T NSCLC cells. GEN has a 26-fold binding affinity preference for ERβ versus ERα and activates ERβ with nearly 7-fold greater potency than ERα [21]. DPN has a 70-fold binding affinity preference for ERβ versus ERα and activates ERβ with 80-fold greater potency than ERα [22]. As a control, we also evaluated the effects of the ERα-selective agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) in our studies, which has a >400-fold binding affinity preference for ERα versus ERβ [23].

2. Materials and methods

2.1. Cell lines

201T cells were produced previously from primary non-small cell lung cancer (NSCLC) tumors as described [24]. The human estrogen-dependent breast cancer cell line MCF-7 and the human NSCLC cell lines H23 and A549 were purchased from ATCC (Manassas, VA). 201T and A549 cells were maintained in Basal Medium Eagles (GIBCO Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine (GIBCO) and 100-units/mL penicillin–streptomycin (GIBCO). MCF-7 and H23 cells were maintained in RPMI 1640 medium (Mediatech) supplemented with 10% FBS and 100-units/mL penicillin–streptomycin. All cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere and were mycoplasma-free. For all experiments involving ER ligands, cells were washed in sterile PBS and then cultured in phenol red-free medium with either charcoal-stripped serum (CSS) or no serum for at least 24 h prior to addition of ligand.

2.2. Chemicals and plasmids

E2 and GEN were purchased from Sigma Aldrich (St. Louis, MO). PPT, DPN and fulvestrant were purchased from Tocris (Ellisville, MO). The ligands were prepared either as 1×10^{-2} M stocks in 100% ethanol (E2, PPT, DPN and fulvestrant) or as a 1×10^{-1} M stock in DMSO (GEN) and stored at −20 °C. Prior to use in an experiment, each of the ligands was further diluted into tissue culture medium so as to keep vehicle concentrations constant across all treatment groups. CMV-ERα contains the full-length coding sequence of human ERα (595 amino acids). CMV-ERß contains the full-length coding sequence of human ERß (530 amino acids). ERE-tk-luciferase contains a single ERE cloned upstream of the thymidine kinase promoter and the luciferase gene (gift of Dr. Don DeFranco, Department of Pharmacology and Chemical Biology, University of Pittsburgh). CMV-SPORT-βgalactosidase was purchased from Invitrogen (Carlsbad, CA).

2.3. Nuclear and cytosolic protein extraction and immunoblot analysis

Cells were grown to 85% confluence in T75 flasks in complete tissue culture media. 201T and A549 nuclear and cytosolic extracts were prepared using the Nuclear Extraction Kit (Panomics, Redwood City, CA) according to the manufacturers' instructions. H23 nuclear, membrane, and cytosolic extracts were prepared using the Proteoextract Subcellular Proteome Kit (Calbiochem, San Diego, CA) according to the manufacturers' instructions. Proteins were quantified using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL). Equivalent amounts of protein from each sample were separated by electrophoresis through pre-cast 10% Tris–HCl polyacrylamide gels (BioRad, Hercules, CA) and transferred to PVDF membranes. Nonspecific binding sites were blocked by incubation in 1× TBS-T (0.05% Tween 20, 150 mM NaCl, 10 mM Tris, pH 7.4)/5% milk for 1 h at room temperature, followed by incubation overnight at 4 °C with a 1:1000 dilution of anti-ERα antibody (sc-544 from Santa Cruz Biotechnology, Santa Cruz, CA), a 1:500 dilution of anti-ERα antibody (sc-543 from Santa Cruz Biotechnology), or a 1:1000 dilution of anti-ERβ antibody (05-824 from Upstate, Lake Placid, NY). The blots were then washed in 1× TBS-T and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Piscataway, NJ). Immune complexes were detected using ECL

chemiluminescent substrate (Pierce Biotechnology), followed by exposure to autoradiography film. Blots were stripped with Restore Stripping Buffer (Pierce Biotechnology) and reprobed with a 1:1000 dilution of anti-PARP antibody (sc-7150 from Santa Cruz Biotechnology) or a 1:10,000 dilution of a pan actin antibody (MAB1501 from EMD Biosciences San Diego, CA).

To study MAPK phosphorylation, 201T cells were grown to 75% confluence in T75 flasks. Cells were washed two times with 1× PBS followed by serum deprivation for 48 h in phenol red-free media. E2, PPT, or DPN was added for the times indicated. Whole cell protein extracts were prepared using ice-cold radioimmunoprecipitation buffer as described previously [12]. Equal amounts of protein (35 μg) for each sample were separated by size on a pre-cast 10% SDS–tricine polyacrylamide gel (Invitrogen). Nonspecific binding sites were blocked as described above. The primary antibody was a 1:1000 dilution of anti-phospho-p44/p42 mitogen activated protein kinase (MAPK) monoclonal antibody (Cell Signaling Technology, Beverly, MA) and secondary antibody was a 1:2000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare Life Sciences, Piscataway, NJ). Washes and detection were performed as described above. Blots were stripped and reprobed for total MAPK protein. Quantitation of the signals was done by densitometric scanning and ImageQuaNT analysis (Molecular Dynamics ImageQuaNT software version 5.2, Sunnyvale, CA).

2.4. Transfections

For transient transfection assays in which the activity of exogenous receptor was measured, cells were plated in 6-well dishes at a density of 4×10^5 cells per well in phenol red-free medium containing 10% CSS. The next day, the cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfections contained 0.75 μg of ERE-tk-luc reporter plasmid, 0.1 μg of CMV-ER expression vector, and 0.5 μg of CMV-β-gal. After 16 h, the medium was replaced and the cells were treated with vehicle or ligand for an additional 24 h. Cells were harvested and assayed for luciferase as indicated below.

For transient transfection assays in which the activity of endogenous receptor was measured, cells were plated in 6-well dishes and transfected using Lipofectamine, as above. Transfections contained 1.5 μg of ERE-tk-luc reporter plasmid, 0.5 μg of CMV-β-gal, and 0.5 μg of empty vector. After 5 h, the medium was replaced with fresh medium containing vehicle or ligand. The next day, cells were harvested and assayed for luciferase as indicated below.

