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**14. ABSTRACT**
Estrogen signaling is primarily mediated by two estrogen receptors (ERs), ERα and ERβ. Triple negative breast cancer (TNBCs) is an aggressive breast cancer sutype that lacks expression of several therapeutic targets. Based on in vitro and clinical data, it is hypothesized that estrogen receptor (ER) β could be targeted with selective ligands to inhibit the growth of TNBCs. The goal of the work completed over the course of this training program aimed to better understand the role of ERβ in TNBC and develop tools to target and detect ERβ in TNBCs. First, reporter cell lines with inducible ERα or ERβ expression and an estrogen responsive luciferase reporter were developed to identify and characterize subtype selective estrogenic ligands. Second, a tumorigenic TNBC cell line was engineered with inducible ERβ expression to determine the effects of ERβ on the growth of TNBC cells in vitro and in vivo. These cells were also used to globally identify the ligand dependent and independent ERβ target genes using RNA sequencing. Finally, ERβ immunohistochemistry was optimized using xenografts and applied to a cohort of TNBCs to assess associations with clinicopathologic features. Not only does this work provide a foundation for further research into the role of ERβ in TNBC, it resulted in several publications, presentations, and a rich training experience for a future career in breast cancer research.

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## Table of Contents

**Page**

## INTRODUCTION

Triple negative breast cancers (TNBCs) account for ~10-15% of breast cancers and are associated with poor clinical outcomes and worse disease-free and overall survival [1]. Thus, there is a critical need to identify new therapeutic targets for this breast cancer subtype. TNBCs lack of expression of three receptors that are commonly used to determine the prognosis and treatment options for patients: estrogen receptor (ER) α, progesterone receptor, and human epidermal growth factor receptor 2 (HER2). A second estrogen receptor (ERβ) has been detected in some TNBCs [2-4] and exogenous expression of ERβ inhibits the growth of breast cancer cells [5-8]. Given the anti-proliferative activity of ERβ, this receptor may be an effective therapeutic target for triple negative breast cancers that express ERβ. *The hypothesis underlying this project is that ERβ is an effective therapeutic target and ERβ selective ligands can inhibit the growth of triple negative breast cancers when ER*β *is expressed.*

Three aims were originally proposed to test this hypothesis: 1) characterize the growth inhibotry effects of ERβ-selective ligands in TNBC cells; 2) identify new ERβ-selective ligands using high throughput screening; and 3) determine the effects of ERβ-selective ligands on the growth of TNBC in xenograft models. Based on the data collected and feedback from both the committee and the reviewers of the original proposal, some of the original aims and experiments were re-designed and a revised statement of work (SOW) was approved in 2012. This revised SOW incorporated 2 new aims: 1) characterize the growth inhibitory effects of ERβ and identify ERβ target genes using RNA sequencing (RNA-seq); 2) assess ERβ expression in a cohort of TNBC clinical samples. This report will detail the results of each aim from the original SOW and the revised SOW. Key research accomplishments and reportable outcomes will also be described.

## BODY

Estrogen signaling is primarily mediated by two estrogen receptors (ERs), ERα and ERβ. Despite sharing several structural similarities, ERα and ERβ have many unique features with respect to ligand binding and gene regulation. ERα and ERβ are expressed in a variety of tissues and have both overlapping and distinct tissue distributions, and there are several ERβ isoforms that can modulate the estrogen response. ERs can regulate gene expression in both ligand dependent and independent manners. ER ligands are structurally diverse, and some ligands show selectivity for ERα or ERβ due to differences in the ligand binding pockets of the two receptors. Ultimately, ligands can elicit tissue- and cell type-specific responses, as exemplified by selective estrogen receptor modulators (SERMs) such as tamoxifen, which is used to target ERα for breast cancer treatment. ERα is expressed in ~70% of breast cancers, but ~10-15% of breast cancers are classified as triple negative breast cancers (TNBCs). TNBCs lack therapeutic targets and there is a need to identify new treatment strategies for this particularly aggressive breast cancer subtype. ERβ is expressed in a subset of TNBCs, and previous *in vitro* data suggested that ERβ is growth inhibitory in ERα-negative breast cancers. Based on the results of previous studies, it was hypothesized that ERβ may be a therapeutic target in a subset of ERα-negative breast cancers, including some TNBCs.

**Aim 1: Characterize the inhibitory effects of ERβ-selective compounds in TNBC**

The goals of this aim were to: 1) determine the effects of cosmosiin and liquiritigenin on the growth of Hs578T-ERβ cells; 2) identify additional models of ERβ-positive TNBC; and 3) identify downstream targets of ERβ that mediate growth inhibitory effects. Much of the work towards this aim has been published [9, 10] (Appendix B) and was described in the previous annual reports. Briefly, cosmosiin and liquiritigenin inhibited the growth of Hs578T-ERβ cells (a TNBC cell line with inducible ERβ expression) only when ERβ was expressed. This inhibitory effect is likely due to a cell cycle arrest because these compounds did not induce apoptosis (data not shown) and 17b-estradiol (E2), the most prevalent estrogen in women of reproductive age, was shown to induce a cell cycle arrest in Hs578T-ERβ cells [11].

Next, these results were confirmed in another model of ERβ-positive TNBC. Because additional models of TNBC with detectable endogenous ERβ could not be identified, a tumorigenic TNBC cell line (MDA-468) was engineered to express ERβ after doxycycline (Dox) treatment. These MDA468-ERβ cells were described in the last annual report, and since then have been described extensively in a recent publication [10] (Appendix B). Briefly, E2 induced a G0/G1 cell cycle arrest in MDA468-ERβ cells only when ERβ was expressed, and surprisingly, ERβ expression alone induced cell cycle arrest, although to a lesser degree. These results suggested that these cells could be used to identify ligand-dependent and ligand-independent ERβ target genes in TNBC cells.

In order to identify downstream targets of ERβ that mediate growth inhibition, RNA-seq was performed in MDA468-ERβ cells in the presence and absence of E2 [10]. This approach allowed the first global identification of ligand-dependent and ligand-independent ERβ target genes in the absence of ERα, thereby providing a rich resource for the scientific community. Since this work is published [10], only a few of the results will be highlighted in this report. First, ERβ induced the expression of the cyclin dependent kinase inhibitor p21, which mediates the progression of the cell cycle. Second, several common ERβ target genes were identified in Hs578T-ERβ and MDA468-ERβ suggesting that the receptor can mediate a common set of genes in different TNBC cell lines. Finally, in collaboration with a biostatistician, ERβ expression was found to be associated with the expression of several of its target genes in TNBC gene expression data from the Cancer Genome Atlas database. Overall, this is the most comprehensive study to date regarding target gene regulation by ERβ. In addition, this study allowed me to build a bioinformatics collaboration and develop the skills necessary to perform large-scale genomics studies. These skills will be critical for my future career in breast cancer research.

**Original Aim 2: Identify new ERβ-selective ligands using high throughput screening (HTS)**

The goals of this aim were to: 1) optimize luciferase assays with Hs578T-ERβLuc, 2) screen additional Hs578T-ERαLuc cells, and 3) conduct a screen and counterscreen with small molecule libraries using Hs578T-ERαLuc and Hs578T-ERβLuc cells. Although significant effort went into the high throughput screening optimization, this aim was ultimately deleted because of the variability in the screening assay. As described in the previous annual reports, the assay could not be optimized for a 384 well. However, these reporter cell lines were highly sensitive to estrogenic ligands, and this part of the project ultimately resulted in two first-author publications [9, 12].

**Original Aim 3: Determine the effects of ERβ-selective ligands on the growth of TNBC in xenograft mouse models**

The original goals of this aim were to: 1) graft Hs578T-ERαLuc and Hs578T-ERβLuc cells into nude mice; 2) inject mice with ERβ ligands and monitor tumor growth using bioluminescent imaging; and 3) assess luciferase expression and tumor histology after treatments. Although several attempts were made to graft Hs578T-ERαLuc and Hs578T-ERβLuc into nude mice, palpable tumors did not consistently form and this part of the aim was revised.

As an alternative approach, MDA468-ERβ cells were labeled with luciferase and injected into the fatpad of nude mice to track the growth of the tumor after treatment with Dox and/or E2. The results of this study are published [10], and the growth inhibitory effects of ERβ were confirmed *in vivo*.

