Estradiol-induced proliferation of papillary and follicular thyroid cancer cells is mediated by estrogen receptors α and ß

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Abstract. Premenopausal women are at highest risk for papillary and follicular thyroid carcinoma, implicating a role for estrogens in thyroid cancer. The expression of estrogen receptors α and ß (ER), the effects of estradiol (E_2), selective estrogen receptor modulators (SERMs) 4-hydroxytamoxifen and raloxifene, and ER subtype selective agonists were examined in NPA87 and KAT5 papillary and WRO follicular thyroid carcinoma cell lines. All three thyroid cancer cell lines expressed full-length ERα and ERß proteins with cytoplasmic localization that was unaffected by E_2. ICI 182,780 (Fulvestrant, an ER antagonist), and inhibitors of non-genomic E_2-activated MAPK and PI3K signaling blocked E_2-induced cell proliferation. SERMs acted in a cell line-specific manner. No E_2-induced estrogen response element (ERE)-driven reporter activity was observed in transiently transfected thyroid cancer cells. However, E_2 increased transcription of established endogenous E_2-target genes, i.e., cathepsin D in WRO and cyclin D1 in both KAT5 and WRO cells in an ER-dependent manner as validated by inhibitor and siRNA experiments. In contrast, E_2 did not increase progesterone receptor expression in the thyroid cancer cell lines. E_2 stimulated phosphorylation of ERK1/2 in KAT5 and WRO cells and siERα or siERß inhibited E_2-induced ERK phosphorylation. Expression of the putative membrane estrogen receptor GPR30 was detected in WRO, but not NPA87 or KAT5 cells. GPR30 expression was lower in WRO than MCF-7 human breast cancer cells. Overall, these findings suggest E_2-mediated thyroid cancer cell proliferation involves ERα and ERß transcriptional and non-genomic signaling events.

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Introduction

Thyroid cancers are among the most common neoplasms affecting the endocrine system (1-4). In 2007, there were 33,550 new thyroid cancer cases in the United States (NCI, http://seer.cancer.gov/). Histological characterization of thyroid tumors indicated that 88% were papillary, 9% follicular, and 3% poorly differentiated thyroid cancer (5). Thyroid cancer is ~2.7 times more frequently diagnosed in reproductive age women compared to similar aged men (5,6). Further, the incidence of all types of thyroid cancer decreases after menopause (7). An increased risk of thyroid cancer has been documented in women who take estrogen for gynecological reasons (8). Together these studies indicate that the gender difference in thyroid cancer incidence may involve estrogens (4,9).

A number of peptide hormones, growth factors, and steroids regulate the proliferation and function of normal and neoplastic thyroid tissue (10-12). Epidemiological studies have indicated estrogens promote growth in number of tissues (13). Estrogens regulate cell proliferation by binding to specific receptors: estrogen receptors α and ß (ERα and ERß) (14). ERß shares high homology with ERα and these two ER subtypes differentially regulate gene expression and cell proliferation in a cell type- and gene-specific manner with ERα considered 'proliferative' whereas ERß is 'antiproliferative' (15).

In the classical, genomic estrogen signaling pathway, estradiol (E_2)-activated ERα translocates to the nucleus, dimerizes, and binds to the 15-bp palindromic estrogen response element (ERE) or interacts with other transcription factors in target genes, recruits coactivators, and stimulates gene transcription thereby promoting cell proliferation (16). ERα interacts with a number of coactivators and corepressors in a ligand-dependent manner (16). The subcellular localization of ERα is cell-type and hormonal milieu-dependent. For example, in some breast cancer cells, ERα interacts with metastasis-associated protein-1 (MTA1), a component of histone deacetylase and nucleosome remodeling complexes (HDAC and NURD) and represses ERα activity (17). A short variant of MTA1 called MTA1s, containing a novel 33 aa insert, binds and sequesters ERα in the cytoplasm, thus

blocking ERα-mediated transcription (18). Another mechanism of estrogen action is more rapid and is termed 'non-genomic' or 'membrane-initiated' because it involves E_2 activation of plasma membrane associated ERα or ERß and leads to rapid activation of intracellular signaling pathways, e.g., ERK1/2 and PI3K/Akt (19). Non-genomic estrogen action is mediated by ERα or ERß interactions with signaling proteins in caveolae (20). GPR30 is a novel membrane estrogen receptor that also activates ERK1/2 and PI3K/Akt signaling, although its role in estrogen action remains controversial (20-23).

The expression of ER has been demonstrated in normal and neoplastic thyroid tissue by mRNA (24), immunohistochemistry (IHC), estradiol (E_2) binding assays (4,13,25), enzyme-immunoassays (26), and immunoblots (9). ERα expression was relatively higher in the thyroid tumors compared to normal thyroid tissue (4). Despite these studies, only a few investigators have examined the function of estrogens or selective ER modulators (SERMs), e.g., tamoxifen and raloxifene, in thyroid cancer cells. These studies demonstrated that E_2 and the ERα-selective agonist PPT stimulated whereas the ERß-selective agonist DPN inhibited KAT5 papillary thyroid cancer cell proliferation (27,28).

Previous studies reported that E_2 rapidly activates ERK1/2 in thyroid cancer cells through the non-genomic estrogen signaling pathway mediated by the membrane estrogen receptor GPR30 (22). E_2-activation of this pathway increased WRO follicular thyroid cancer cell proliferation by increasing c-fos, cyclin A, and cyclin D1 expression (22). These and other recent data indicate that the signaling mechanisms explaining estrogen action are far more complex than initially appreciated because it involves multiple forms of estrogen receptors (22,29).

Although considerable progress has been made in our understanding of the molecular mechanisms of thyroid cancer in recent years, the specific nature of ER signaling leading to increased cell proliferation is poorly understood. The objective of this study was to determine the effect of E_2 and other ER ligands in different types of thyroid cancer cells. We compared the proliferative and transcriptional responses of follicular (WRO) and papillary (NPA87 and KAT5) thyroid cancer cells. Using siRNA specific for ERα and ERß, we examined the effect of E_2 on the expression of ER-regulated gene targets cathepsin D (*CTSD*) and cyclin D1 (*CCND1*). Our results indicate that E_2 increases the proliferation of thyroid cancer cells through mechanisms independent of the classical genomic activity of ER.

Materials and methods

Reagents. Estradiol (E_2), Raloxifene (RAL), and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). ICI 182,780 (ICI), 4,4',4'-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT, an ERα-selective agonist), and 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN, an ERß-selective agonist) were purchased from Tocris Chemicals (Ellisville, MO, USA). The selective ERα agonist/ERß antagonist R,R-tetrahydrochrysene (R,R-THC) was a generous gift from Dr John A. Katzenellenbogen (30). PD98059, pertussis toxin (PTX), and Wortmannin were purchased from Sigma-Aldrich. E_2, 4-OHT, and R,R-THC were dissolved in ethanol and ICI, PPT, and DPN were dissolved in dimethylsulfoxide (DMSO).

Cell culture. Human papillary thyroid carcinoma cells (NPA87) were generously provided by Dr James A. Fagin (Memorial Sloan-Kettering Cancer Center, New York, NY). Human papillary thyroid carcinoma cells (KAT5) and follicular carcinoma cells (WRO and TPC1) were a kind gift from Dr Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY). These cell lines were grown in RPMI-1640 with 10% fetal calf serum, 2 mM L-glutamine, and 50 U/ml of penicillin and streptomycin. For WRO cells, 1x non-essential amino acids and 1 mM sodium pyruvate (Mediatech Inc., Herndon, VA) were added to the medium. FTC133 cells were generously provided by Dr Electron Kebebew (The University of California, San Francisco, CA) and were routinely cultured in DMEM/F12 (Mediatech) with 10% fetal bovine serum (FBS), 200 mM L-glutamine, 10 mIU/ml human thyrotropin, and 10 μg/ml insulin (31). All cell cultures were maintained at 37˚C and 5% CO_2 atmosphere in a humidified cell culture chamber with growth medium changed each 3-4 days. Breast cancer cell line MCF-7 (expressing wild-type ERα) was used as a positive control in many experiments. MCF-7 cells were grown in IMEM supplemented with penicillin and streptomycin and 10% FBS. At 70-80% confluence of cells, the growth medium was replaced with hormone and phenol red-free IMEM medium with 2% dextran charcoal stripped FBS (DCC-FBS) for 48 h before stimulation with E_2 or other ER ligands. FTC133 cell experiments were carried out in H5 media: phenol red-free DMEM/F12 supplemented with 2% DCC-FBS, 200 mM L-glutamine, 10 μg/ml insulin, 5 μg/ml transferrin, 10 mg/ml somatostatin, 2 ng/ml gly-his-lys, and 360 pg/ml hydrocortisone (31). For the indicated experiments, 100 nM ICI, 4-OHT, or R,R-THC was added 1 h before addition of 10 nM E_2. For other studies, cells were preincubated for 3 h with 50 μM PD98059 (MEK1 inhibitor), 100 nM Wortmannin (PI3K inhibitor), or 100 ng/ml pertussis toxin (PTX, a Gα inhibitor) prior to adding 10 nM E_2 for the indicated time.

Cell proliferation assay. For quantitative proliferation assays, 2,000 cells were seeded in 96-well plates in regular growth medium. Cells were incubated in medium containing 2% DCC-FBS for 48 h, prior to treatment with E_2, 4-OHT, ICI, raloxifene, DPN, PPT, and R,R-THC as indicated in the text and figures with the medium containing the treatment renewed every 48 h. Cell proliferation was measured using the BrdU Cell Proliferation kit (Roche, Indianapolis, IN) according to the manufacturer's specifications. Quadruplicates were performed for each treatment. Experiments were performed at least three times and the relative proliferation values are given in comparison to EtOH/DMSO (vehicle).