2.5. Luciferase assay

Cells were harvested and washed twice with PBS. The washed cell pellet was lysed in 100 μL of 1× Reporter Lysis Buffer (Promega, Madison, WI). Complete cell lysis was achieved using one freeze–thaw cycle that consisted of a 10 min incubation at −80 °C followed by a rapid thaw at 37 °C. Cell extracts were transferred to a microcentrifuge tube and clarified by centrifugation for 3 min at 12,000 × *g* at room temperature. The supernatants were transferred to a fresh tube and assayed using the Luciferase Assay System (Promega). For each assay, 30 μL of extract was diluted with 70 μL of 1× Reporter Lysis Buffer. Luminescence was read using an AutoLumat LB953 luminometer (Berthold, Pforzheim, Germany).

The luciferase activity was normalized to the β-gal activity to control for transfection efficiency and cell recovery. β-Gal activity was measured by diluting a fixed volume of cell extract in 1× Reporter Lysis Buffer to a final volume of 0.1 mL. The resulting samples were mixed with 0.1 mL of 2× β-gal Assay Buffer (200 mM $NaPO_4$ buffer pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/mL *ortho*-nitrophenyl-β-galactoside) and incubated at 37 °C until yellow color developed. The reactions were terminated by addition of 0.7 mL of 1 M Na_2CO_3 solution, and the optical density was measured at 420 nm.

2.6. MTT assays

201T cells were seeded in complete tissue culture medium in 96-well plates. After overnight attachment, the tissue culture medium was removed. The adherent cells were washed twice with sterile PBS, and the cells were cultured for an additional 48 h in phenol red-free tissue medium supplemented with 0.1% CSS. The cells were then treated with the indicated ligands for a total of 48 h. The effect of treatment on cell growth was determined by MTT assay. The MTT assay kits were purchased from ATCC, and assays were conducted essentially as described by us previously [25]. The % cell growth was calculated using the following equation: % cell growth = $100[(O.D._{treatment} - O.D._{blank})/(O.D._{vehicle} - O.D._{blank})]$.

2.7. In vivo tumor xenograft model

Female ovariectomized nude mice (10 per group) were treated with 500 μg PPT, 250 μg DPN or vehicle control by s.c. injection 5 times per week. Ligand doses were chosen based on those reported by Frasor et al. [26]. All compounds were dissolved in DMSO then diluted 1:10 in corn oil for injection in 100 μl volume. Following 1 week of ligand treatment, 201T NSCLC cells (2×10^6) were injected into the flank of each animal. Ligand treatment was continued for 4 additional weeks. Tumor volume was measured two times per week using digital calipers and reported as an average relative tumor volume (mm^3). Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and were conducted in strict compliance with institutional guidelines.

2.8. Statistical analysis

Data were graphed and analyzed using GraphPad Prism 4.0 software. The Student's *t* test or ANOVA was used for the statistical analysis of the *in vitro* studies. All values are expressed as the mean ± SD. Significance tests were performed with two-sided significance level 0.05. For the *in vivo* study, we used a repeated measures (mixed effects regression) model to analyze the data. Based on the Bayes Information Criterion, the best model fit was achieved when tumor volume was logarithmically transformed, and when predictor variables included a linear term for the day and the interaction between day and treatment group. We also evaluated a quadratic model using the raw outcome value without transformation.

3. Results

As a first step toward dissection of the relative roles of ERα and ERβ in mediating genomic and non-genomic signaling, fractionation studies were conducted to determine the subcellular localization of each receptor in NSCLC cells. We used 201T adenocarcinoma cells, H23 adenocarcinoma cells, and A549 bronchioloalveolar cells for these studies because their *in vivo* growth is increased by E2 and decreased by therapeutic agents that inhibit estrogen signaling [11–13,27]. MCF-7 breast cancer cells which express both ERα and ERβ were included as a positive control for receptor expression. Cells were grown to near-confluence and then were harvested and fractionated. Expression of ERα and ERβ was analyzed in each fraction by immunoblot. Full-length ERα protein (66 kDa) was detected in the nuclear fraction of MCF-7 cells but not in 201T or A549 cells (Fig. 1A). Full-length ERα was also not detected in H23 cells, even when a different fractionation procedure and two antibodies that recognize distinct ERα epitopes (hinge and C-terminus) were