**Revised Aim 3: Determine the effects of ERβ expression and activation on the growth of TNBC in xenograft mouse models and assess ERβ expression in TNBC clinical samples**

The first part of this aim is described above. In order to address concerns of the reviewers and thesis committee, a second part of the aim was incorporated into the revised statement of work. A major weakness of the original proposal was that it is not widely accepted that ERβ is expressed in TNBCs. Many studies aimed at assessing ERβ expression have yielded inconsistent results (reviewed in [13]). In order to address the issues of antibody specificity, immunohistochemistry (IHC) protocols for ERβ detection were optimized using MDA468-ERβ xenograft tissues in which the expression of ERβ was regulated by Dox exposure. As shown in Figure 1, ERβ was specifically detected in the xenograft tissues obtained from mice exposed to Dox (+ERβ) and this signal was eliminated by preabsorbing the antibody with peptide corresponding to ERβ. These IHC protocols were then applied to two cohorts of TNBC from the Marshfield Clinic in collaboration with the Translational Research Initiatives in Pathology (TRIP) lab and Dr. Kari Wisinski (manuscript in preparation) (Figure 2). In order to objectively score ERβ expression in the samples, the VECTRA$^{TM}$ multispectral imaging instrument was used to quantify the ERβ signal in the nuclear and cytoplasmic compartments. As shown in Figure 3, there was a close relationship between the percent positive nuclei and the mean optical density (OD) for ERβ detection. There was also a close relationship between the cytoplasmic and nuclear ERβ expression (Figure 3B and 3C).

The tissues were also stained for Ki67, an indicator of proliferation, to assess whether ERβ expression was associated with proliferation. Surprisingly, there was a significant positive correlation between ERβ and Ki67 (Figure 4A and 4B). A survival analysis revealed that there was no association between ERβ expression and survival in this cohort of TNBCs (Figure 5A and 5B). These results are currently being prepared for publication and will surely be published by the end of the year because this is the first objective, quantitative analysis of ERβ expression in TNBC. Although there was a discrepancy between the antiproliferative effects of ERβ in vitro and the positive association between ERβ and Ki67 in clinical samples, this part of the project provides a foundation on which ERβ IHC should be performed in future studies with larger cohorts. In addition, this aspect of the project provided experience in bridging basic and clinical research that is necessary for successful translational research. I learned the challenges associated with collaborating and communicating with clinical, statistical and basic researchers, and this invaluable experience will help me successfully drive these translational projects in the future.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Characterization of growth inhibitory effects of cosmosiin and liquiritigenin in Hs578T-ERβ cells
- Generation of MDA468-ERβ inducible cell lines
- Characterization of the sensitivity and selectivity of Hs578T-ERβLuc and Hs578T-ERαLuc cells
- Optimization of HTS assay for Hs578T-ERβLuc in 96 well format
- Determined that ERβ inhibits MDA468- ERβ cell growth by inducing cell cycle arrest
- Demonstrated that ERβ regulates p21, a key regulator of G1 arrest
- Developed a retroviral delivery system to express ERβ in additional TNBC cell lines
- Identification of ERβ targets using RNA sequencing using RSEM and DESeq
- Identified several functions and pathways that are enriched in ERb target genes and
- validated the ERβ target genes using quantitative PCR
- Developed MDA468- ERβ -FLuc cells for xenografts experiments and *in vivo* imaging
- Assessed the expression of ERβ in a cohort of ERα-negative breast cancer samples
- Determined the associations between ERβ expression and clinicopathologic characteristics in a cohort of TNBC

**REPORTABLE OUTCOMES:**

**Conferences Attended:**
Society of Toxicology Annual Meeting, 2012
Gordon Research Conference on Hormone Action in Development and Cancer, 2011

**Awards:**
RSESS Society of Toxicology 2012 Annual Meeting Travel Award, 2012

**Publications:**

**Shanle E,** Onitilo A, Huang W, Kim K, Zang C, Engel J, Xu W, Wisinski K. Prognostic significance of full length estrogen receptor beta expression in Stage I-III triple negative breast cancer . *In preparation.*

Yarger JG, Babine RE, Bittner M, **Shanle E**, Xu W, Hershberger P, Nye SH, 2012. Structurally similar estradiol analogs uniquely alter the regulation of intracellular signaling pathways. *J Mol Endocrinol*. 50, 43-57.

Sievers C*, **Shanle EK***, Bradfield C, Xu W, 2012. Differential action of monohydroxylated polycyclic aromatic hydrocarbons with estrogen receptors α and β. *Toxicol Sci* [Epub ahead of print]. *Authors contributed equally to this work

Powell E, **Shanle E**, Brinkman A, Li J, Keles S, Wisinski K, Huang W, Xu W. Identification of Estrogen Receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ERα and ERβ. *PLoS ONE*, e30993.

**Shanle E,** Xu W. Function, expression, and detection of estrogen receptor isoforms in normal and malignant tissues, in: Chen, G. (Ed.), Estrogen Receptors: Mechanisms, Structure and Role in Disease. Nova Science Publishers Inc., New York, ISBN 978-1-62257-180-2. (Book Chapter)

**Shanle E,** Xu W. Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands. *Biochem Pharmacol* **82**, 1940 (2011).

**Shanle E,** Xu W. Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. *Chem Res Toxicol* **24**, 6 (2010). (review)

**Poster Presentations:**

Blanke, K., **Shanle, E.** Exploring Toxicology: Designing learning goals and evaluation strategies for outreach activities; Accepted for oral presentation in the K-12 Toxicology Outreach Activities: Regional Chapter Successes and Resources Informal Specialty Section. Society of Toxicology Annual Meeting, March 13, 2013.

**Shanle, E.,** Xu, W. Stable reporter cell lines for the identification of subtype selective estrogenic ligands. Society of Toxicology Annual Meeting, March 11-15, 2012. San Francisco, CA. Poster Presentation.

**Shanle, E.**, Xu, W. Stable reporter cell lines for the identification of subtype selective estrogenic ligands. Gordon Conference for Hormone Action in Development and Cancer, July 30-August 4, 2011. Bryant University, Smithfield, RI.

**Shanle, E.**, Powell, E., Eastlund, V., Xu, W. Growth inhibitory effects of natural phytoestrogens on breast cancer cells mediated by ERβ. Great Lakes Nuclear Receptor Conference, October 22-23, 2010. University of Michigan, Ann Arbor, MI.

**CONCLUSION**

At the completion of the Department of Defense Breast Cancer Research Program Pre-Doctoral Traineeship, several significant accomplishments were made. Each aim was successfully completed over the course of the training period, resulting in 3 first-author primary publications, one book chapter, two review articles, and an additional first-author primary publication to be submitted soon. I successfully defended my thesis and received numberous post-doctoral research position offers from institutions including MD Anderson Cancer Center, the University of Colorado, and the University of North Carolina – Chapel Hill. This training program ultimately prepared me for an independent research career and enabled me to pursue a competitive postdoctoral fellowship at the University of North Carolina in Chapel Hill.

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**Appendix A: Figures and Tables**

Figure 1: Xenograft tissues with inducible ERβ expression are useful for optimizing ERβ IHC. MDA468-ERβ cells were injected into the mammary fat pads of nude mice. After tumors formed, mice were treated with either vehicle (1% sucrose) (A, B, C) or Dox (D, E, F). IHC was performed with the PA1-313 ERβ antibody (A, D). For controls, the antibody was pre-absorbed with ERβ peptide (B, E) or the primary antibody was excluded entirely (C, F). The brown staining that indicates reactivity toward ERβ is only observed in tissues from mice exposed to Dox (+ERβ) (A). Scale bars = 100 μm.