Transient transfection and luciferase assays. KAT5, NPA87, and WRO cells (15,000 cells/well) were plated into 24-well plates with 500 μl of regular growth medium. After overnight incubation, the growth medium was replaced with serum-free medium for transfection using FuGENE 6 reagent as

recommended by the manufacturer (Roche). Each well was transfected with a mixture containing 250 ng of pGL3-2ERE-pro-luciferase reporter plasmid (32) and 5 ng of pRL-tk, *Renilla* luciferase reporter from Promega, and 100 ng or pcDNA3.1 (Promega), ERα, or ERß expressing plasmids (33). Eighteen hours post-transfection, the cells were treated with EtOH, 10 nM E_2, 100 nM ICI, 100 nM 4-OHT, 100 nM RAL, 10 nM DPN, or 10 nM PPT for 30 h. Cells were lysed using Promega passive lysis buffer. Firefly and *Renilla* luciferase activities were measured with the dual luciferase kit (Promega, Madison, WI) according to the manufacturer's recommendations in a Plate Chameleon luminometer (Hidex Oy, Finland) (34). Firefly luciferase values were normalized to *Renilla* luciferase activity. The normalized relative light unit values obtained in EtOH-treated MCF-7 cells was set as 1. Experiments were repeated at least three times.

Small inhibitory RNA (siRNA) transfection. Cells were plated in 6-well plates in antibiotic-free RPMI medium. For the siRNA studies, On-Target plus SMARTpool of siRNA against ERα (L-003401-00), or ERß (L-003402-00) were purchased from Dharmacon Tech (Lafayette, CO). As a negative control, the universal negative siRNA was purchased from Invitrogen. siRNAs were transfected into the cells according to the manufacturer's instructions. Briefly, cells were transfected with 100 nM siRNA duplexes using DharmaFECT 1 (Dharmacon Tech). After overnight incubation, the cells were then grown for 2 days in culture medium supplemented with 3% DCC-FBS. Cells were harvested at 48 or 72 h after transfection and processed for RNA and protein analysis, respectively. To allow the same total time of siRNA transfection (48 h), the transfected cells were treated with E_2 or ICI at 39, 42, 45 and 47 h as needed for 9, 6, 3, and 1 h of E_2 treatment. RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The ERα and ERß mRNA and protein expression levels were analyzed using q-RT-PCR and Western blotting, as described below.

Preparation of whole cell extracts (WCE) and Western blotting. Thyroid cancer cells were washed with cold PBS and were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.20, 1% SDS, 1% Nonidet P-40, 0.25% Na-deoxycholate, 5 mM EDTA, 2 mM NaF, 1 mM $VaPO_3$), and protease inhibitor cocktail (Roche), 100 mM PMSF and 1 mM Na_3VO_4 were added to lysis buffer before adding to the cells. Lysed cells were sonicated using Branson 250 Sonicator (Branson, Danbury, CT) three times at 20 amplitude for 15 sec on ice and were sedimented at 12,000 rpm for 30 min. Protein concentrations in WCE were determined using Bio-Rad Detergent Compatible (DC) protein assay (Hercules, CA, USA).

WCE (50 μg of protein) were mixed with 4X electrophoresis sample buffer (SB) and boiled for 5 min prior to separation on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane (Whatman, Florham Park, NJ). The membrane was blocked for 1 h in 5% non-fat dried milk in TBS-Tween at room temperature (RT). The membranes were then incubated with the following primary antibodies overnight at 4˚C: HC-20 (1:200, rabbit polyclonal raised against ERα C-terminal, Santa Cruz Biotechnologies, Santa Cruz, CA), AER320 (1:150, mouse monoclonal antibody generated against ERα 495 to 595 C-terminal (Neomarkers/Labvision, Freemont, CA), H150 (1:150 rabbit polyclonal antibody raised against human ERß N-terminal 1-150 amino acids (Santa Cruz), MTA1 (1:100, mouse monoclonal, Sigma-Aldrich). The membranes were then incubated in a horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibodies, or rabbit anti-goat secondary antibody (Amersham Biosciences, Piscataway, NJ) at 1:5,000 dilutions for 1 h at room temperature. Following ER or other primary protein target detection, the membranes were stripped and re-probed with an antibody to ß-actin (Cell Signaling) for normalization. The protein bands were detected by chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL). Most of the Western blot signals were captured using Kodak BioMax MS film (Eastman Kodak, Rochester, NY). In some cases, the Western signal was recorded with a FluorChem FC2 (Alpha Innotech, San Leandro, CA). The molecular weights (MW) of the proteins were estimated by comparing migration against the dual color MW standard (Invitrogen, Carlsbad, CA). Sizes and density of immunoreactive protein bands were quantified by densitometry using Un-Scan-It (Silk Scientific, Orem, UT). Relative ERα or ERß protein expression was normalized by ß-actin within each blot.

Immunofluorescence staining. Cells were allowed to grow on sterile glass cover slip and were maintained in 2% serum-free medium for 3 days and then were treated with 10 nM E_2 for 45 min. The cells were fixed in 4% paraformaldehyde for 30 min. After paraformaldehyde removal, the cells were washed three times with PBS, and were permeabilized with 0.05% Triton X-100 in PBS for 30 min. Then the cells were incubated with 5% bovine serum albumin for 60 min to block the non-specific binding sites. Immunocytochemical staining for ERα and ERß was performed using AER320 and H150 as the primary antibodies, respectively. The secondary antibodies used for fluorescence detection were Alexa Fluor 488 goat anti-mouse IgG for ERα and Alexa Fluor 568 goat anti-rabbit IgG for ERß (Invitrogen). The cells were washed 3 times with PBS and once with deionized water before the cover slip were mounted onto a slide containing 10 μl of Vectashield mounting media containing DAPI for staining DNA (Vector Laboratories, Burlingame, CA). The fluorescence were detected in Olympus iX50 inverted fluorescence microscope and the images were captured using QCapture (Quantitative Imaging Corp., Surrey, BC, Canada) and the captured images were color coded using Northern Eclipse software (Empix Imaging, Inc. Cheektowaga, NY). All the images were captured at magnification x200 with exposure time of 0.5 sec for the blue, 3 sec for the red, and 6 sec for the green fluorescent dye.

Q-PCR for progesterone receptor (PR), cathepsin D (CTSD), cyclin D1 (CCND1), and GPR30. Total RNA was isolated from untreated cells or cells after 1, 3, 6 and 9 h following vehicle [ethanol (EtOH)], 10 nM E_2, or 100 nM ICI treatment using TRIzol reagent (Invitrogen). The quality and quantity of RNA was assessed by measuring the A260/A280 ratio using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (3 μg) was converted to

cDNA using a high capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA) according to the procedure provided by the manufacturer. TaqMan Gene Expression Assays were used to quantitate mRNA levels for progesterone receptor (Hs00172183_m1), cathepsin D (Hs00157201_m1), cyclin D1 (Hs00277039_m1), GPR30 (Hs00173506_m1). Quantitative real-time PCR (Q-PCR) was performed in a 7300 ABI Real-Time System (Applied Biosystems). Relative target gene expression was determined using the ΔC_t method employing the formula: relative expression = $2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ control]}$ (35) where Ct refers to the threshold cycle, sample indicates the gene of interest and control indicates the endogenous house-keeping gene (18S). Within each experiment, samples were run in triplicate and the experiments were repeated at least three times.

Statistical analysis. Statistical analyses were performed using Prism 4.0 (GraphPad Software Inc. San Diego, CA) for One way ANOVA followed by the Student-Newman-Keuls test, or Student's t-test. P-values <0.05 were considered statistically significant.

Results

E_2 and SERMs affect thyroid cancer cell proliferation. To address the role of estrogens in the proliferation of the three major types of thyroid cancer, three thyroid cancer cell lines representing papillary (KAT 5 and NPA87) and follicular (WRO) cancers were incubated with increasing concentrations of E_2 and fixed concentrations of the SERMs 4-OHT and RAL or the selective estrogen receptor down-regulators (SERD) ICI 182,780 (Faslodex/Fulvestrant, ICI), alone or in combination. Cell proliferation was measured by BrdU incorporation (Fig. 1). E_2 increased KAT5, NPA87, and WRO thyroid cancer cell proliferation in a concentration-dependent manner. The KAT5 data are consistent with a previous report (27).

4-OHT, the classical SERM with its cell line-specific ER antagonist/agonist activity (36), increased proliferation of KAT5 and WRO cells, but not NPA87. The effect of the SERM RAL was also examined because it has less agonist activity than 4-OHT (37). Like 4-OHT, RAL stimulated WRO cell proliferation, but had no effect in KAT5 or NPA87 cells. ICI, the SERD considered as a pure antiestrogen (38), had no significant effect on cell proliferation, but blocked E_2-induced cell proliferation in all 3 thyroid cancer cell lines, indicating that the effect of E_2 on cell proliferation is ER-mediated (Fig. 1). Neither 4-OHT nor ICI inhibited basal proliferation, reflecting the modest effect of E_2 on thyroid cell proliferation, consistent with previous reports (27,28). When combined with E_2, 4-OHT inhibited E_2-induced proliferation in all 3 thyroid cancer cell lines, but RAL only inhibited E_2-induced BrdU incorporation in NPA87 cells, similar to 4-OHT (Fig. 1B).