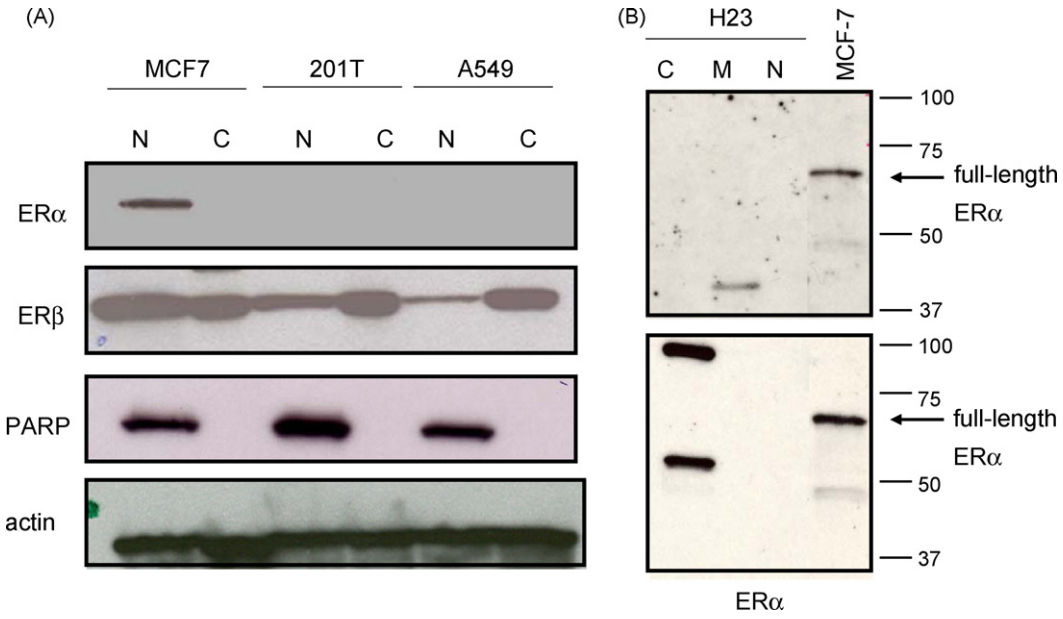

Fig. 1. Subcellular localization of ER protein in NSCLC cells. (A) MCF-7, 201T, and A549 cells were grown to near-confluence in complete tissue culture medium. The cells were harvested and nuclear (N) and cytosolic (C) fractions prepared as described in Section 2. Equivalent amounts of protein (25 μg) from each extract were resolved on a 10% polyacrylamide gel and transferred to PVDF membranes. The membranes were probed for ERα or ERβ. The ER blots were stripped and reprobed for either PARP (as a control for nuclear fractionation) or actin (as a control for protein loading and quantitation). (B) H23 cells were grown to near-confluence and then nuclear (N), membrane (M), or cytosolic (C) fractions were prepared as described in Section 2. Equivalent amounts of protein (60 μg) from each extract were analyzed for ERα expression by immunoblot using antibodies that detect either the hinge domain (top panel) or C-terminus (bottom panel) of the protein. The nuclear fraction from MCF-7 cells was included as a positive control for ERα expression. Arrows indicate the position of the full-length ERα protein.

utilized (Fig. 1B). Smaller immunoreactive bands of ≈42 kDa and ≈54 kDa were observed in the membrane and cytosolic fractions of H23 cells, respectively. Although we cannot exclude the possibility that these represent variant forms of ERα, they do not share common epitopes or subcellular localization and may represent nonspecific cross-reactive bands. In contrast to ERα, full-length ERβ protein (59 kDa) was detected in the nuclear and cytosolic fractions of 201T and A549 cells. The localization of ERβ to both the nuclear

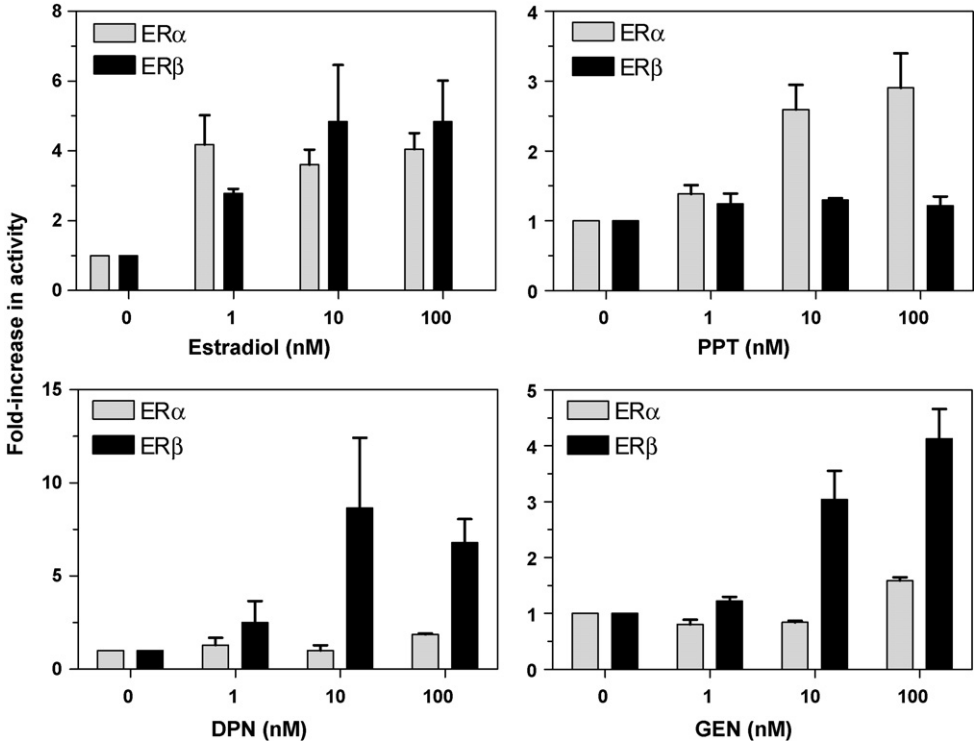

Fig. 2. Receptor selectivity of subtype-specific ligands in 201T cells. 201T NSCLC cells were cultured for a minimum of 24 h in phenol red-free medium supplemented with 10% charcoal-stripped serum. Then, the cells were transiently co-transfected with either CMV-ERα or CMV-ERβ expression vectors and the ERE-tk-luciferase reporter construct, as described in Section 2. The next day, the medium was replaced with fresh phenol red-free medium supplemented with 10% CSS containing either vehicle or the indicated concentrations of E2, PPT, DPN or GEN. After 24 h, the cells were harvested and assayed for luciferase activity. The fold-increase in activity was calculated by assigning the luciferase activity in control (vehicle-treated) cells a value of 1.0. Results are the mean ± SD for replicate wells.

and cytosolic fractions suggests it has the potential to impact both genomic and non-genomic signaling pathways in lung cancer cells.

We planned to use well-characterized ER subtype selective agonists to determine the functional consequences of selective activation of endogenous ERβ (or ERα) in lung cancer cells. We first confirmed that the ligands displayed the expected receptor selectivity in 201T cells by measuring transcriptional responses in transient transfection assays. To do this, 201T cells were co-transfected with an expression vector that encodes either ERα or ERβ and an ERE-tk-luciferase reporter construct. Following transfection, the cells received vehicle (controls) or were treated with increasing concentrations of E2, the ERα-selective agonist PPT, or the ERβ-selective agonists DPN or GEN (Fig. 2). In these assays, luciferase reporter gene activity serves as a measure of ER-mediated transcription. E2 stimulated reporter gene activity in 201T cells in the presence of both ERα and ER β, whereas PPT activated transcription only in the presence of ERα. A maximal ERα driven transcription response was observed at 10 nM PPT, although receptor specificity was maintained up to the highest concentration of PPT tested (100 nM). DPN and GEN selectively increased ERβ transcription at ligand concentrations ≤10 nM. However, when the ligand concentration was increased to 100 nM, both DPN and GEN modestly increased ERα transcription. These data indicate that PPT functions as an ERα-selective agonist in 201T cells at ligand concentrations up to 100 nM, whereas DPN and GEN function as ERβ-selective agonists in 201T cells at concentrations ≤10 nM.