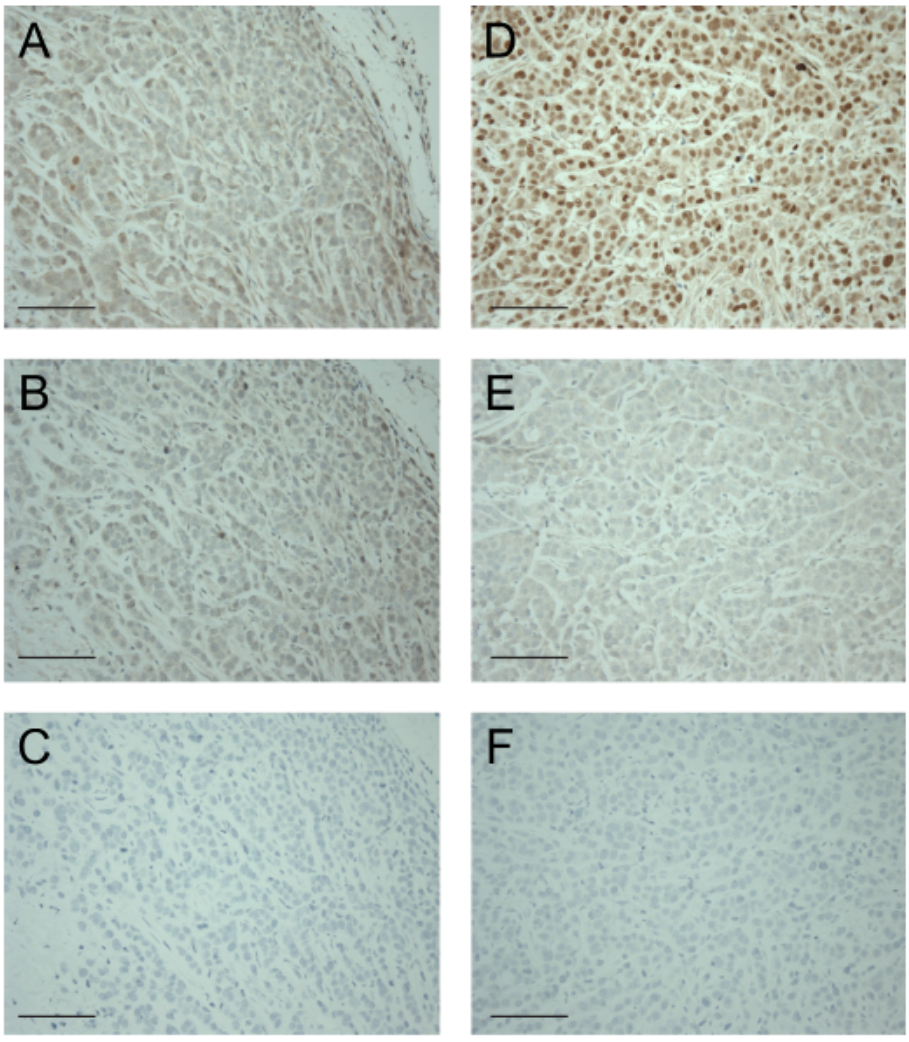


Figure 2: Immunohistochemistry staining for ERβ in TNBCs from the Marshfield Clinic. A and B) Representative images of ERβ-negative tumor tissues (0.33% and 2.6% 1+ or higher nuclei, respectively). C and D) Representative images of ERβ-positive tumor tissues (66.2% and 73.2% 1+ or higher nuclei, respectively). Scale bars = 200 μm.

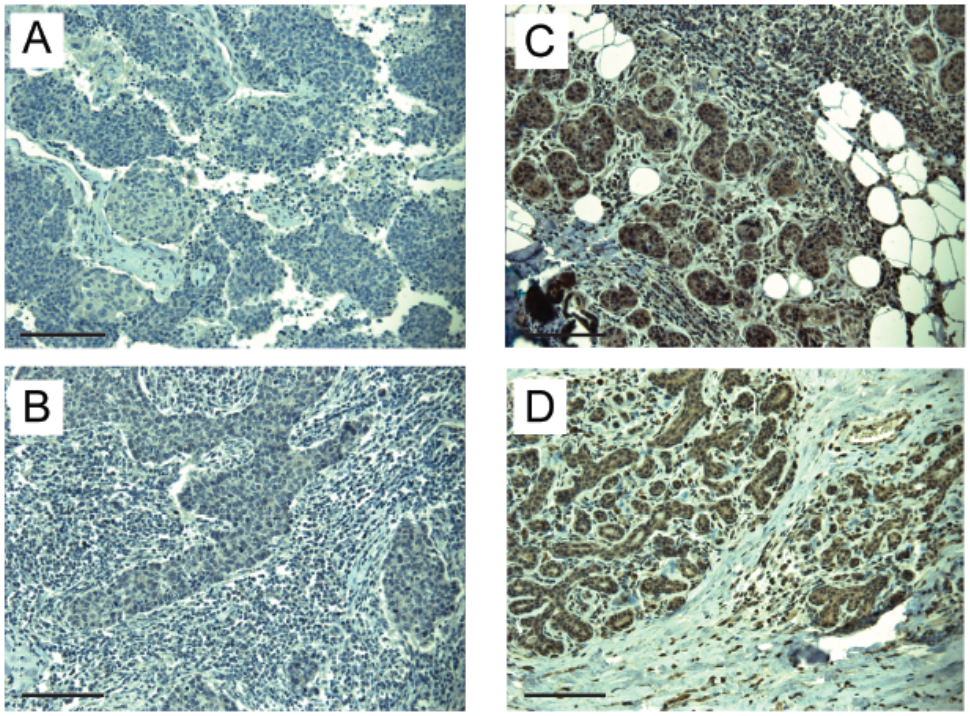


Figure 3: Relationships between ERβ subcellular localization and scoring strategies in the Marshfield cohort. A) A comparison of the nuclear mean OD and percent ERβ-positive nuclei. B) Relationship between the nuclear and cytoplasmic ERβ-positive cells. C) Relationship between the nuclear and cytoplasmic mean OD values.

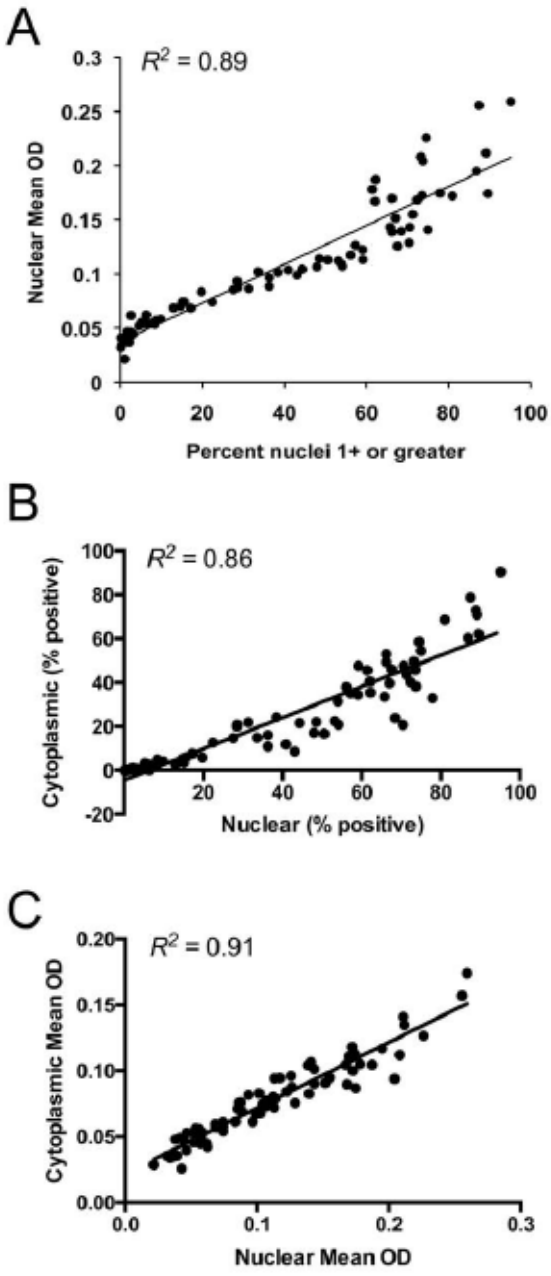


Figure 4: ERβ expression is correlated with Ki67 expression. A scatter plot showing the relationship between the percentage of Ki67-positive nuclei (1+ or greater) and the mean OD for (A) nuclear ERβ expression or (B) cytoplasmic ERβ expression as detected using the PA1-313 antibody. There was a moderately significant positive correlation between the two variables (Spearman correlation, $p<0.0001$ for both comparisons).