Because non-genomic ER activation leads to cell proliferation (28,39), the effect of inhibitors of non-genomic E_2 signaling were tested in the 3 thyroid cancer cell lines. Cells were preincubated with each inhibitor and then E_2 was added. PD98059 (MEK1 inhibitor) inhibited basal proliferation of KAT5 and stimulated basal proliferation in NPA87 cells. PD98059 inhibited E_2-induced proliferation of KAT5 and WRO cells. Wortmannin (PI3K inhibitor) inhibited E_2-induced proliferation of KAT5, NPA87, and WRO cells. PTX (a Gα inhibitor) inhibited E_2-induced proliferation of all 3 thyroid cancer cell lines. Together with the SERM and ICI data, these data indicate that the E_2-induced proliferation of KAT5 and WRO involves ER and MAPK, PI3K, and G-protein coupled pathways. In contrast, while clearly involving ER, PI3K, and G-protein pathways, the MAPK does not appear to play a role in E_2-induced proliferation of NPA87 cells.

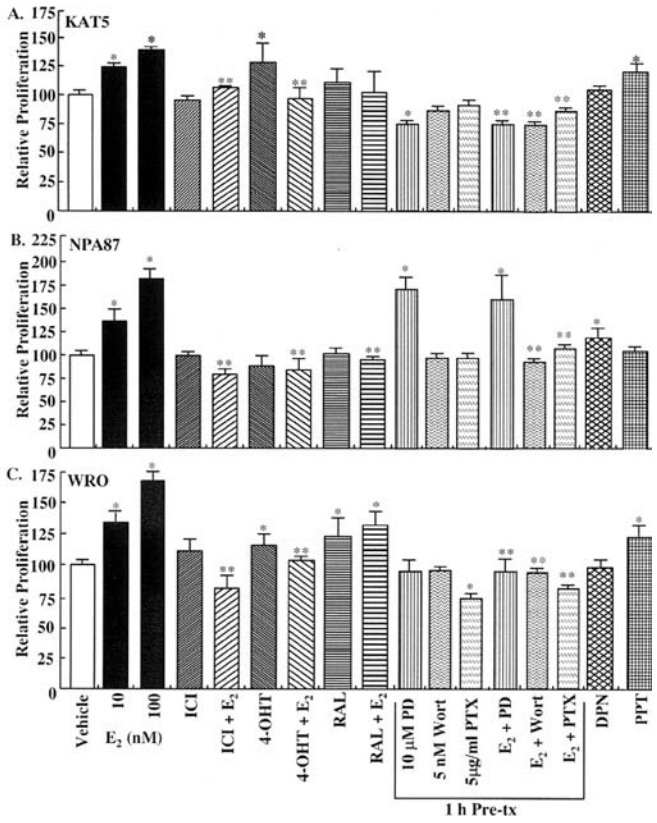

Figure 1. E_2 increases thyroid cancer cell proliferation. The effect of the indicated concentrations of E_2 and 100 nM ICI, 100 nM 4-OHT, 100 nM RAL, 10 nM DPN, 10 nM PPT, alone or in combination with 10 nM E_2 on the proliferation of KAT5 (A), NPA87 (B), and WRO (C) thyroid cancer cells was determined after 48 h of treatment by assaying BrdU incorporation as described in Materials and methods. Where indicated, cells pre-incubated for 3 h with the non-genomic pathway inhibitors at the concentrations shown. Data were normalized to the vehicle (EtOH/DMSO) in each experiment. Values are % of vehicle control and are the mean ± SEM of at least 5 independent experiments. *Significantly different from vehicle (EtOH) or **10 nM E_2 within each cell line, respectively ($p<0.05$).

To address which ER subtype might be mediating the observed effects of E_2 and SERMs on cell proliferation, cells were treated with the ERα agonist PPT (40), ERß agonist DPN (41), or the ERα-selective agonist/ERß-selective antagonist R,R-THC (30) (Fig. 1). DPN increased NPA87 cell proliferation (Fig. 1B), suggesting a role for ERß in the E_2 effect, but not in KAT5 and WRO (Fig. 1A and C) cells. PPT increased KAT5 and WRO cell proliferation (Fig. 1A and C), suggesting involvement of ERα in the E_2 effect. R,R-THC increased cell proliferation in all the three cell lines (Fig. 1),

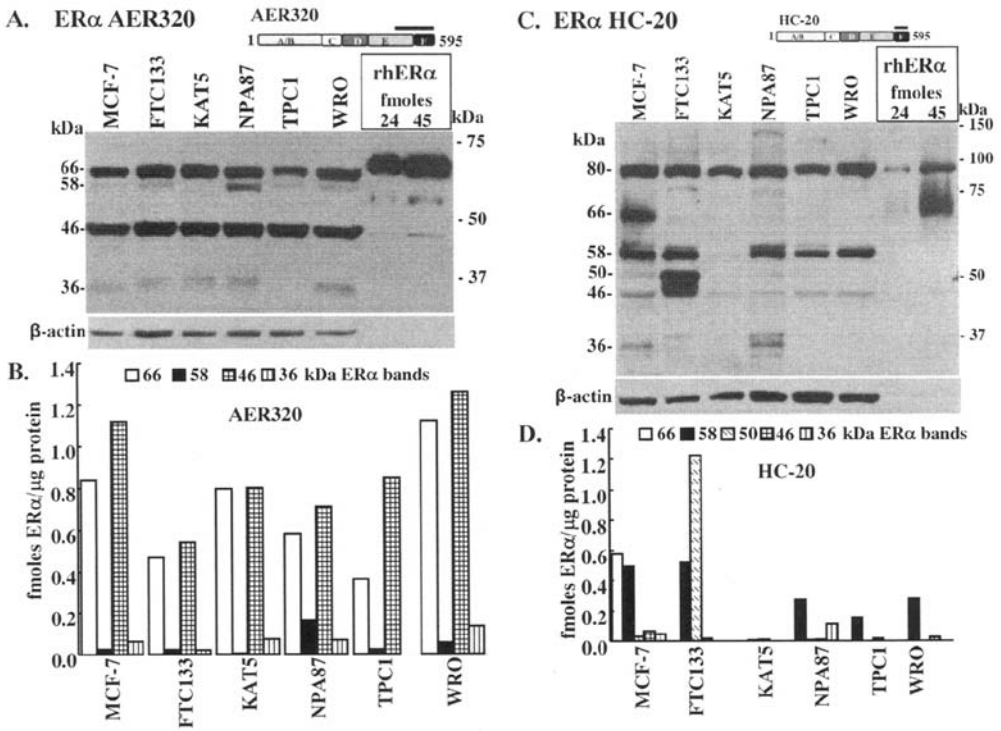

Figure 2. Thyroid cancer cells express ERα. The expression of ERα was examined by Western blotting using 50 μg of WCE protein from the indicated cell lines using ERα monoclonal AER320 (A) or polyclonal HC-20 (C) antibodies as described in Materials and methods. The indicated fmoles of recombinant human (rh) ERα were included as a control. The membrane was stripped and reprobed for ß-actin. The epitopes recognized by each ERα antibody are indicated by the black bar above the E-F domains in the ERα diagram provided at the top right (A and C). The migration of molecular weight (MW) standards is indicated at the right (kDa). The MW sizes of the immunoreactive ERα bands, indicated at the left, were estimated as described in Materials and methods. The bar graphs below each Western blotting (B and D) are a quantitation of the data in each blot. ERα expression was normalized to ß-actin as described in Materials and methods. These data are representative of at least five separate Western blots that show similar patterns of ERα expression.

perhaps reflecting its ERα-agonist activity (30), and blocked the E_2-induced proliferation of NPA87 and WRO (data not shown), but not in KAT5 cells. The inhibition of E_2-induced proliferation by R,R,-THC in NPA87 and WRO suggests a role for ERß in mediating E_2-induced proliferation these cell lines. We also studied the effect of E_2 on the growth of FTC133 and TPC1 follicular and papillary thyroid cancer cell lines, respectively, and found them to be non-responsive to E_2 or any of the SERMs (data not shown).

Expression of ERα and ERβ. To determine the expression of ER subtypes in the three thyroid cancer cell lines, Western blots were performed (Fig. 2). In addition, we examined ER expression in FTC133 and TPC1 thyroid cancer cells. We used MCF-7 human breast cancer cells as a positive control since ERα and ERß are expressed in these cells (34,42,43). Using a monoclonal ERα antibody AER320 which recognizes an epitope in the C-terminus (44), the expression of full length wild-type ERα66 and bands corresponding to the MW of the ERα46 and ERα36 splice variants (42) were detected in MCF-7, FTC133, KAT5, NPA87, TPC1, and WRO cells (Fig. 2A). An additional band of 58 kDa was detected in NPA87, with lower amounts in MCF-7, FTC133, TPC1, and WRO cells (Fig. 2A). ERα46 is the predominant splice variant detected in all cell lines (Fig. 2A). Different MW bands for ERα were observed using polyclonal antibody HC-20, epitope shown in Fig. 2C, compared to those identified by AER320, despite the fact that the epitope for each antibody is the ERα C terminus. The full length ERα66 was not detected in any of the thyroid cancer cell lines with HC-20, but it was detected in MCF-7 cells. In agreement with the data in Fig. 2A, the 58-kDa ERα band was prominent in MCF-7, FTC133, NPA87, TPC1, and WRO cells, but weakly expressed in KAT5 cells (Fig. 2C). An ~50 kDa ERα band was prominent in FTC133 cells. The 46-kDa ERα band was weakly recognized by the HC-20 antibody in all cell lines except FTC133 (Fig. 2C). A 36-kDa ERα band was observed in all cells except KAT5, TPC1, and WRO (Fig. 2C). Previous studies identified ERα36 in breast cancer cells and tumors as a dominant negative ERα splice variant that localizes to the plasma membrane (45,46). The quantification of ERα using the 2 ERα antibodies is in general agreement for MCF-7, but not for the thyroid cancer cell lines (Fig. 2B and C). This is likely due to variability in ERα epitope detection between these antibodies. HC-20 also recognizes an 80-kDa band which the manufacturer (Santa Cruz) terms non-specific, but which also corresponds to an ERα splice variant containing a duplication of exons 6 and 7 (47). However, the presence of this band in the baculovirus-expressed rhERα lane, suggests, in agreement with the manufacturer's web site, that this band is non-specific. Because FTC133 and TPC1 were non-responsive to E_2 in the cell proliferation assay (data not shown), these cell lines were excluded from further analyses.