To confirm that the effects of GEN and DPN on ERE-mediated transcription were dependent on the classical ER, we examined the ability of the pure anti-estrogen fulvestrant [28] to block transcription activation. As above, 201T cells were co-transfected with an ERβ expression vector and the ERE-tk-luciferase reporter construct. The next day, cells were untreated or were treated with E2 ± fulvestrant, GEN ± fulvestrant, or DPN ± fulvestrant (Fig. 3). As expected based on the results presented in Fig. 2, E2, GEN, and DPN treatment induced a statistically significant increase in reporter gene activity compared to vehicle controls. The stimulatory effect of each ligand was abrogated in the presence of fulvestrant. This result confirms that the transcriptional response to GEN and DPN in 201T cells is mediated via the classical ER.

We next determined the effect of the subtype-selective ligands on transcriptional responses mediated by the endogenous ER. To do this, 201T cells were transiently transfected with ERE-tk-luciferase alone. The cells were left untreated or were treated with increasing concentrations of E2, PPT, or GEN (Fig. 4). The concentrations of each ligand that were utilized for these endogenous receptor activation studies were based on the results presented in Fig. 2. Luciferase activity was significantly increased by 10 nM E2 ($p = 0.0085$ versus vehicle) and 10 nM GEN ($p = 0.0068$ versus vehicle) but not by PPT. At a final ligand concentration of 10 nM, reporter gene activity increased a mean 1.6-fold in the presence of E2 and 1.5-fold in the presence of GEN.

In addition to signaling via the classical transcription pathway, the ER has been implicated in a non-classical signaling pathway involving the rapid, non-genomic activation of MAPK [29]. This non-genomic pathway allows for cross-talk between the ER and growth factor signaling pathways that contribute to NSCLC cell growth [12,13]. To determine whether ERα or ERβ mediates non-genomic signaling, 201T cells were cultured for 48 h in phenol red-free, serum-free medium. Then, the cells were incubated for increasing times (0–240 min) with either vehicle, 10 nM E2, 10 nM DPN, or 100 nM PPT. Whole cell extracts were prepared and analyzed by immunoblot for MAPK phosphorylation (Fig. 5A). As shown in Fig. 5B, the magnitude of induction of MAPK phosphorylation was greatest in 201T cells treated with E2 (7.5-fold). However, DPN and PPT also increased MAPK activation (3.7-fold and 2.5-fold, respectively), and the kinetics of activation were similar for E2, DPN and PPT (Fig. 5B). Phosphorylation of MAPK was maximal within 5 min of treatment and returned to near-baseline levels within 30 min. Similar results were obtained in A549 NSCLC cells treated with E2, PPT and DPN and in 201T cells treated with GEN (data not shown).

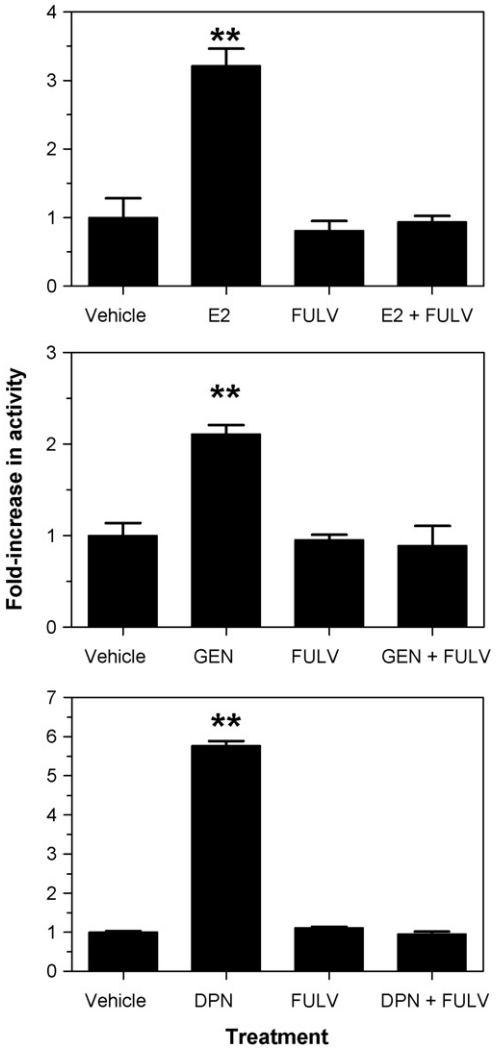

Fig. 3. Effect of fulvestrant on ERβ-mediated transcription in 201T cells. 201T cells were cultured for a minimum of 24 h in phenol red-free medium supplemented with 10% CSS. Then, the cells were transiently co-transfected with the CMV-ERβ expression vector, the ERE-tk-luciferase reporter construct, and CMV-β-gal. The next day, the medium was replaced with fresh phenol red-free medium supplemented with 10% CSS containing either vehicle or the indicated ligands. The final concentration of each ligand was as follows: E2 (10 nM), GEN (10 nM), DPN (10 nM), fulvestrant (100 nM). After 24 h, the cells were harvested and assayed for luciferase and β-gal activity. Luciferase activity was normalized to β-gal activity to control for any variation in transfection efficiency or cell recovery. The fold-increase in activity was calculated by assigning the ratio of luciferase/β-gal in control (vehicle-treated) cells a value of 1.0. Results are the mean ± SD for 3 wells per treatment group. $^{**}p < 0.001$, unpaired Student's *t* test, calculated versus vehicle control.