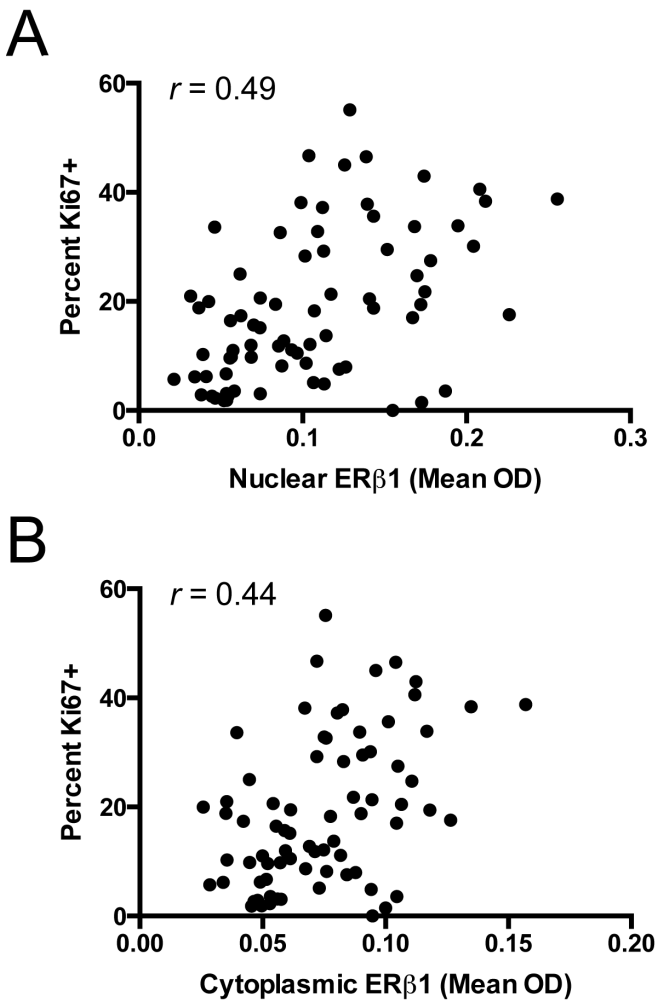
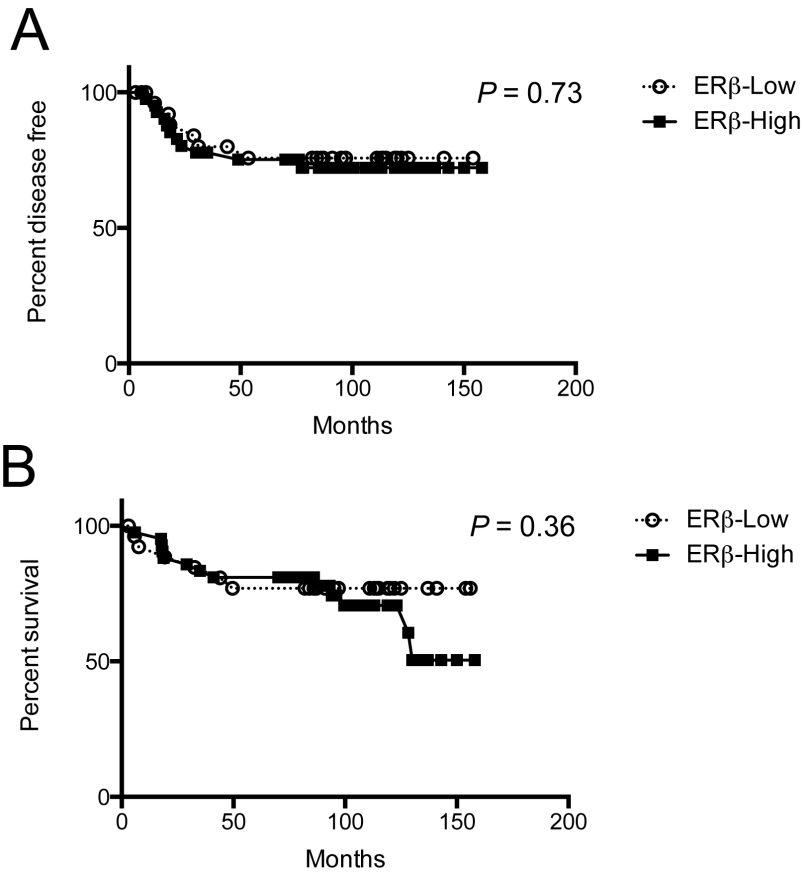


Figure 5: ERβ expression is not significantly associated with disease-free survival or overall survival in the Marshfield cohort. Kaplan-Meier graphs for disease-free survival (A) and overall survival (B) with respect to ERβ low (<20% nuclear staining, n = 42) and ERβ high (>20% nuclear staining, n = 28) expression.



## Appendix B: Research Manuscripts

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# Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands

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## Abstract

Estrogen signaling is mediated by two estrogen receptors (ERs), ERα and ERβ, which have unique roles in the regulation of breast cancer cell proliferation. ERα induces proliferation in response to estrogen and ERβ inhibits proliferation in breast cancer cells, suggesting that ERβ selective ligands may be beneficial for promoting the anti-proliferative action of ERβ. Subtype selective ligands can be identified using transcriptional assays, but cell lines in which ERα or ERβ are independently expressed are required. Of the available reporter cell lines, none have been generated in breast cancer cells to identify subtype selective ligands. Here we describe the generation of two isogenic breast cancer cell lines, Hs578T-ERαLuc and Hs578T-ERβLuc, with stable integration of an estrogen responsive luciferase reporter gene. Hs578T-ERαLuc and Hs578T-ERβLuc cell lines are highly sensitive to estrogenic chemicals and ER subtype selective ligands, providing a tool to characterize the transcriptional potency and subtype selectivity of estrogenic ligands in the context of breast cancer cells. In addition to measuring reporter activity, ERβ target gene expression and growth inhibitory effects of ERβ selective ligands can be determined as biological endpoints. The finding that activation of ERβ by estrogen or ERβ selective natural phytoestrogens inhibits the growth of Hs578T-ERβ cells implies therapeutic potential for ERβ selective ligands in breast cancer cells that express ERβ.

### Keywords

Estrogen receptors; subtype selectivity; phytoestrogens; breast cancer

## 1. Introduction

Estrogens regulate mammary gland growth and differentiation, ovary and uterus maturation, and bone homeostasis [1]. The physiological effects of estrogens are primarily mediated by two estrogen receptors (ERs), ERα and ERβ. Because of the broad range of ER target tissues and the ligand dependent activity of the receptors, synthetic and natural estrogens hold



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therapeutic promise in selectively targeting ERs. Therapies aimed at preventing ERα transcriptional activation are currently used for breast cancer treatment and osteoporosis prevention [2]. Though ERβ is not currently a therapeutic target, accumulating evidence suggests an anti-proliferative role for ERβ in breast cancer [3]. In the mammary gland, ERα and ERβ play opposing roles in regulating growth and differentiation in response to estrogens; ERα promotes proliferation while ERβ inhibits ERα-mediated proliferation [4–6]. Because the anti-proliferative action of ERβ may be enhanced by ligand-dependent activation, the paradigm of ER targeted therapies is expanding towards the development of ER subtype selective ligands [7].

Though ERα and ERβ share many structural and transcriptional features, ligands can display subtype selectivity. In classical ligand dependent transcriptional activation, the receptors dimerize upon ligand binding and undergo conformational changes to allow cofactor recruitment. The receptors directly bind DNA most often at estrogen response elements (EREs), consisting of a consensus GGTCAnnnTGACC sequence. ERα and ERβ have 97% identity within the DNA binding domains, and the receptors bind similar DNA sequences with high affinity. Genome wide binding studies in MCF7 breast cancer cells expressing ERα or ERβ independently have shown that ERα and ERβ bind similar sites in response to 17β-estradiol (E2); ~60% of ER binding sites contain full EREs and ~25% contain half EREs [8].

The ligand binding pockets of ERα and ERβ are relatively large, and the receptors bind a wide array of chemicals. The ligand binding domains of ERα and ERβ have 59% identity, and the receptors bind E2 with similar affinities. Despite similarities in their ligand binding domains, several ligands have modest selectivity for ERα or ERβ [9], and some synthetic ligands maintain high selectivity. For example, propyl pyrazole triol (PPT) is an ERα selective agonist that displays a 400-fold higher binding affinity for ERα compared to ERβ [10]. Estrogenic chemicals produced in plants, known as phytoestrogens, often display subtype selectivity for ERβ. For example, liquiritigenin is a flavanone derived from *Glycyrrhizae uralensis* that has been shown to have 20-fold higher binding affinity for ERβ and even greater selectivity in transcriptional assays [11]. Compounds such as liquiritigenin often show low binding affinities relative to E2, and ERβ selective ligands with higher affinity and greater selectivity are needed to fully elucidate the anti-proliferative role of ERβ in breast cancer.

Mammalian cell lines have been developed to enable screening for subtype selective ligands. HeLa cervical carcinoma cells have been used to create HELN-ERα and HELN-ERβ, two cell lines in which ERα or ERβ, respectively, are constitutively expressed with stable integration of a luciferase reporter downstream of an ERE [12]. Human embryonic kidney cells, HEK293, have also been created using a similar strategy in which ERα or ERβ are constitutively expressed and human placental alkaline phosphatase downstream of the vitellogenin ERE is stably integrated [13]. The only available breast cancer reporter cell line is T47D-KBLuc in which three tandem EREs upstream of a luciferase reporter have been stably integrated [14]. However, identification of subtype selective ligands is prohibited because T47D cells express both ERα and ERβ.