To examine the expression of ERß in thyroid cancer cells, Western blots were performed with polyclonal ERß antibody H150 that recognizes the N-terminus of ERß,

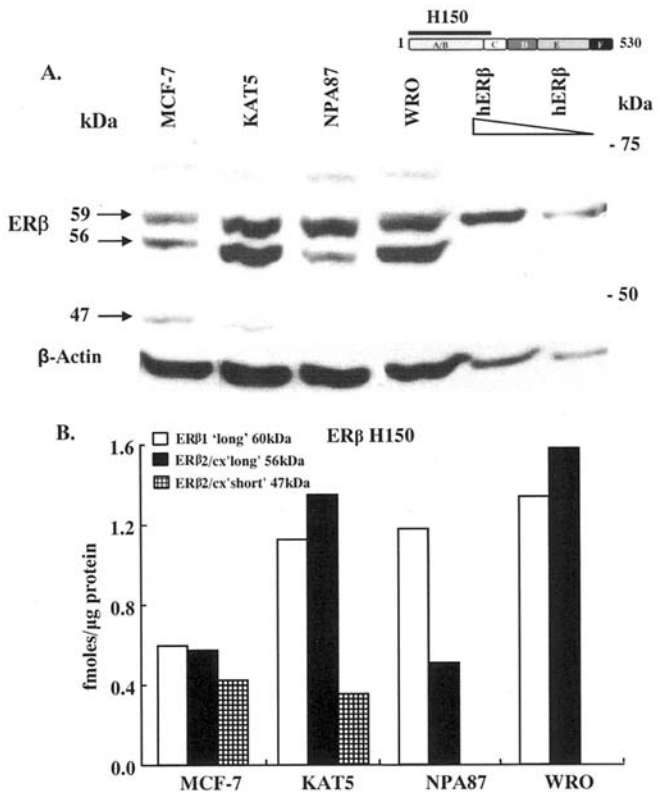

Figure 3. KAT5, NPA87, and WRO thyroid cancer cells express ERß. The expression of ERß was examined by immunoblotting using 40 μg of WCE protein and using ERß polyclonal H150 antibody. H150 recognizes an epitope in the N terminus of ERß, as indicated by the black bar above the A/B domain in the ERß diagram provided at the top right. One and a half fmoles of recombinant human (rh) ERß was included as a control, as indicated. The membrane was stripped and reprobed for ß-actin. The migration of MW standards is indicated at the right (kDa). The MW sizes for the immunoreactive bands, indicated at the left by arrows, were estimated as described in Materials and methods. The bar graph is a quantitation of the data in each blot. This is the result of a single experiment and is representative of at least three separate Western blots that show similar patterns of ERß expression.

(epitope indicated in Fig. 3). Full length ERß1 (59 kDa) and its apparent splice variants were identified in all 3 thyroid cancer cell lines and in MCF-7 cells (Fig. 3). The identity of the apparent ERß splice variants was inferred from their MW size (48). The full length ERß1 'long' isoform is the 59 kDa band that was expressed in MCF-7 (positive control), KAT5, NPA87, and WRO cells and its identity was confirmed by identical migration of baculovirus-expressed rhERß (49) (Fig. 3). Notably, all three thyroid cancer cell lines express higher amounts of ERß1 than MCF-7 cells. More ERß2/cx 'long' form, 56 kDa, was seen in KAT5 and WRO cells compared to MCF-7 cells. The relative levels of the ERß1 were similar in all 3 thyroid cancer cell lines. The total expression of ERß2/cx 'long' was similar in KAT5 and WRO cells whereas ERß2/cx 'long' was only detected in KAT5 and MCF-7 cells. Based on calculations using known amounts of rhERα and rhERß, the protein expression of ERß was lower than ERα in these 3 thyroid cancer cell lines and MCF-7 cells (Figs. 2B and D, and 3B).

Intracellular localization of ERα and ERß. The cellular localization of ERα and ERß in the 3 thyroid cancer cell lines was examined by immunofluorescent staining with and without a 45-min treatment with 10 nM E_2. Again, MCF-7 cells were used as a positive control. In untreated KAT5, NPA87, and WRO thyroid cancer cells, ERα and ERß were in the cytoplasm and enriched in the perinuclear regions and, as expected (50), in the cytoplasm of MCF-7 cells (Fig. 4A). The yellow in the merged images indicates an overlap in the localization of ERα and ERß in all cell lines (Fig. 4A). Following 45 min of E_2 treatment, ERα and ERß in KAT5, NPA87 and WRO cells remained in the cytoplasm. In contrast, both ERα and ERß were, as anticipated (50,51), translocated to the nucleus in MCF-7 cells (Fig. 4B). Thus, ERα and ERß exhibit different cellular responses to E_2 in thyroid cancer cells versus MCF-7 breast cancer cells. Specifically, E_2 fails to stimulate the translocation of ERα and ER, to the nucleus in the 3 thyroid cancer cell lines.

Transcriptional ER activity in transiently transfected thyroid cancer cells. To examine the transcriptional activity of endogenous ERα and ERß in the thyroid cancer cells, each cell line was transiently transfected with a reporter plasmid containing two tandem copies of a consensus ERE (33,52) and the ability of E_2, ICI, 4-OHT, and RAL, to stimulate luciferase activity was examined. E_2 had no effect on luciferase activity in KAT5, NPA87, and WRO cells (Fig. 5A). The same concentration of E_2 elicited a 2-fold stimulation of luciferase activity in MCF-7 cells (Fig. 5A), indicating that the lack of response was not due to inactivity of the ERE-luciferase reporter. The thyroid cells were transfected since the Firefly and *Renilla* luciferase readings were ~300,000 and ~90,000 (data not shown). 4-OHT, ICI, and RAL had no significant effect on luciferase activity in KAT5, NPA87, and WRO cells (Fig. 5A). In contrast, both 4-OHT and ICI inhibited E_2-induced luciferase activity in MCF-7 cells. These data indicate that although ERα and ERß are present in KAT5, NPA87, and WRO thyroid cancer cells, they do not activate reporter gene transcription. These data are also in agreement with the lack of nuclear ERα and ERß in these 3 cell lines (Fig. 4).

Two possible explanations for the lack of reporter gene activity in the thyroid cancer cells are: i) the lack of nuclear ERα and ERß (Fig. 4B); or ii) inactivity of the expressed ERs. Thus, we examined the effect of transient transfection of ERα and ERß on ERE-driven luciferase activity in each cell line (Fig. 5B). E_2-induced transcriptional response in ERα and ERß-transfected KAT5 and WRO cells (Fig. 5B). Basal reporter activity was increased in NPA87 transfected with ERα and no further induction was detected with E_2 treatment (Fig. 5B). Transfection with ERß increased E_2-induced luciferase activity in NPA87 cells. As expected, transfection of ERα and ERß increased E_2-induced luciferase activity in MCF-7 cells (Fig. 5B). Thus, the lack of ERE-driven luciferase activity in the thyroid cancer cells (Fig. 5A) may be due to low ERα expression (Fig. 2) or the presence of dominant negative ERα splice variants that may inhibit endogenous ER transcriptional activity on the transfected ERE-reporter (42).