Finally, we examined the effect of the receptor subtype selective ligands on the growth of 201T cells. For *in vitro* studies, we determined whether the ligands stimulated cell growth as single agents and whether they cooperated with EGF to increase cell growth. To do this, the cells were cultured for 48 h in phenol red-free medium with 0.1% CSS. Then the cells were treated with vehicle, 10% FBS (as a positive control), EGF, each of the ER ligands alone, or each of the ER ligands plus EGF. Effects on growth were determined 48 h post-treatment by MTT assay (Fig. 6). Compared to vehicle control, FBS and EGF significantly increased cell growth, but E2 alone, DPN alone, and PPT alone had no consistent stimulatory effect (Fig. 6A). When

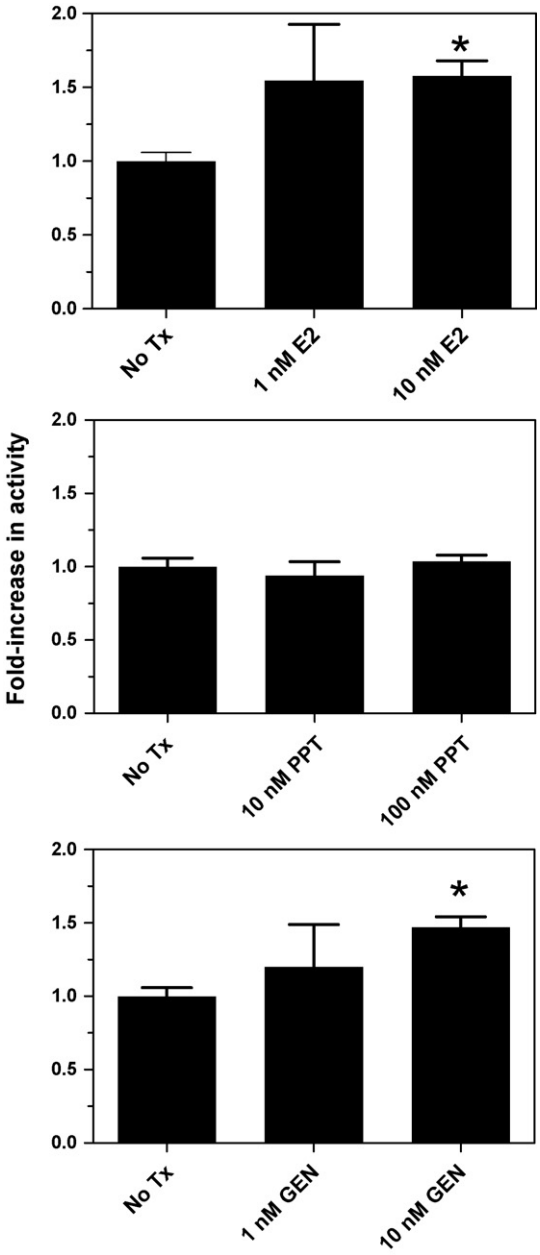

Fig. 4. Regulation of transcription by endogenous ER in 201T cells. 201T cells were cultured for a minimum of 24 h in phenol-red free medium supplemented with 10% CSS. Then, the cells were transiently co-transfected with the ERE-tk-luciferase reporter construct and CMV-β-gal. After 5 h, the medium was replaced with fresh phenol red-free medium supplemented with 10% CSS containing vehicle or E2, GEN, or PPT at the indicated final concentrations. After 24 h, the cells were harvested and assayed for luciferase and β-gal activity. Luciferase activity was normalized to β-gal activity to control for any variation in transfection efficiency or cell recovery. The fold-increase in activity was calculated by assigning the ratio of luciferase/β-gal in control (vehicle-treated) cells a value of 1.0. Results are the mean ± SD for 3 wells per treatment group. The results shown were all obtained in the same experiment and are representative of 9 independent experiments with E2, 3 independent experiments with GEN and 2 independent experiments with PPT.

combined with EGF, E2 and DPN but not PPT mediated a significant, further increase in cell growth (Fig. 6B). These data support the conclusion that ERβ cooperates with EGF to promote the proliferation of 201T cells. We also tested the ability of PPT and DPN to promote lung tumor growth compared to control treated animals in an *in vivo* xenograft model (Fig. 6C). When 201T cells were implanted into female, nude, ovariectomized mice, tumor growth was stimulated by DPN but not PPT ($p = 0.05$; DPN versus vehicle control). By day 33 of ligand treatment, a 1.5-fold increase in tumor volume was observed in mice treated with DPN versus control treated mice.

4. Discussion

Although the role of estrogen as a proliferative stimulus in breast cancer is well established, its possible role in lung cancer has only more recently been studied. Several biological responses to E2 have been observed in lung cancer cells including growth stimulation, alteration of gene expression, and phosphorylation/activation of MAPK [7,8,10,12]. In addition, the estrogen signaling pathway has been found to interact with the epidermal growth factor receptor signaling pathway [12,13] or with tobacco carcinogens to stimulate the proliferation of small airway epithelial cells [30] and lung cancer cells [9]. The growth-promoting effects of estrogen on lung cancer precursors and lung cancer cells provide a rationale for the further evaluation of agents that disrupt estrogen signaling pathways in lung cancer chemoprevention and treatment models.

Theoretically, more precise therapeutic targeting could be achieved by the identification of the ER subtype that mediates the effects of estrogen in lung cancer. However, ERα and ERβ expression in lung cancer cells has been variably reported, making it difficult to identify the receptor that is responsible. To assist in the identification of the receptor responsible for genomic and non-genomic signaling in NSCLC cells, we used immunoblot assays to examine the subcellular distribution of each receptor and studied the effect of ligands that selectively activate either ERα or ERβ. Consistent with our previously published data using whole cell extracts [8], we did not observe expression of full-length ERα protein in nuclear or cytoplasmic extracts prepared from NSCLC cells (Fig. 1). In contrast, ERβ was detected in both the nuclear and cytoplasmic fractions of two different E2-responsive cell lines, suggesting that it may participate in both genomic and non-genomic signaling. Consistent with this idea, we find that ERβ-selective ligands significantly stimulate reporter gene transcription and increase MAPK phosphorylation. Together, this data leads us to conclude that endogenous levels of ERβ are sufficient to generate both the genomic and non-genomic response to estrogen in 201T lung cancer cells.