Here, we describe the generation of two isogenic reporter cell lines, Hs578T-ERαLuc and Hs578T-ERβLuc, that provide a tool to characterize the transcriptional potencies and subtype selectivity of estrogenic compounds in the context of breast cancer cells. These cell lines are highly sensitive to estrogenic ligands and subtype selective ligands and can be used to validate ER transcriptional activation by analysis of endpoints such as endogenous target gene regulation. Further, ERβ selective ligands are shown to induce ERβ-mediated reporter gene expression, endogenous gene regulation, and growth inhibition, suggesting that

Hs578T-ERβLuc cells may be used to isolate ERβ selective ligands with desired biological effects.

## 2. Materials and Methods

### 2.1 Cell lines and reagents

Cosmosiin (apigenin 7-glucoside), dimethyl sulfoxide (DMSO), E2, and diethylstilbestrol (DES) were obtained from Sigma (St. Louis, MO); DPN, PPT, and ICI 182,780 were obtained from Tocris (Ellinsville, MO); liquiritigenin was obtained from Chromadex (Irvine, CA). Doxycycline (Dox) was obtained from Clontech. Hygromycin B, blasticidin S, zeocin, NaCl, sodium dodecyl sulfate (SDS), and dithiothreitol (DTT) were obtained from Research Products International (Mount Prospect, IL). Triton X-100 was obtained from Fisher (Fair Lawn, NJ); protease inhibitors were obtained from Roche Scientific (Basel, Switzerland); benzonase was obtained from Novagen (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell culture media were obtained from Invitrogen (Carlsbad, CA). MCF7 and HEK293 cells were cultured in DMEM + 10% fetal bovine serum (FBS; Gemini Bio Products, West Sacramento, CA) at 37 °C and 5% $CO_2$. Hs578T-ERα and Hs578T-ERβ were previously created by Secreto and coworkers [15]. These cells were cultured at 37 °C and 5% $CO_2$ in DMEM/F12 supplemented with L-glutamine, 10% Tet-system approved FBS (Clontech Mountain View, CA), 500 mg/L Zeocin and 5 mg/L Blasticidin S.

### 2.2 Generation of Hs578T-ERαLuc and Hs578T-ERβLuc reporter cell lines

Stable reporter cell lines were created using a modified pGL4.32 reporter (Promega, Madison, WI) which contains the *luc2P* reporter and hygromycin resistance. The pGL4.32 vector was digested with *Nhe*1 and *Hind*III (New England Biolabs, Ipswich, MA) and three consensus EREs spaced by three nucleotides were cloned upstream of *luc2P* using the following oligonucleotides: 5′ –CTA GCG GTC ACA GTG ACC TGC GAG GTC ACA GTG ACC TGC GAG GTC ACA GTG ACC TGC GA – 3′ and 5′ – AGC TTC GCA GGT CAC TGT GAC CTC GCA GGT CAC TGT GAC CTC GCA GGT CAC TGT GAC CG – 3′. Successful cloning was verified by complete sequencing and the vector was designated pGL4.3xERE. Estrogen responsiveness was validated by batch transfecting HEK293 cells with 2 ng of CMX-ERα or CMX-ERβ, 45 ng pGL4.3xERE vector, and 40 ng CMX-β-galactosidase per well of a 48 well plate. Cells were incubated 24 hr to allow protein expression before the addition of the indicated ligands. After 24 hr of ligand treatment, cells were lysed, firefly luciferase substrate (Promega) was added, and luminescence was measured on a Victor X5 microplate reader (Perkin Elmer, Waltham, Massachusetts) using luminescence detection and a 700 nm filter. To normalize data for transfection efficiency, β-galactosidase expression was analyzed using the Tropix β-galactosidase detection kit (Applied Biosystems, Foster City, CA). Luciferase counts were normalized to β-gal counts in each well.

After characterizing the pGL4.3xERE stable reporter vector, Hs578T-ERα and Hs578T-ERβ cells were transfected with 10 μg of the vector and selected in 125 μg/mL hygromycin B for 4 weeks. Individual colonies were selected using 3 mm cloning discs, expanded, and screened for estrogen induced luciferase expression. One clone from each cell line was selected for further characterization, referred to here as Hs578T-ERαLuc and Hs578T-ERβLuc.

### 2.3 Quantitative western blots and ligand binding assays

For quantitative western blots, cells were split in phenol red free DMEM/F12 + 5% SFS and treated with 50 ng/mL Dox or vehicle (water) 24 hr later. After 48 hr treatment, cells were collected by trypsinization, washed with Dulbecco's phosphate buffer saline (Invitrogen), and lysed by suspension in lysis buffer (50 mM Tris pH 8.0, 400 mM NaCl, 10% glycerol, 0.5% triton X-100, protease inhibitors, and benzonase). After centrifugation, total protein was quantified using BioRad Protein Assay (BioRad), and 40 ug of protein was resolved using SDS-PAGE and 8% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane for 1.5 hr at 0.35 A. Membranes were blocked with 5% nonfat milk and incubated overnight with 1:1000 anti-FLAG-M2 antibody (Sigma) or 1:5000 anti-β-Actin (Sigma) at 4°C. Membranes were then incubated with IRDye 800CW goat-anti-mouse IgG secondary antibody (Licor Biosciences, Lincoln, NE) for 1 hr at room temperature and visualized on a Licor Odyssey near-infrared gel reader (Licor Biosciences).

For ligand binding assays, Hs578T-ERαLuc and Hs578T-ERβLuc cells were cultured in phenol red free DMEM/F12 + 10% 6x charcoal stripped FBS (SFS) for 3 days prior to the assay to remove residual estrogens from the cells. At 90% confluence, cells were collected, resuspended in phenol red free DMEM/F12 + 5% SFS, and plated at a density of $10^5$ cells/well on a 24 well plate in the presence or absence of 50 ng/mL Dox. After 24 hr, cells were labeled in triplicate with 20 nM [$^3$H]-E2 (89.2 Ci/mmol specific activity, Perkin Elmer) in the presence or absence of 450 μM DES cold competitor for 2 hr at 37 °C and 5% $CO_2$. Labeled cells were washed 3 times with cold PBS + 0.1% BSA and lysed with 500 μL SDS lysis buffer (0.5% SDS, 0.05 M Tris-HCl pH 8.0, and 1 mM DTT). Total cell lysate (400 μL) was mixed with 5 mL liquid scintillation cocktail and [$^3$H] bound radioactivity was liquid scintillation counted for 5 min. Two additional wells of each condition were used to count the cell number and determine the total protein using RC DC protein assay (BioRad, Hercules, CA).

### 2.4 Luciferase assays

Hs578T-ERαLuc and Hs578T-ERβLuc cells were cultured in phenol red free DMEM/F12 + 10% SFS for 3 days prior to the assay to remove residual estrogens from the cells. Cells were seeded in triplicate at a density of $10^4$ cell/well on white 96 well tissue culture plates (Fisher) in phenol red free DMEM/F12 + 5% SFS treated with 50 ng/mL Dox. After 24 hr of Dox treatment, media were replaced with treated media containing vehicle (0.15% DMSO) or a range of serially diluted ligands. All treatments were conducted in the presence and absence of 100 nM ICI 182,780. After treatment for 24 hr, cells were washed with PBS and lysed with 35 μL lysis buffer (100 mM $K_2HPO_4$, 0.2% triton X-100, pH 7.8). Lysate (30 μL) was mixed 1:1 with luciferase substrate (Promega) and luminescence was measured on a Victor X5 microplate reader (Perkin Elmer, Waltham, Massachusetts) using luminescence detection and a 700 nm filter. Total protein (5 μL) was quantified using BioRad Protein Assay (BioRad). $EC_{50}$ values were calculated using GraphPad Prism Software (Version 5.04, GraphPad Software Inc., San Diego, CA) and a three parameter log versus response nonlinear regression. Two tailed t-tests performed with GraphPad Prism Software were used to determine statistically significant differences from control treatments.