MTA1 expression in thyroid cancer cells. MTA1 is a corepressor of ERα-driven gene transcription in breast cancer (17), and its short form variant, MTA1s, interacts with ERα and

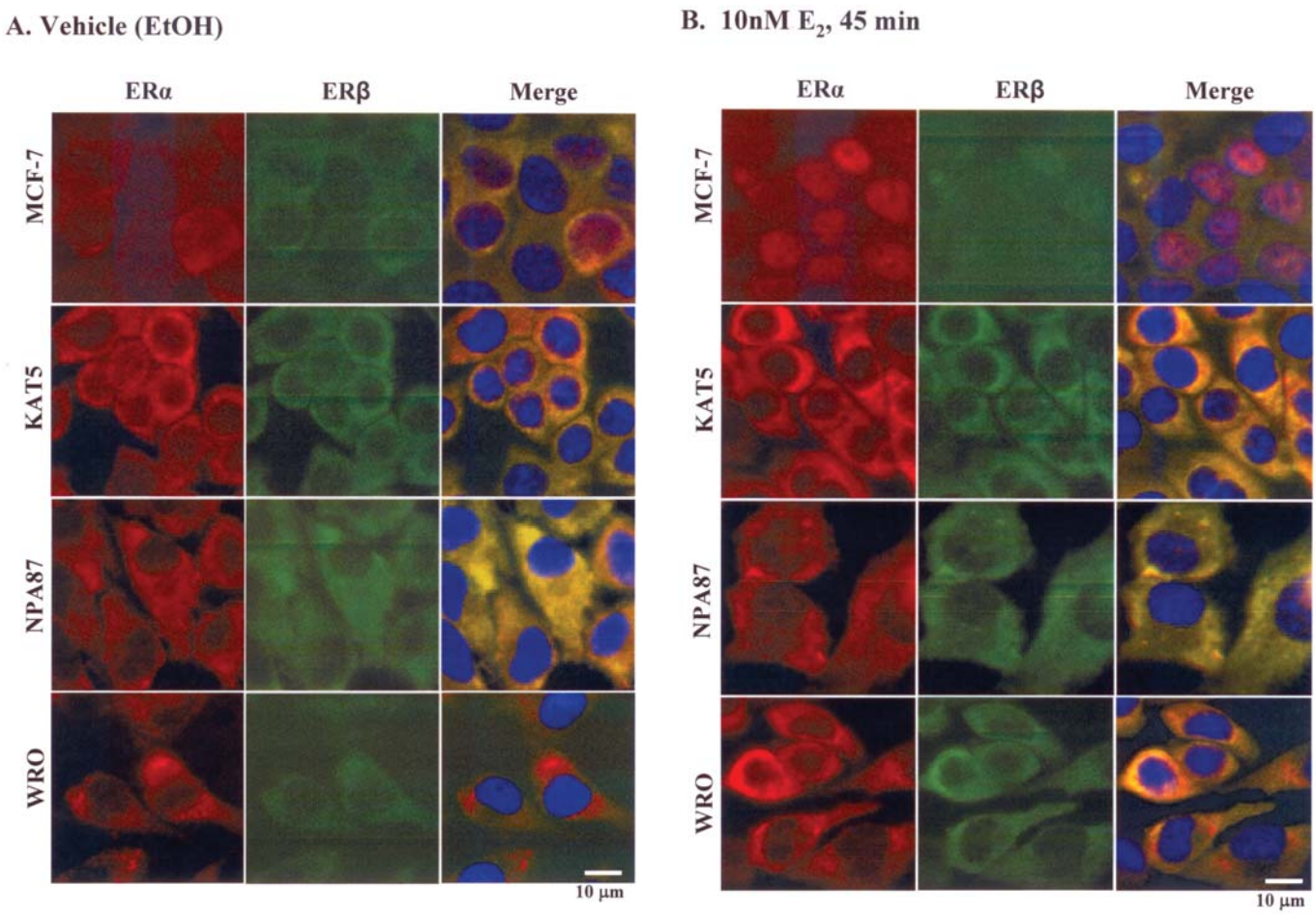

Figure 4. Cellular localization of ERα and ERß. MCF-7 human breast cancer cells (as a positive control) or the indicated thyroid cancer cell lines were treated with vehicle [EtOH (A) or 10 nM E_2 for 45 min (B) and immunostained for ERα (red) and ERß (green) using an anti-ERα (AER320) and anti-ERß (H150) antibodies as described in Materials and methods]. Cell nuclei were counterstained with DAPI. Merged images are shown in the third column. Images were captured using Olympus iX50 inverted fluorescence microscope as described in Materials and methods. Scale bar, 10 μm.

sequesters ERα in the cytoplasm, thus inhibiting transcriptional activity (18). We tested the hypothesis that the lack of endogenous ER transcriptional activity in the thyroid cancer cell lines is due to higher MTA1s expression in the thyroid cancer cell lines compared in MCF-7. Western blotting revealed that all three thyroid cancer cell lines express levels of MTA1 comparable to MCF-7 cells (data not shown). We used three different antibodies in an attempt to identify MTA1s (54 kDa) in these cells, but did not detect this variant (data not shown). Thus, we conclude that higher MTA1s is not the reason for the lack of endogenous genomic ER activity in these three thyroid cancer cell lines.

Effect of E_2 on endogenous estrogen responsive gene transcription. To further address the activity of endogenous ER in thyroid cancer cells, we examined whether E_2 increases mRNA expression of PR, cathepsin D, and cyclin D1, well-established estrogen target genes (53), in the 3 thyroid cancer cell lines and again, MCF-7 cells served as a positive control for E_2 responses. Real-time quantitative PCR (Q-RT-PCR) showed that PR was increased 19-fold in response to E_2 in MCF-7 cells (Fig. 6 inset). In contrast, PR mRNA was undetectable in KAT5, NPA87, and WRO cells even after E_2 treatment (Fig. 6 inset). These data agree with a previous report that PR was not detected in normal thyroid or in thyroid carcinomas by enzyme immunoassay (11). We conclude that PR is not an endogenous ER target gene in KAT5, NPA87, and WRO thyroid cancer cells.

Cathepsin D is a lysosomal protease involved in proteolytic degradation, cell invasion, and apoptosis (54) which is highly expressed in thyroid carcinomas as compared to normal thyroid gland (11,54,55). Cathepsin D expression is stimulated by E_2 in breast cancer cells (54,55). As expected, E_2 increased cathepsin D (*CTSD*) mRNA expression in MCF-7 cells in an ER-dependent manner since ICI inhibited E_2-induced *CTSD* expression (Fig. 6). Basal *CTSD* expression was higher in NPA87 and WRO than MCF-7 and KAT5 cells (data not shown). KAT5 had the lowest *CTSD* expression (data not shown). E_2 increased *CTSD* expression in WRO cells and this was blocked by ICI (Fig. 6). We thus conclude that *CTSD* gene transcription is not E_2-regulated in KAT5 or NPA87 cells, whereas E_2 increased *CTSD* transcription in WRO cells by an ER-dependent mechanism.

Cyclin D1 expression in E_2 treated thyroid cancer cells. Cyclin D1 is a major regulator of the G_1-S transition of the

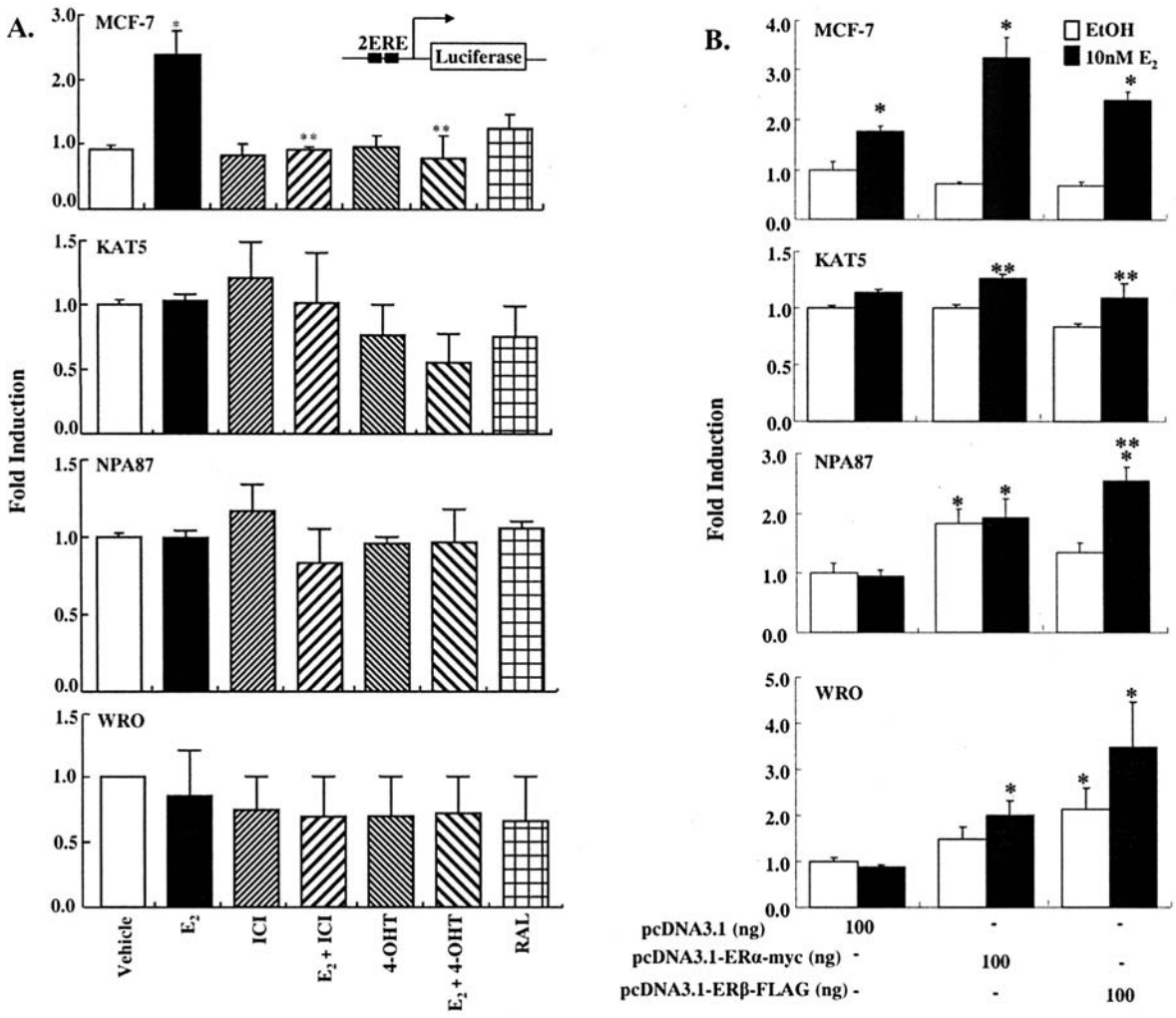

Figure 5. ER transcriptional activity in thyroid cancer cells. (A) MCF-7 breast, or KAT5, NPA87, and WRO thyroid cancer cells were transiently transfected with pGL3-pro-2EREc38 luciferase and a *Renilla* reporter for a dual luciferase reporter assay as described in Materials and methods. Twenty-four hours post-transfection, the cells were treated with 10 nM E_2, 100 nM ICI, 100 nM 4-OHT, 10 nM RAL, 10 nM DPN, and 10 nM PPT for 24 h. (B) MCF-7 breast, or KAT5, NPA87, and WRO thyroid cancer cells were transiently transfected with pGL3-pro-2EREc38 luciferase and a *Renilla* reporter for a dual luciferase reporter assay as described in Materials and methods. In addition, the cells were transfected with an empty expression vector (pcDNA3.1), or with the ERα or ERß expression plasmids, as indicated. The transfected cells were treated with EtOH or 10 nM E_2 for 30 h. For (A and B), the luciferase response was normalized to vehicle (EtOH/DMSO). Values are the mean ± SEM of 3 independent experiments. *Significantly different compared to vehicle (EtOH) ($p<0.05$). **Significantly different from the E_2 value (MCF-7 data in A).