To evaluate the ability of endogenous ER to mediate genomic signaling, we used a luciferase reporter plasmid that contains a single ERE in the promoter region. With this construct, we observed a significant and reproducible increase in transcription in response to E2 addition (Fig. 4 and data not shown). The magnitude of E2 induction of reporter gene activity in 201T cells was modest (1.5-fold), but was consistent with what was observed in a different lung cancer cell line that expresses only ERβ [31]. In contrast to our findings, Zhang et al. recently reported that E2 does not activate transcription from an ERE-luciferase reporter plasmid in NSCLC cells that express ERβ but not ERα [32]. We speculate that the greater fraction of nuclear ERβ protein we observe in our cell lines may (A) contribute to the disparate findings and (B) be more representative of the clinical situation because 45–69% of lung cancer cases (analyzed across 4 studies that included at least 100 patients each) express a nuclear pool of the receptor [17–20].

We are unable to make any conclusions regarding the contribution of endogenous ERα signaling to the cellular response to estrogen because our cell lines do not express detectable levels of ERα protein (Fig. 1). However, one other study examined the relative roles of endogenous ERα or ERβ in supporting lung cancer cell proliferation by transiently transfecting siRNA duplexes targeting each receptor into H23 cells [13]. Unlike our findings, their H23 cells express both ERα and ERβ. Complete siRNA-mediated suppression of mRNA corresponding to either receptor resulted in approximately 20% inhibition of cell growth, suggesting that ERα and ERβ contribute equally to the growth of these H23 cells. The ability of the siRNA to attenuate either the genomic or non-genomic

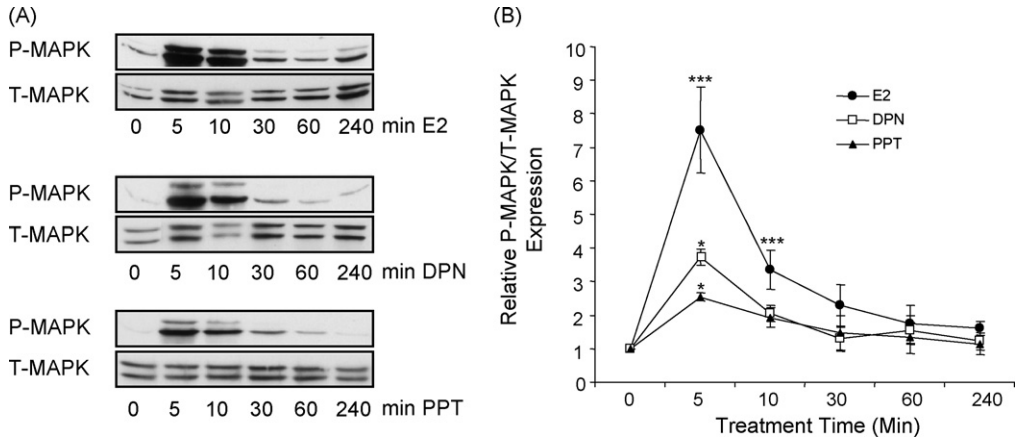

Fig. 5. Effects of ER subtype-specific ligands on MAPK activation in 201T cells. 201T cells were serum deprived for 48 h followed by addition of vehicle, E2 (10 nM), DPN (10 nM), or PPT (100 nM) for the times indicated. Whole cell extracts were prepared and analyzed by immunoblot for MAPK phosphorylation. The blot was stripped and reprobed with an antibody against total MAPK. (A) Representative immunoblots are shown. (B) Quantitation was done using densitometry and ImageQuant analysis. No treatment was set to 1.0 and represents the ratio of phospho-MAPK expression to total MAPK expression. The quantitative data was derived from 3 independent experiments. $^{***}p < 0.0001$; $^{*}p < 0.05$ by ANOVA.

responses to E2 was not examined, so that it is not possible to state whether the two receptors have redundant or independent functions with regard to these activities.

Surprisingly, we observed that PPT induced MAPK activation in 201T cells despite the absence of any detectable full-length ERα protein. Because we used two antibodies that were raised against distinct epitopes of ERα to analyze protein expression (Fig. 1A and B), we consider it unlikely that there is a small functional pool of ERα protein that escaped detection in our immunoblot studies. Although PPT is an ERα-selective ligand, it binds ERβ with low affinity [33]. Possibly, this binding is sufficient to nominally activate non-genomic signaling from ERβ by dissociating the receptor from the repressive HSP90 complex, allowing for some MAPK phosphorylation. Although PPT may bind ERβ and activate non-genomic