### 2.5 Gene expression analysis

For analysis of reporter induction by cosmosiin, Hs578T-ERαLuc and Hs578T-ERβLuc cells were split in phenol red free DMEM/F12 + 5% SFS and treated with 50 ng/mL Dox for 48 hr followed by treatment with DMSO (0.1%), 1 nM E2, or 1 μM cosmosiin for 4 or 24 hr. Total RNA was extracted using RNEasy Plus Kit according to manufacturer protocol (Qiagen, Valencia, CA). RNA (2 μg) was reverse transcribed using Superscript II RT according to manufacturer protocol (Invitrogen), and firefly luciferase (FLuc) expression

was determined by reverse-transcription polymerase chain reaction using primers shown in Table 1.

For quantitative real-time PCR analysis of endogenous target gene expression, Hs578T-ERα and Hs578T-ERβ cells were cultured in phenol red free DMEM/F12 + 10% SFS for 3 days prior to the assay to remove residual estrogens from the cells. Cells were split in phenol red free DMEM/F12 + 5% SFS and treated with 50 ng/mL Dox for 48 hr prior to ligand treatment. Cells were treated with Dox and ligands or vehicle (0.1% DMSO) for 24 hr, and total RNA was extracted using RNEasy Plus Kit according to manufacturer protocol (Qiagen). RNA (2 μg) was reverse transcribed as above, and quantitative PCR was performed using TaqMan Prime Time custom designed assays (IDT, Coralville, IA), FastStart Universal Probe Master Mix (Roche Scientific), and a CFX96 instrument (BioRad). Primer and probe sequences are shown in Table 1. Data were analyzed using the ΔΔCq method calculated by the CFX Manager Software (BioRad). Two tailed t-tests performed with GraphPad Prism Software were used to determine statistically significant differences from control treatments using data from three biological replicates.

### 2.6 Cell counting assays

Hs578T-ERα and Hs578T-ERβ cells were cultured in phenol red free DMEM/F12 + 10% SFS for 3 days prior to the assay to remove residual estrogens from the cells. Cells were seeded at a density of 15,000 cell/well in phenol red free DMEM/F12 + 5% SFS in triplicate in 6 well tissue culture dishes in the presence or absence of 50 ng/mL Dox. After 24 hr, the cells were treated with DMSO (0.1%) or compound in the presence or absence of 50 ng/mL Dox. Media were refreshed every 48 hr, and cells were counted after trypan blue exclusion using an automated cell counter (BioRad) according to manufacturer protocol.

## 3. Results

### 3.1 Generation of Hs578T-ERαLuc and Hs578T-ERβLuc reporter cell lines

In order to generate stable reporter breast cancer cell lines, we first cloned a construct encoding a selection marker and a luciferase reporter linked to EREs. The pGL4.32 vector (Promega) contains the *luc2P* gene and was modified to contain 3 tandem consensus EREs upstream of the minimal promoter (pGL4.3xERE, Fig. 1A). Upon complete sequencing, the estrogen responsiveness of the vector was validated in ER-negative HEK293 cells transfected with full length ERα (Fig. 1B) or ERβ (Fig. 1C). The pGL4.3xERE reporter showed extremely low background with a 65-fold induction in cells transfected with ERα. The ER antagonist ICI 182,780 abolished estrogen induced expression, reducing the luciferase signal to that of vehicle treated cells. Cells transfected with ERβ showed a 15-fold induction of luciferase upon E2 treatment; ICI 182,780 inhibited luciferase expression in both vehicle and estrogen treated cells. The minimal background luciferase expression and the selection marker conferred by the pGL4.3xERE vector made the vector suitable for creating stable reporter cells lines for the identification and characterization of ER selective agonists.

In order to create stable ER reporter breast cancer cell lines, an ER negative breast cancer cell line engineered to express either ERα or ERβ was necessary. Previously, Secreto and coworkers created such lines using Hs578T cells [15], a triple negative breast cancer cell line with a basal-like gene expression profile [16]. Hs578T cells lack expression of ERα and ERβ providing a clean background in which to express ERα or ERβ. Using the tetracycline inducible system, two cell lines were created in which ERα or ERβ are inducibly expressed (Hs578T-ERα and Hs578T-ERβ cells, respectively) [15]. Hs578T-ERα and Hs578T-ERβ cells were transfected with the pGL4.3xERE vector, and individual clones were isolated

after hygromycin selection. Over 20 clones were screened for estrogen induced luciferase expression (data not shown). One clone from each cell line was selected for further characterization, referred to here as Hs578T-ERαLuc and Hs578T-ERβLuc. Additional ERα and ERβ reporter clones were used to verify reporter data obtained from Hs578T-ERαLuc and Hs578T-ERβLuc cells.

Hs578T-ERαLuc and Hs578T-ERβLuc cells were first characterized by assessing luciferase induction by ER ligands in the presence or absence of the full antagonist ICI 182,780 (Figure 2). Cells were treated with vehicle, 1 nM E2, 10 nM DPN (a reported ERβ selective agonist), or 10 nM PPT (a reported ERα selective agonist). PPT selectively activated luciferase expression in Hs578T-ERαLuc, but DPN activated the reporter in both Hs578T-ERαLuc and Hs578T-ERβLuc cells, though to a lesser extent in Hs578T-ERαLuc cells. Co-treatment with ICI 182,780 blocked luciferase induction in both cell lines (Fig. 2), and luciferase was not induced in the absence of Dox treatment (data not shown).

Basal and E2-induced luciferase signals were much higher in Hs578T-ERαLuc cells when compared to Hs578T-ERβLuc cells, a trend observed in all luciferase assays. On average, Hs578T-ERβLuc cells expressed 630 luciferase units per mg protein and Hs578T-ERαLuc expressed 2900 luciferase units per mg protein at saturating E2 concentrations (0.1 nM or greater). A range of luciferase signals was observed among the clones screened (data not shown), suggesting the accessibility of the reporter in the chromatin may be responsible for differences in luciferase expression. In order to verify Hs578T-ERαLuc and Hs578T-ERβLuc cells had similar ER expression levels at the Dox concentration used throughout the study (50 ng/mL), quantitative western blots were used to compare ER expression in the parent cell lines and reporter cell lines (Fig. 3A). Western blots with FLAG antibody demonstrated similar ER expression in Hs578T-ERαLuc and Hs578T-ERβLuc cells and also confirmed expression levels similar to the parent cell lines. In addition, whole cell ligand binding assays were used to quantify the active receptor in each cell line (Fig. 3B). ERα positive MCF7 breast cancer cells expressed ~150,000 receptors/cell which was very similar to reported values [17]. Both Hs578T-ERαLuc and Hs578T-ERβLuc cells expressed ~120,000 receptors/cell after 50 ng/mL Dox treatment. The comparable number of ERs per cell suggests that differences in ER expression do not account for the higher luciferase signal observed Hs578T-ERαLuc cells. Higher luciferase expression in Hs578T-ERαLuc cells may be due to the accessibility of the reporter in the chromatin or the enhanced transcriptional activity of ERα, in agreement with previous findings that the transcriptional activity of ERα is greater than that of ERβ on ERE-containing reporters [18]. Finally, the reporter cell lines did not have an altered morphological phenotype compared to the parent cell lines (Fig. 3C), and no other phenotypic changes due to the integration of the luciferase reporter were observed in Hs578T-ERαLuc and Hs578T-ERβLuc cells.

### 3.2 Ligand selectivity of Hs578T-ERαLuc and Hs578T-ERβLuc reporter cell lines

We next assessed ligand subtype selectivity using these isogenic reporter cell lines. All luciferase data were normalized to the luciferase signal induced by a saturating concentration of E2 (0.1 nM) and expressed as the percent transactivation relative to 0.1 nM E2. Dose-response curves were obtained for E2, DPN, and PPT to characterize the sensitivity of the reporter cells to ER ligands (Fig. 4). Cells were treated with 10-fold dilutions of ligands and approximate $EC_{50}$ concentrations for each ligand were calculated from 3 independent experiments (Table 2). The ratios of $EC_{50}$ values obtained from Hs578T-ERαLuc cells and Hs578T-ERβLuc cells are also presented in Table 2 and provide a measure of the selectivity of the ligands. Higher α/β ratios indicate selectivity for ERβ.

Both cell lines were highly sensitive to estrogen (Fig. 4A). Hs578T-ERαLuc cells showed $EC_{50}$ values near 1 pM; four additional Hs578T-ERαLuc clones showed similar sensitivities

(data not shown). Hs578T-ERβLuc cells also showed $EC_{50}$ values for estrogen in the pM range, though the average $EC_{50}$ was 6.5-fold higher than that of Hs578T-ERαLuc cells. Similar differences in estrogen sensitivities have been observed in other ERE-luciferase reporter cell lines expressing ERα or ERβ [12–14], suggesting the difference in E2 sensitivity between Hs578T-ERαLuc and Hs578T-ERβLuc cells is due to differences in the transactivation of ERα and ERβ.