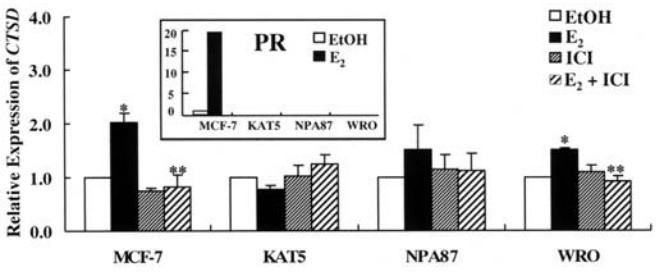

Figure 6. Endogenous E_2 target gene transcription in thyroid cancer cells. MCF-7 or the indicated thyroid cancer cells were treated with vehicle (EtOH), 10 nM E_2, 100 nM ICI, or the combination for 6 h. Q-RT-PCR analysis of PR (inset) and cathepsin D (*CTSD*) were normalized to 18S and the fold comparison was against MCF-7 treated with EtOH as described in Materials and methods. E_2 did not induce PR in any of the thyroid cancer cell lines and induced cathepsin D only in WRO cells. Values are the mean ± SEM of 3 independent experiments. *Significantly different from the EtOH value ($P<0.05$). **Significantly different from the E_2 alone value in that cell line ($P<0.05$).

cell cycle (56). E_2 induces cyclin D1 (*CCND1*) transcription (57). We examined the effect of E_2 and ICI on *CCND1* expression in the 3 thyroid cancer cell lines and MCF-7, as a positive control, at various times (Fig. 7). As expected, E_2 stimulated *CCND1* transcription at 1 h in MCF-7 and ICI blocked the E_2-induced *CCND1* expression, indicating ER-dependence (Fig. 7A). In KAT5, E_2 increased *CCND1* expression at 1 h and ICI blocked E_2-induced *CCND1*, indicating ER-dependence (Fig. 7B). ICI stimulated *CCND1* transcription at the 9 h time-point in KAT5 and E_2 reduced this stimulation, suggesting an ER-mediated response. In NPA87, basal *CCND1* transcription was reduced at 1, 3 and 9 h with EtOH (vehicle) treatment. The potential mechanism for this effect of EtOH was not evaluated and we compared the effects of the ER ligands relative to the basal *CCND1* transcript level with EtOH treatment at each time-point. E_2 increased *CCND1* transcription at 1 and 9 h. ICI alone had no

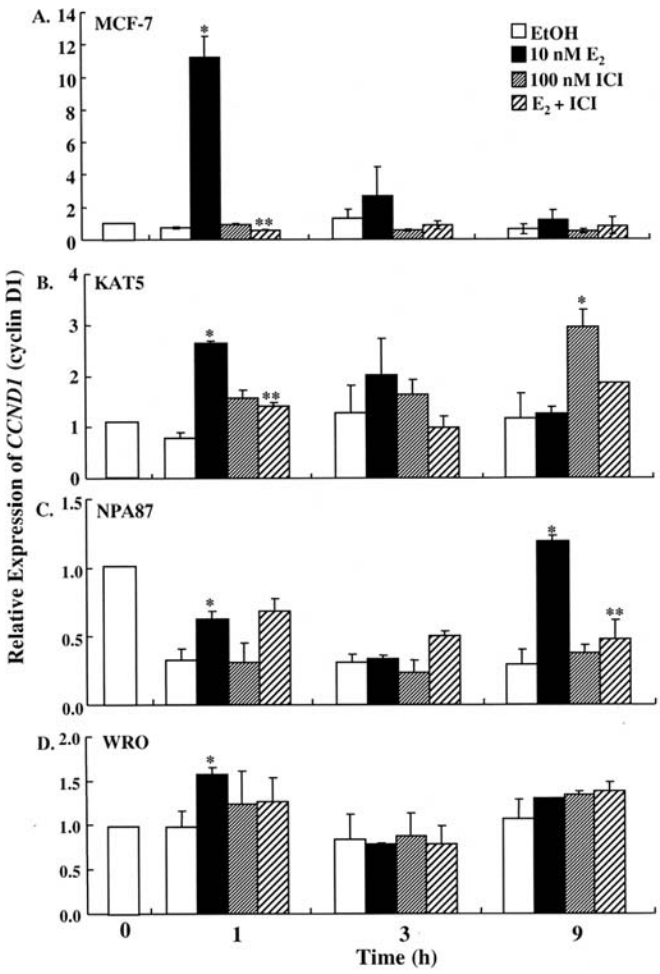

Figure 7. E_2 induces cyclin D1 (*CCND1*) transcription in thyroid cancer cells. MCF-7 (A) or the indicated thyroid cancer cells (B-D) were treated with vehicle (EtOH), 10 nM E_2, 100 nM ICI, or the combination for the indicated time. Q-PCR analysis of *CCND1* was normalized to 18S and the fold comparison was against EtOH time zero for each cell line as described in Materials and methods. Values are the average ± SEM of three separate experiments. *Significantly different ($P<0.05$) from the EtOH value at that time. **Significantly different from the 10 nM E_2 value at that time. Differences were examined within each cell line.

effect on *CCND1* transcription. ICI did not block E_2-induced *CCND1* at 1 h but blocked E_2 induced *CCND1* at 9 h (Fig. 7C). In WRO, E_2 increased *CCND1* transcription, but this was not abrogated by ICI (Fig. 8D). Overall, we conclude that E_2 regulates *CCND1* transcription in KAT5, NPA87, and WRO cells with different cell-line-specific kinetics by classical, genomic ER.

Knockdown of ERα or ERß reduces E_2-upregulated CCND1 and CTSD transcription in KAT5 and WRO cells. KAT5 and WRO were selected as representative papillary and follicular thyroid cancer cells for ER knockdown studies. To determine the involvement of ERα and ERß in the E_2 induction of cyclin D1 in KAT5 and WRO cells and cathepsin D in WRO (Fig. 7), ERα and ERß were specifically knocked-down using siRNA. Because of the potential effects of EtOH on basal *CCND1* transcription in NPA87 (Fig. 7C), further experiments were not pursued in this cell line. Experiments to optimize ERα and ERß knockdown revealed that 100 nM siRNA was the most effective concentration to silence the expression of either ER subtype without affecting cell viability (data not shown). siERα reduced ERα mRNA by 75% in KAT5 cells with no effect on ERß (Fig. 8A) and ERα protein was reduced ~55%, likewise with no effect on ERß protein (Fig. 8B). siERß reduced ERß mRNA by 83% in KAT5 cells, but ERα mRNA was reduced ~50% as well (Fig. 8A). ERß protein was reduced ~60% with siERß in KAT5 cells and a <10% change in ERα protein was detected (Fig. 8B). siERα reduced ERα mRNA by 65% in WRO cells with an ~15% reduction in ERß mRNA (Fig. 8C) and ERα protein was reduced ~60% with 50% increase in ERß protein level (Fig. 8D). siERß reduced ERß mRNA by 50% in WRO cells and ERα was increased by ~40% in these cells (Fig. 8C). In WRO cells, siERß reduced ERß protein by 60%, but, in contrast to the increase in ERα mRNA, no change in ERα protein was detected (Fig. 8D).

As seen in Fig. 8E, E_2 induced *CCND1* in KAT5 cells transfected with the siControl and this induction was ablated by ICI. Knockdown of ERα resulted in elevated basal levels of *CCND1* in KAT5 cells. However, siERα blocked further induction of *CCND1* transcription by E_2, thus reducing *CCND1* relative to basal. In contrast, knockdown of ERß in KAT5 cells did not alter basal *CCND1* expression, but inhibited E_2-induced *CCND1* transcription. ICI reduced basal *CCND1* in the cells in which ERß was reduced with siERß (Fig. 8E). These data suggest that basal expression of *CCND1* is regulated by ERα whereas E_2-induced expression of *CCND1* in KAT5 cells is controlled by both ERα and ERß. Further, the knockdown of ERß 'unmasks' the ability of ICI-occupied ERα to inhibit basal *CCND1* transcription. In WRO cells, ERα knockdown blocked E_2-induced *CTSD* transcription (Fig. 8F) As seen for KAT5, siERα resulted in elevated basal *CCND1* in WRO cells (Fig. 8G). Likewise, ERα knockdown abrogated the E_2-induced increased expression of *CCND1* but, in contrast to KAT5, ERß knockdown did not inhibit E_2-induced *CCND1* expression (Fig. 8G). Together these data indicate a role for ERα in the E_2 increased expression of *CCND1* in both KAT5 and WRO and a role for ERß in E_2-regulation of this gene in KAT5.