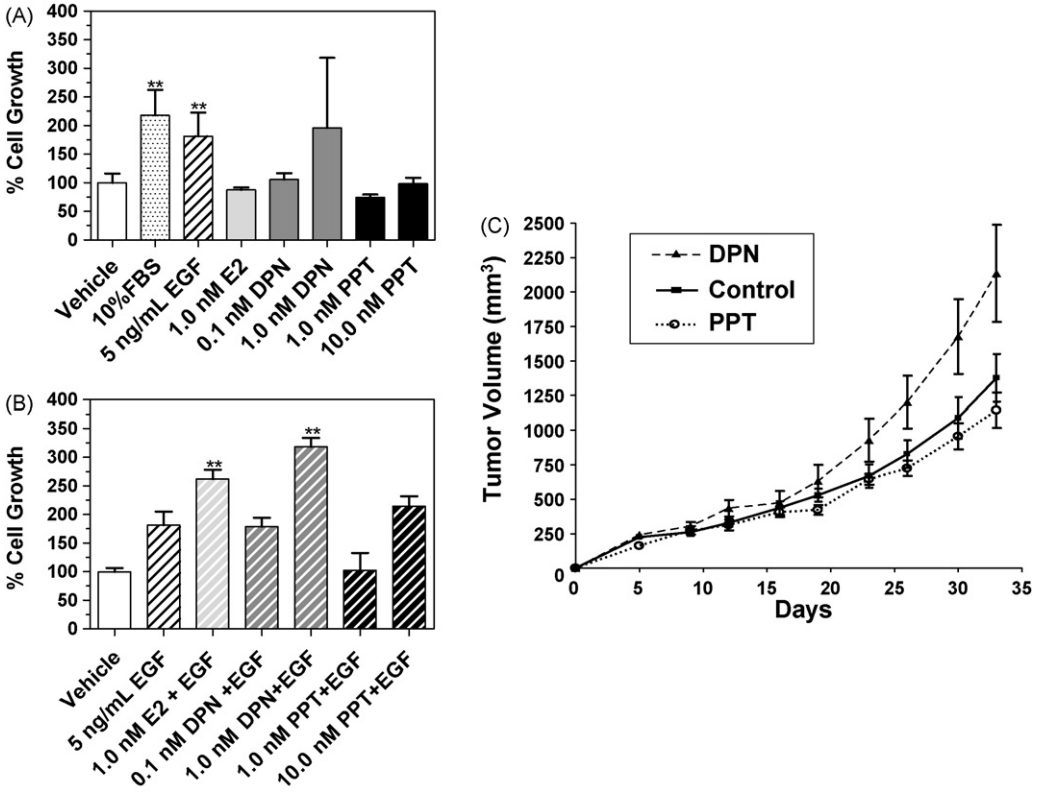

Fig. 6. Effects of ER subtype-specific ligands on the growth of 201T cells *in vitro* and *in vivo*. 201T cells (2×10^3 per well) were cultured for 48 h in phenol red-free medium containing 0.1% CSS. Then, the cells were treated with vehicle, 10% FBS (as a positive control), or (A) the indicated ligands individually or (B) the indicated ligands in combination with 5 ng/mL EGF. Cell growth was assessed 48 h post-treatment by MTT assay. Bars represent the mean ± SD for triplicate wells. In (A) $^{**}p < 0.005$, unpaired Student's *t* test, calculated versus vehicle control. In (B) $^{**}p < 0.05$, unpaired Student's *t* test, calculated versus EGF alone. The results are representative of 3 independent experiments. (C) Female ovariectomized nude mice ($n = 10$ per group) were treated with 500 μg PPT, 250 μg DPN or vehicle control by s.c. injection 5 times per week (in 0.1 mL volume). After 1 week of ligand treatment, 201T cells (2×10^6) were injected into the flank of each animal. Ligand treatment was continued for 4 additional weeks. Tumor volume was measured two times per week. Day 0 represents the day of tumor implantation. Data represent the average tumor volumes per treatment group. When a repeated measures (mixed effects regression) model was used to analyzed the data, the rate of volume increase was marginally significantly different between DPN and control ($p = 0.05$).

signaling, it clearly does not activate transcription from an ERE in the presence of ERβ (Fig. 2B and Fig. 4 [21,33]). PPT also does not cooperate with EGF to support 201T cell growth *in vitro* (Fig. 6A) nor does it stimulate the growth of 201T cells *in vivo* (Fig. 6C). A model consistent with all of our data is that activation of non-genomic signaling (without a corresponding genomic response) is insufficient to induce a proliferative response to estrogen in these lung cancer cells.

In conclusion, our data provide evidence that endogenous levels of ERβ are sufficient to generate genomic and non-genomic responses to estrogen in 201T cells and suggest that activation of both of these pathways may be necessary to support cell proliferation. To determine the *in vivo* significance of these findings, studies are in progress to quantify the effect of ER subtype-specific ligands on gene activation and MAPK phosphorylation in the 201T xenograft model.