Next, dose responses to two highly selective ERα and ERβ agonists, PPT and DPN respectively, were analyzed using Hs578T-ERαLuc and Hs578T-ERβLuc cells. PPT showed nearly 1000-fold selectivity for ERα (Fig. 4B). Surprisingly, PPT could activate reporter expression in Hs578T-ERβLuc cells at concentrations greater than 100 nM, although it could not induce luciferase expression to the same extent as E2. It has been reported that PPT was unable to induce an estrogen responsive reporter in HEC-1 cells transfected with ERβ [10] or in HELN-ERα cells [12]. DPN was not as selective as PPT and could maximally activate luciferase expression Hs578T-ERαLuc cells at 100 nM (Fig. 4C). DPN fully activated ERβ at 10 nM. Though DPN has been shown to have a 50 to 70-fold higher binding affinity for ERβ [12, 19], comparison of $EC_{50}$ values showed approximately 30-fold selectivity for ERβ in these reporter assays.

Next, the subtype selectivity of two natural phytoestrogens, liquiritigenin and cosmosiin, were analyzed using Hs578T-ERαLuc and Hs578T-ERβLuc cells (Fig. 5). Liquiritigenin is a phytoestrogen derived from *Glycyrrhizae uralensis* and the most active estrogenic component of MF101, an herbal supplement with therapeutic potential [11]. In the initial characterization of liquiritigenin, Mersereau and coworkers found liquiritigenin showed minimal activation of ERα at concentrations up to 2.5 μM in transcriptional assays in U2OS, HeLa, or WAR5 prostate cancer cells transfected with ERα [11]. Binding assays demonstrated that liquiritigenin had a 20-fold higher affinity for ERβ and selectivity was proposed to be due to selective recruitment of co-activators to ERβ, namely SRC-2 [11]. Comparison of $EC_{50}$ values showed liquiritigenin had a 3.6-fold selectivity for ERβ, and maximal reporter induction was obtained by 100 nM liquiritigenin in Hs578T-ERβLuc cells and 1 μM in Hs578T-ERαLuc (Fig. 5A, Table 2).

Cosmosiin, or apigenin 7-glucoside, is a flavone found in chamomile [20] that was identified as an ER agonist that selectively induces ERα/β and ERβ/β dimers as measured by bioluminescence resonance energy transfer (BRET) assays (unpublished data). It has a 3-fold higher binding affinity for ERβ as measured by competitive ligand binding assays ($IC_{50}$ ERα 15.9 μM, $IC_{50}$ ERβ 3.3 μM, unpublished data). Interestingly, cosmosiin induced luciferase expression to a much greater extent than E2, an effect described as supramaximal induction [21]. Even at concentrations up to 10 μM, cosmosiin did not saturate the luciferase output, and $EC_{50}$ values could not be reasonably calculated (Fig. 5B). Another Hs578T-ERβLuc clone treated with cosmosiin also showed supramaximal induction (data not shown). Cosmosiin did not induce luciferase expression in Dox-treated cells co-treated with ICI 182,780 or cells not treated with Dox (data not shown), indicating the supramaximal induction was due to ERβ activation. To determine if the supramaximal induction truly represented enhanced transcriptional activation, the transcript levels of luciferase were assessed after 4 and 24 hr treatments of E2 and cosmosiin (Fig. 5C). Cosmosiin did not induce luciferase expression to a greater extent than E2 in either Hs578T-ERαLuc or Hs578T-ERβLuc cells, indicating alternative mechanisms are responsible for the supramaximal effect.

### 3.3 Selective regulation of ERα and ERβ target genes by ERβ selective ligands

We next sought to validate the subtype selectivity of DPN, PPT, liquiritigenin and cosmosiin by assessing regulation of endogenous ER target genes. Estrogen responsive target genes of

ERα and ERβ were previously identified in Hs578T-ERα and Hs578T-ERβ cells [15], and two ERβ target genes and one ERα target gene were selected for analysis. Cells were treated with 50 ng/mL Dox for 48 hr to induce expression of the receptors and further treated with the corresponding ligands for 24 hr. Complement component 3 (*C3*, NM_000064) was up-regulated in Hs578T-ERβ cells upon E2 treatment (Fig. 6A). DPN and liquiritigenin were capable of inducing *C3* expression to a comparable level as E2 at concentrations that fully activate ERβ with minimal ERα activation, as measured by reporter assays (Fig. 6A). Cosmosiin induced *C3* expression at 1 μM, but not to the same extent as E2, demonstrating cosmosiin does not fully activate the receptor at this concentration. PPT slightly induced *C3* expression compared to DMSO in Hs578T-ERβ cells, although PPT induced expression of *C3* to a much lesser degree compared to E2. Repression of the ERβ target gene Jagged 1 (*JAG1*, NM_000214) occurred to a similar degree by E2, DPN, liquiritigenin and cosmosiin, although 100 nM liquiritigenin and 1 μM cosmosiin do not fully repress *JAG1* expression compared to E2, DPN or 1 μM liquiritigenin (Fig. 6B). Although the ERα selective agonist PPT slightly induced *C3* expression in Hs578T-ERβ cells, it had no effect on *JAG1* repression, demonstrating incomplete ERβ activation by PPT. To further validate the subtype selectivity observed in reporter assays, expression of the ERα target gene alpha-6 integrin (*ITGA6*, NM_000210) was determined after treatment of Hs578T-ERα cells with E2, DPN, PPT, liquiritigenin and cosmosiin. As shown in Figure 6C, *ITGA6* was up-regulated by E2 and PPT treatment, but DPN and liquiritigenin did not fully activate its expression at concentrations that showed selectivity in reporter assays (10 nM and 100 nM, respectively). At 1 μM, liquiritigenin and cosmosiin were capable of activating ERα, and *ITGA6* expression was induced in Hs578T-ERα cells.

Therefore, the subtype selectivity of DPN and liquiritigenin observed in reporter cell lines was validated by subtype selective regulation of endogenous target genes. Cosmosiin, however, activated expression of an Hs578T-ERα endogenous gene target at concentrations that only slightly activated luciferase reporter expression in Hs578T-ERαLuc cells.

### 3.4 Growth inhibition of Hs578T-ERβ cells by liquiritigenin and cosmosiin

We next characterized the growth effects of liquiritigenin and cosmosiin in Hs578T-ERα and Hs578T-ERβ cells. It was previously shown that E2 inhibits the growth of Hs578T-ERβ cells [15], supporting the notion that the anti-proliferative action of ERβ may be activated by estrogenic ligands. We tested whether 100 nM liquiritigenin, a concentration at which ERβ was selectively activated, and 1 μM cosmosiin could also inhibit the growth of Hs578T-ERβ cells. Hs578T-ERα and Hs578T-ERβ cells were treated with vehicle (DMSO), 1 nM E2, 100 nM liquiritigenin or 1 μM cosmosiin in the presence or absence of 50 ng/mL Dox (with or without ER, respectively) for a total of 5 days. When ERα and ERβ were not expressed (-Dox), the compounds had no effect on the growth of the cells (Fig. 7A, B). In contrast, E2, liquiritigenin, and cosmosiin inhibited the growth of Hs578T-ERβ cells when ERβ was expressed (+ Dox, Fig. 7D), and there was an approximately 50% reduction in the number of cells after 5 days of treatment with all three compounds (Fig. 7F). Hs578T-ERα cells showed slight inhibition with E2 and liquiritigenin treatment when ERα was expressed (Fig. 7C), but there was not a statistically significant effect after 5 days of treatment as measured by 2 independent experiments (Fig. 7E). However, ERα expression in ER negative cells often leads to growth inhibition [22, 23], and it is likely that activation of ERα inhibits the growth of Hs578T-ERα cells. This suggests that 100 nM liquiritigenin partially activates ERα despite minimal regulation of *ITGA6* at this concentration.

## 4. Discussion

ERα is an established therapeutic target for breast cancer treatment, but the development of subtype selective estrogenic ligands has gained interest with the identification of ERβ [1].