Knockdown of ERα or ER, inhibits E_2-induced ERK1/2 phosphorylation in KAT5 and WRO cells. Having established that non-genomic E_2 signaling through MEK and PI3K play a role in E_2-induced cell proliferation (Fig. 1) and that knockdown of either ERα or ERß abrogates the E_2-induced cyclin D1 (*CCND1*) transcription in both KAT5 and WRO cells (Fig. 8E and G), we evaluated the impact of ER subtype-specific knockdown on E_2-induced non-genomic signaling by examining ERK1/2 phosphorylation (p-ERK). Previous investigators reported that KAT5 cells required at least 1 h E_2 treatment to increase p-ERK (27). Hence, 1 h E_2 was the time selected for our knockdown experiments. E_2 increased p-ERK/total ERK in both KAT5 and WRO cells and this increase was blocked by ICI and abrogated by knockdown of ERα or ERß, indicating ER-mediated ERK activation (Fig. 9). In WRO cells, the knockdown of ERα increased basal p-ERK compared to vehicle control, indicating a potential role for ERα in regulating

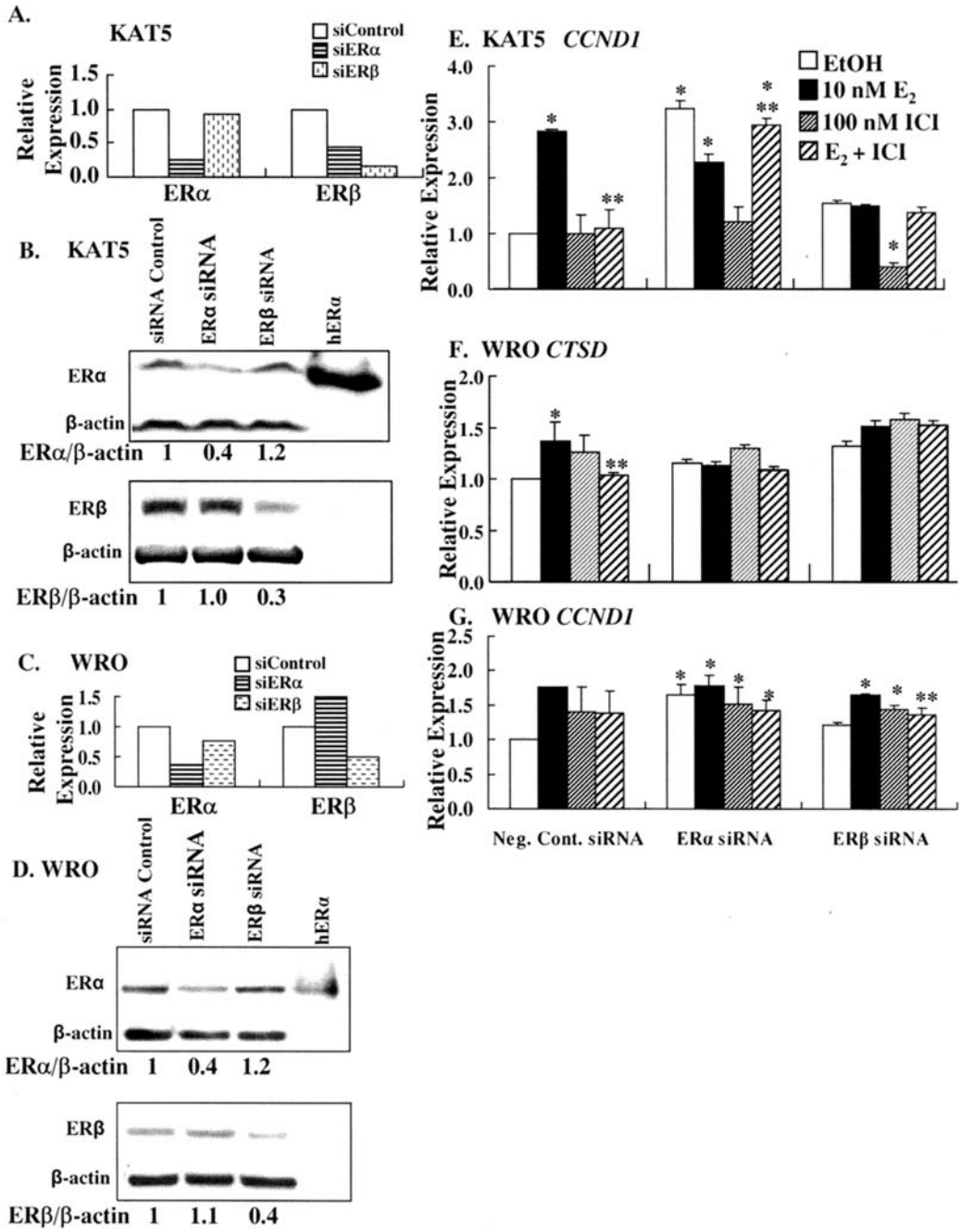

Figure 8. Knockdown of ERα and ERß in KAT5 and WRO cells reduces endogenous E_2-target gene transcription. Transfection of KAT5 (A-B) and WRO (C-D) with siRNA against ERα and ERß selectively reduced ER subtype-specific mRNA expression 48 h after transfection and protein levels 72 h after transfection. Q-PCR and Western blots were performed and analyzed as described in Materials and methods. *ESR1* and *ESR2* mRNA values were normalized to 18S and the fold comparison was against siRNA negative control as described in Materials and methods. Quantification of the ER protein bands was performed as described in Materials and methods and presented relative to ß-actin with siRNA-transfected control set to 1. (E-F) Effect of knockdown of ER on ER target gene transcription. Cells were treated with 10 nM E_2 or 100 nM ICI for 1 h for *CCND1* (E and G) and 6 h for *CTSD* (F). The expression of *CCND1* (E and G) and *CTSD* (F) was determined by Q-PCR, normalized to 18S, and the fold comparison was against EtOH as described in Materials and methods. Values are the average ± SEM of three separate experiments. *Significantly different from EtOH ($P<0.05$). **Significantly different from the E_2 alone value for that gene in that cell line ($P<0.05$).

basal ERK activity. Together, these data indicate roles for ERα and ERß in mediating E_2-induced ERK1/2 phosphorylation in KAT5 and WRO thyroid cancer cells.

GPR30 expression is low in KAT5 and NPA87 papillary carcinoma cells. GPR30 was identified as a novel membrane estrogen receptor in 2000 (58). E_2 was reported to increase WRO proliferation by activating GPR30 (22). No one has evaluated GPR30 expression in KAT5 or NPA87 thyroid cancer cells. We examined mRNA levels of GPR30 in the 3 thyroid cancer cell lines (Fig. 10). GPR30 expression was higher in WRO follicular than either KAT5 or NPA87 papillary thyroid carcinoma cells. MCF-7 cells had higher GPR30 than WRO cells. Because of the low or absent expression of

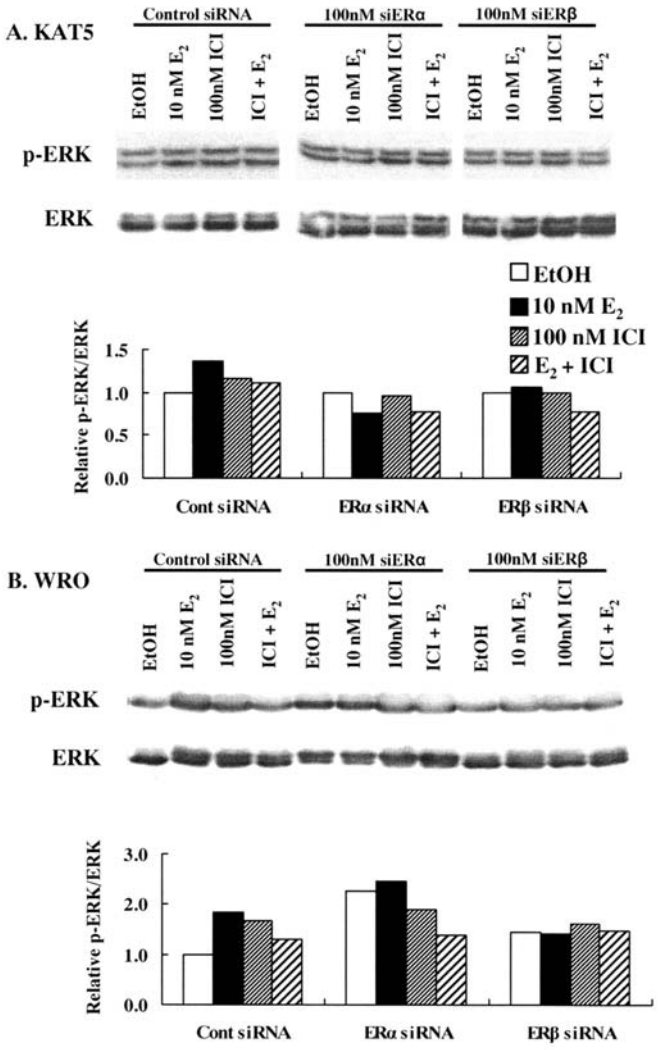

Figure 9. E_2-induced ERK phosphorylation in thyroid cancer cell lines. KAT5 (A) and WRO (B) cells that had been transfected with control siRNA or siERα or siERß for 48 h were treated with 10 nM E_2 or 100 nM ICI for 1 h. WCE were analyzed for ERK phosphorylation (p-ERK) and the blots were stripped and reprobed for total ERK (ERK). Densitometric values for p-ERK were normalized to ERK and control vehicle was set to 1.