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References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, Cancer statistics, 2007, CA Cancer J. Clin. 57 (1) (2007) 43–66.
[2] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics, 2002, CA Cancer J. Clin. 55 (2) (2005) 74–108.
[3] C. La Vecchia, Hormone replacement therapy in menopause and lung cancer: an update, Eur. J. Cancer Prev. 15 (3) (2006) 189–190.
[4] A.B. Oton, C. Belani, C. Cai, T. Owonikoko, W. Gooding, J. Siegfried, R. Ramalingam, Comparison of survival for non-small cell lung cancer between premenopausal and postmenopausal women: an analysis of the National Surveillance, Epidemiology and End Results (SEER) Database, J Clin Oncol. ASCO Annu. Meet. Proc. 24 (18S) (2006) 7038.
[5] K.S. Albain, J. Unger, C.C. Gotay, A.M. Davies, M. Edelman, R.S. Herbst, K. Kelly, S. Williamson, A.J. Wozniak, D.R. Gandara, Toxicity and survival by sex in patients with advanced non-small cell lung carcinoma on modern Southwest Oncology Group (SWOG) trials, J. Clin. Oncol. ASCO Annu. Meet. Proc. 25 (18S) (2007) 7549.
[6] H. Ross, F.B. Oldham, B. Bandstra, L. Sandalic, J. Bianco, P. Bonomi, J.W. Singer, Serum-free estradiol (E2) levels are prognostic in men with chemotherapy-naive advanced non-small cell lung cancer and performance status (PS) 2, J. Clin. Oncol. ASCO Annu. Meet. Proc. 25 (18S) (2007) 7683.
[7] Z. Hammoud, B. Tan, S. Badve, R.M. Bigsby, Estrogen promotes tumor progression in a genetically defined mouse model of lung adenocarcinoma, Endocr Relat Cancer 15 (2) (2008) 475–483.
[8] P.A. Hershberger, A.C. Vasquez, B. Kanterewicz, S. Land, J.M. Siegfried, M. Nichols, Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands, Cancer Res. 65 (4) (2005) 1598–1605.
[9] M.J. Jarzynka, P. Guo, I. Bar-Joseph, B. Hu, S.Y. Cheng, Estradiol and nicotine exposure enhances A549 bronchioloalveolar carcinoma xenograft growth in mice through the stimulation of angiogenesis, Int. J. Oncol. 28 (2) (2006) 337–344.
[10] R.J. Pietras, D.C. Márquez, H.W. Chen, E. Tsai, O. Weinberg, M. Fishbein, Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells, Steroids 70 (5–7) (2005) 372–381.
[11] L.P. Stabile, A.L. Gaither Davis, C.T. Gubish, T.M. Hopkins, J.D. Luketich, N. Christie, S. Finkelstein, J.M. Siegfried, 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, Cancer Res. 62 (2002) 2141–2150.
[12] L.P. Stabile, J.S. Lyker, C.T. Gubish, W. Zhang, J.R. Grandis, J.M. Siegfried, Combined targeting of the estrogen receptor and the epidermal growth factor receptor in non-small cell lung cancer shows enhanced anti-proliferative effects, Cancer Res. 65 (2005) 1459–1470.
[13] D.C. Márquez-Garbán, H.W. Chen, M.C. Fishbein, L. Goodglick, R.J. Pietras, Estrogen receptor signaling pathways in human non-small cell lung cancer, Steroids 72 (2) (2007) 135–143.
[14] J. Matthews, J.A. Gustafsson, Estrogen signaling: a subtle balance between ERα and ERβ, Mol. Interv. 3 (5) (2003) 281–292.
[15] C. Patrone, T.N. Cassel, K. Pettersson, Y.-S. Piao, G. Cheng, P. Ciana, A. Maggi, M. Warner, J.A. Gustafsson, M. Nord, Regulation of postnatal lung development and homeostasis by estrogen receptor β, Mol. Cell Biol. 23 (2003) 8542–8552.
[16] A. Morani, R.P. Barros, O. Imamov, K. Hultenby, A. Arner, M. Warner, J.A. Gustafsson, Lung dysfunction causes systemic hypoxia in estrogen receptor beta knockout (ERbeta−/−) mice, Proc. Natl. Acad. Sci. U.S.A. 103 (18) (2006) 7165–7169.
[17] C.T. Wu, Y.L. Chang, J.Y. Shih, Y.C. Lee, The significance of estrogen receptor beta in 301 surgically treated non-small cell lung cancers, J. Thorac. Cardiovasc. Surg. 130 (4) (2005) 979–986.
[18] A.G. Schwartz, G.M. Prysak, V. Murphy, F. Lonardo, H. Pass, J. Schwartz, S. Brooks, Nuclear estrogen receptor beta in lung cancer: expression and survival differences by sex, Clin. Cancer Res. 11 (20) (2005) 7280–7287.
[19] H. Kawai, A. Ishii, K. Washiya, T. Konno, H. Kon, C. Yamaya, I. Ono, Y. Minamiya, J. Ogawa, Estrogen receptor alpha and beta are prognostic factors in non-small cell lung cancer, Clin. Cancer Res. 11 (14) (2005) 5084–5089.
[20] B.G. Skov, B.M. Fischer, H. Pappot, Oestrogen receptor beta over expression in males with non-small cell lung cancer is associated with better survival, Lung Cancer (2007).
[21] A. Escande, A. Pillon, N. Servant, J.P. Cravedi, F. Larrea, P. Muhn, J.C. Nicolas, V. Cavailles, P. Balaguer, Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta, Biochem. Pharmacol. 71 (10) (2006) 1459–1469.
[22] M.J. Meyers, J. Sun, K.E. Carlson, G.A. Marriner, B.S. Katzenellenbogen, J.A. Katzenellenbogen, Estrogen receptor-beta potency-selective ligands: structure–activity relationship studies of diarylpropionitriles and their acetylene and polar analogues, J. Med. Chem. 44 (24) (2001) 4230–4251.
[23] S.R. Stauffer, C.J. Coletta, R. Tedesco, G. Nishiguchi, K. Carlson, J. Sun, B.S. Katzenellenbogen, J.A. Katzenellenbogen, Pyrazole ligands: structure–affinity/activity relationships and estrogen receptor-alpha-selective agonists, J. Med. Chem. 43 (26) (2000) 4934–4947.
[24] J.M. Siegfried, N. Krishnamachary, A. Gaither Davis, C. Gubish, J.D. Hunt, S.P. Shriver, Evidence for autocrine actions of neuromedin B and gastrin-releasing peptide in non-small cell lung cancer, Pulmonary Pharmacol Ther. 12 (1999) 291–302.
[25] R. Parise, M.J. Egorin, B. Kanterewicz, M. Taimi, M. Petkovich, A.M. Lew, S.S. Chuang, M. Nichols, T. El-Hefnawy, P.A. Hershberger, CYP24, the enzyme that catabolizes the anti-proliferative agent vitamin D, is increased in lung cancer, Int. J. Cancer 119 (2006) 1819–1828.
[26] J. Frasor, D.H. Barnett, J.M. Danes, R. Hess, A.F. Parlow, B.S. Katzenellenbogen, Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) α activity by ER β in the uterus, Endocrinology 144 (7) (2003) 3159–3166.
[27] O.K. Weinberg, D.C. Márquez-Garbán, M.C. Fishbein, L. Goodglick, H.J. Garban, S.M. Dubinett, R.J. Pietras, Aromatase inhibitors in human lung cancer therapy, Cancer Res. 65 (24) (2005) 11287–11291.
[28] C.K. Osborne, A. Wakeling, R.I. Nicholson, Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action, Br. J. Cancer 90 (Suppl. 1) (2004) S2–S6.
[29] S.R. Hammes, E.R. Levin, Extranuclear steroid receptors: nature and actions, Endocr. Rev. 28 (7) (2007) 726–741.
[30] M. Majidi, H.A. Al-Wadei, T. Takahashi, H.M. Schuller, Nongenomic beta estrogen receptors enhance beta1 adrenergic signaling induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human small airway epithelial cells, Cancer Res. 67 (14) (2007) 6863–6871.
[31] Y. Omoto, Y. Kobayashi, K. Nishida, E. Tsuchiya, H. Eguchi, K. Nakagawa, Y. Ishikawa, T. Yamori, H. Iwase, Y. Fujii, M. Warner, J.A. Gustafsson, S.-I. Hayashi, Expression, function, and clinical implications of the estrogen receptor β in human lung cancers, Biochem. Biophys. Res. Commun. 285 (2001) 340–347.
[32] G. Zhang, X. Liu, A.M. Farkas, A.V. Parwani, K.L. Lathrop, D. Lenzner, S.R. Land, H. Srinivas, Estrogen receptor beta functions through nongenomic mechanisms in lung cancer cells, Mol. Endocrinol. 23 (2) (2009) 146–156.
[33] D.M. Kraichely, J. Sun, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-α and estrogen receptor-β: correlations with biological character and distinct differences among SRC coactivator family members, Endocrinology 141 (10) (2000) 3534–3545.