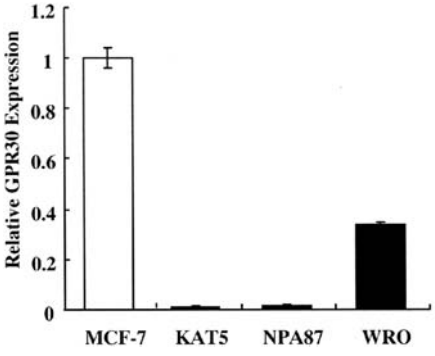

Figure 10. GPR30 expression is lower in thyroid cancer cells than MCF-7 cells. Q-PCR was performed using ABI TaqMan primer/probes on untreated cells. The Ct values were normalized to 18S and normalized to MCF-7. Values are the average of 3 determinations ± SEM.

GPR30 mRNA in the three thyroid cancer cell lines, further studies were not pursued.

Discussion

Although thyroid cancer is predominantly a disease affecting premenopausal women, relatively few studies have examined the roles of E_2 and ER in thyroid cancer cell proliferation (4,22,27,28,59,60). Previous studies reported that E_2 stimulated cell proliferation in FRTL5, a mouse thyroid cell line (61), FRO and WRO human follicular thyroid carcinoma cells (22), TT medullary thyroid carcinoma cells (62), papillary KAT5 cells (27,59,60) and HTC-TSHr thyroid carcinoma cells (9). Despite these reports, the mechanisms involving E_2-induced cell proliferation in these thyroid cancer cell lines are not clearly understood. Here we demonstrated that KAT5, NPA87, and WRO thyroid cancer cell lines responded proliferatively to E_2 and that ICI 182,780 (Fulvestrant) blocked E_2-induced proliferation, indicating that the E_2-response was ER mediated. The E_2-induced proliferation in KAT5 is similar to that reported previously (27,59,60). In contrast, we found that follicular FTC133 and papillary TPC1 thyroid cancer cells were not responsive to E_2 (data not shown), despite expressing ERα. These data conflict with a report that E_2 stimulated FTC133 cell proliferation as determined by cell counting (63). The reason for this disparity is unknown, but differences in cell maintenance and assay conditions are likely involved. Our studies using ER subtype-selective agonists and antagonists (Fig. 1) indicate that ERα is responsible for the E_2-induced proliferation in KAT5 and WRO cells whereas ERß appears to play a role in both NPA87 and WRO cells. In contrast to a previous report (64), we observed that 4-OHT stimulated KAT5 and WRO proliferation, but had no significant effect on NPA87. Notably, we used 100 nM 4-OHT and the previous reports used higher concentration, i.e., 1.5 μM tamoxifen (64,65). Our WRO data consistent with another report demonstrating that 4-OHT increased cell proliferation in WRO through GPR30 (22) since we detected GPR30 mRNA only in WRO and not in KAT5 or NPA87 cells. Here we observed that RAL stimulated WRO, but not KAT5 or NPA87 cell proliferation and we again speculate a role for GPR30 in this response. Both 4-OHT or RAL inhibited E_2-induced proliferation of WRO and NPA87, but RAL did not inhibit E_2-activated proliferation of KAT5. These data reflect the established cell-specificity of SERM pharmacology, i.e., 4-OHT and RAL are ER agonists in some cells and antagonists in others (66).

We demonstrated that KAT5, NPA87, and WRO thyroid cancer cells express full length ERα and ERß, as well as a number of previously reported (14), subtype-specific splice variants. Our data are in agreement with a previous report that ERα46 and ER, 59 kDa are expressed in WRO cells (22) and that KAT5 cells have full length ERα (27,59,60). The 36 and 58 kDa ERα protein splice variants have not been reported in the thyroid cancer cells and their roles remain to be resolved. Because higher ERα46 expression was seen compared to the full length ERα in each of the three thyroid cancer lines used in this study (Fig. 2A), it is possible that dominant negative activity of ERα46, reported in breast cancer cells (67,68), and ERα36, also reported in breast cancer cell lines and transfected HEK-293 cells (45,46), and may inhibit genomic ER signaling in thyroid cancer cells, thus offering an explanation for our transient transfection and Q-PCR data.

Further studies will be required to dissect the role of ERα splice variants in E_2 responses in thyroid cancer cells.

The classical estrogen signaling paradigm involves activation of ER by binding of E_2 leading to ER hetero- or homodimerization, translocation to the cell nucleus, interaction with DNA and coregulators leading to alteration in gene transcription that stimulates cell cycle progression (52,69). To our surprise, despite the fact that KAT5, NPA87, and WRO express full length ERα and ERß, E_2 did not stimulate luciferase activity from a transiently transfected ERE reporter in any of these cell lines. In addition to the presence of dominant negative splice variants, another explanation for our results is the predominant cytoplasmic localization of ERα and ERß in the thyroid cancer cells and the lack E_2 stimulation of redistribution of ER to the nucleus. Our immunofluorescent studies agree with a previous report that ERα is exclusively cytoplasmic following E_2 treatment of WRO cells (22), but conflict with a report that ERα is cytoplasmic and nuclear in untreated KAT5 cells (60). It is possible that differences in KAT5 culture conditions account for these disparities between labs. It is generally believed that ER signaling involves ligand-activated translocation to nucleus (70), although ERα is also nuclear in the absence of ligand (71). Although a short splice variant of MTA1, i.e., MTA1s, was reported to sequester ERα in the cytoplasm of breast cancer cells (72), thus blocking transcriptional activity, we did not detect MTA1s in the thyroid cancer cell lines or in MCF-7 cells. Thus, we conclude that MTA1s expression does not provide a mechanism to account for either the predominant cytoplasmic localization of ERα in the three thyroid cancer cell lines, regardless of E_2 treatment, or the lack of E_2-induced reporter gene expression in the transiently transfected cells.

Other studies in WRO cells reported that E_2 increased cell proliferation by activating GPR30 (22). GRP30 has been reported to convey estrogenic signaling in a variety of cell types, independent of ER expression (73). We detected low GPR30 mRNA expression in WRO relative to MCF-7 breast cancer cells and report for the first time that neither KAT5 nor NPA87 cells express GPR30. While GPR30 was reported not be a *bone fide* membrane ER, its role as a collaborator with ERα and ERß (23), remains to be resolved. E_2 was reported to activate MAPK signaling in KAT5 cells (27). Here, based on inhibitor studies, we demonstrated that the E_2-induced proliferation of KAT5, NPA87, and WRO cells involved non-genomic ER activation of MAPK, PI3K, and Gα proteins. E_2 rapidly activates PI3K/AKT and ERK1/2 in a variety of cell types (19). Our data demonstrating that knockdown of ERα and ERß expression in KAT5 and WRO cells inhibited E_2-induced phosphorylation of ERK1/2 implicates a role for both subtypes in ERK1/2 activation. The integration of non-genomic and genomic ER signaling pathways in breast cancer is complex (74) and is likely to be equally complex in thyroid cancer. Further experiments are required to elucidate the form(s) of ER, including splice variants and interactions of ERs with GPR30, and other plasma membrane receptors, e.g., EGFR, in thyroid cancer cells.

Despite the lack of nuclear ERα and ERß localization, E_2 increased cathepsin D (*CTSD*) transcription in WRO cells and expression was inhibited by ICI and by siRNA-mediated knockdown of ERα and ERß. Cathepsin D is a classical E_2 target gene regulated by Sp1-ERα promoter binding (75). Interestingly, the basal cathepsin D protein expression was higher in each of the 3 cell thyroid cancer cell lines than in MCF-7 cells. It is well-established that cathepsin D expression is elevated in thyroid tumors and correlates with disease aggression (76). Cathepsin D mRNA levels were unaffected by E_2 in KAT5 and NPA87 cells, likely reflecting cell line-specific gene responses to E_2.

The expression of another classical E_2 target gene, *CCND1*, cyclin D1 (77), was stimulated by E_2 in all three thyroid cancer cell lines and co-treatment with ICI as well as siERα and siERß experiments revealed roles for ERα and ERß in regulating cyclin D1 transcription. E_2 regulation of cyclin D1 transcription involved ERα-Sp1 interaction (78) and AP-1-ERα or AP-1-ERα interaction (79). Thus, the induction of cyclin D1 transcription in response to E_2 indicates intact genomic ER signaling in KAT5, NPA87, and WRO thyroid cancer cells.

Overall, this study provides evidence that although KAT5, WRO, and NPA87 thyroid cancer cells express ERα and ERß, genomic ER activity is reduced compared to that observed in MCF-7 breast cancer cells. Further, in agreement with previous reports (22,27,28), we observed non-genomic E_2 activation of ERK1/2 and cell proliferation. Clearly, additional studies are necessary to elucidate the roles of ERα and ERß and their splice variants in both transcriptional and non-genomic signaling activates in response to E_2 and other ER ligands including SERMs in thyroid cancer. Such studies may lead to a new understanding of the pathogenesis of thyroid cancer and its female bias.

Acknowledgements

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