ERβ opposes the actions of ERα suggesting that it may be a potential therapeutic target. Exogenous ERβ expression in ERα positive breast cancer cells impaired E2 stimulated proliferation [24] and tumor growth in xenografts [25]. In support of the anti-proliferative role of ERβ, MCF7 cells were more proliferative when ERβ was knocked down [6]. Activation of ERβ by subtype selective ligands may enhance ERβ growth repression without stimulating proliferation through ERα; indeed ERβ selective ligands inhibited growth of HC11 mouse mammary cells [5]. Here, we have also shown that ERβ ligands can inhibit the growth of breast cancer cells when ERβ is expressed. In breast cancer, however, ERβ expression is thought to decline during progression [26–28] so ligands aimed at targeting ERβ must be highly selective and used only in patients that lack ERα or those with low ERα:ERβ ratios of expression. The rate of ERβ positivity in breast cancer has been reported to range from 13% to 83% [29–32]. In order to effectively target ERβ for cancer treatment, there is an imminent need to: a) identify ERβ selective ligands with minimal side effects and better *in vivo* efficacy and selectivity, and b) design clinical trials to recruit patients with low ERα:ERβ ratios in earlier stages of disease progression.

Although ERβ selective ligands have not yet been used for cancer treatment, the therapeutic value of ERβ has been assessed in other diseases. Two of the most promising ERβ selective therapies are the ERβ selective ligand ERB-041 and the herbal extract MF-101 [33]. Clinical trials have been completed to determine the efficacy of ERB-041 for treatment of Crohn's disease, endometriosis, interstitial cystitis, and rheumatoid arthritis. Although results have not been published for most of the clinical trials, results of the rheumatoid arthritis trial showed ERB-041 was well tolerated but did not improve arthritis symptoms [34]. MF-101 also showed a relatively safe profile and reduced the frequency of hot flashes in a phase II clinical trial for treatment of post-menopausal symptoms [35]. Liquiritigenin is the most active estrogenic component of MF-101[11], suggesting ERβ selective ligands may prove useful for treating post-menopausal symptoms.

Strategies to identify ER subtype selective ligands include competitive ligand binding, dimerization, transcriptional reporter, and proliferation assays [21, 36]. Competitive ligand binding assays provide insight into binding affinities and are useful for high throughput small molecule screening [37], but they are limited because ligands can act as agonists or antagonists and binding affinity does not often reflect transcriptional potency. BRET assays to measure receptor dimerization have been used to identify subtype selective ligands [38], but also cannot differentiate between agonists or antagonists [39]. Agonists can be characterized using proliferation assays in MCF7 cells, which are highly sensitive and provide a biologically relevant endpoint in the context of estrogen-sensitive cells [40]. However, this assay is limited by a lack of specificity, as non-estrogenic mitogens can stimulate proliferation, and cannot be used to detect subtype selective agonists.

Transcriptional assays can differentiate between agonists and antagonists, overcoming limitations of binding and dimerization assays. Mammalian reporter cell lines useful for identifying subtype selective ligands have been created from HeLa cervical carcinoma cells [12] and HEK293 kidney cells [13]. HELN-ERα and HELN-ERβ were generated from HeLa cells in two steps: 1) stable integration of ERE-luciferase to generate HELN cells, 2) stable expression of ERα or ERβ to generate HELN-ERα and HELN-ERβ [12]. 293/hERα and 293/hERβ cells were generated by a similar strategy. Only one breast cancer reporter cell line, T47D-KBLuc, is available to characterize agonists in the context of breast cancer cells [14], but both ERα and ERβ are expressed, preventing identification of subtype selective ligands.

In this report, we described the development of a new set of breast cancer reporter cell lines to characterize subtype selective estrogenic ligands. Hs578T-ERαLuc and Hs578T-ERβLuc cells were highly sensitive to E2 with $EC_{50}$ values of 1 pM and 6.5 pM, respectively (Fig.

4A). Similar E2 sensitivity was observed in T47D-KBLuc cells, which showed an approximate $EC_{50}$ of 3 pM [14]. Hs578T-ERαLuc and Hs578T-ERβLuc cells were more sensitive to E2 than HELN-ER and 293/ER reporter cells, but all reporter cell lines showed greater E2 sensitivity in ERα expressing cells. HELN-ERα cells were approximately 3 times more sensitive to E2 than HELN-ERβ cells ($EC_{50}$ of 0.017 nM and 0.068 nM, respectively) [12] and 293/hERα cells were approximately 4 times more sensitive to E2 than cells expressing ERβ ($EC_{50}$ of 50 pM and 200 pM, respectively) [13]. Although Hs578T-ERαLuc and Hs578T-ERβLuc cells were not created using the same strategy as HELN-ER or 293/hER reporter cells and likely have unique genomic integration of the reporter, similar sensitivities observed in all reporter cell lines suggest that this does not inhibit comparison of subtype selectivity.

Reporter assays with two ER subtype selective ligands confirmed that Hs578T-ERαLuc and Hs578T-ERβLuc cells could be used to differentiate between ERα and ERβ selective ligands. The ERβ selective agonist DPN maintained 33-fold selectivity in Hs578T-ERLuc cells ($EC_{50}$ of 0.26 nM for ERβ and 8.5 nM for ERα, Table 2). Dose response assays with the ERα selective agonist PPT revealed the sensitivity of Hs578T-ERβLuc cells (Fig. 4B). Although PPT was unable to activate reporter expression in HEC-1 cells transfected with ERβ [12], PPT did activate reporter expression in Hs578T-ERβLuc cells at high concentrations, although not to the full extent induced by E2. PPT reporter activation was blocked by ICI 182,780 co-treatment (Fig. 2A) and did not occur in the absence of Dox treatment (data not shown), verifying reporter activation was mediated by ERβ. Despite activation of ERβ at high concentrations, PPT could not fully activate reporter expression in Hs578T-ERβLuc cells and maintained 1000-fold selectivity for ERα.

Subtype selectivity of two natural phytoestrogens, cosmosiin and liquiritigenin, was also assessed in Hs578T-ERαLuc and Hs578T-ERβLuc cells. Liquiritigenin maintained selectivity for ERβ but to a lesser extent than expected, as it has been shown to minimally activate ERα in other cell lines [11]. The discrepancy in the selectivity of liquiritigenin may be due to the enhanced sensitivity of Hs578T-ERαLuc cells, differences in cofactor expression in Hs578T cells, or purity of the compound (our studies utilized commercially available liquiritigenin and Mersereau and coworkers [11] used extract from *G. uralensis*). The selectivity of cosmosiin could not be assessed using luciferase assays due to supramaximal induction (Fig. 5B). Supramaximal activation of estrogen responsive reporters have been described in many systems [21]. Here, we showed that supramaximal induction by cosmosiin was not due to enhanced transcriptional activation of the reporter (Fig. 5C). Despite limitations of the reporter system, the subtype selectivity of cosmosiin could be characterized by assessing target gene regulation in Hs578T-ERα and Hs578T-ERβ cells. While DPN and liquiritigenin maintained similar extents of selectivity as measured by reporter assays, cosmosiin activated both ERα and ERβ as measured by endogenous gene regulation (Fig. 6). Cosmosiin and liquiritigenin induced similar growth inhibitory effects as E2 in Hs578T-ERβ cells, indicating the phytoestrogens could elicit ERβ activation to a similar extent as E2 (Fig. 7).

Hs578T-ERαLuc and Hs578T-ERβLuc cells have several advantages for identifying ERβ selective agonists in comparison to available mammalian reporter cell lines. First, the Hs578T reporter cell lines have inducible expression of ERα and ERβ, allowing determination of off-target reporter activation by assessing reporter expression in the absence of Dox. Second, Hs578T-ERαLuc and Hs578T-ERβLuc cells are highly sensitive to estrogenic ligands. Third, endogenous gene regulation can be used to validate subtype selectivity. Finally, growth inhibition assays using Hs578T-ERβ cells in the presence and absence of Dox can be used to determine the biological endpoint of ERβ activation and validate specificity of ligands to ensure they do not have off-target cytotoxic effects. High

throughput screening may be possible using Hs578T-ERαLuc and Hs578T-ERβLuc cells, and luciferase assay optimization using Hs578T-ERβLuc cells has shown a Z factor of 0.5 (data not shown), an acceptable range for high throughput screening [41]. Therefore, Hs578T-ERαLuc and Hs578T-ERβLuc cells are useful for the identification and characterization of ER subtype selective ligands that may hold therapeutic promise.

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## Abbreviations

| | |
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| **BRET** | bioluminescence resonance energy transfer |
| **Cos** | cosmosiin |
| **Dox** | doxycycline |
| **DPN** | diarylpropionitrile |
| **E2** | 17β-estradiol |
| **ER** | estrogen receptor |
| **PPT** | propyl pyrazole triol |
| **ERE** | estrogen response element |
| **ICI** | ICI 182,780 |
| **Liq** | liquiritigenin |

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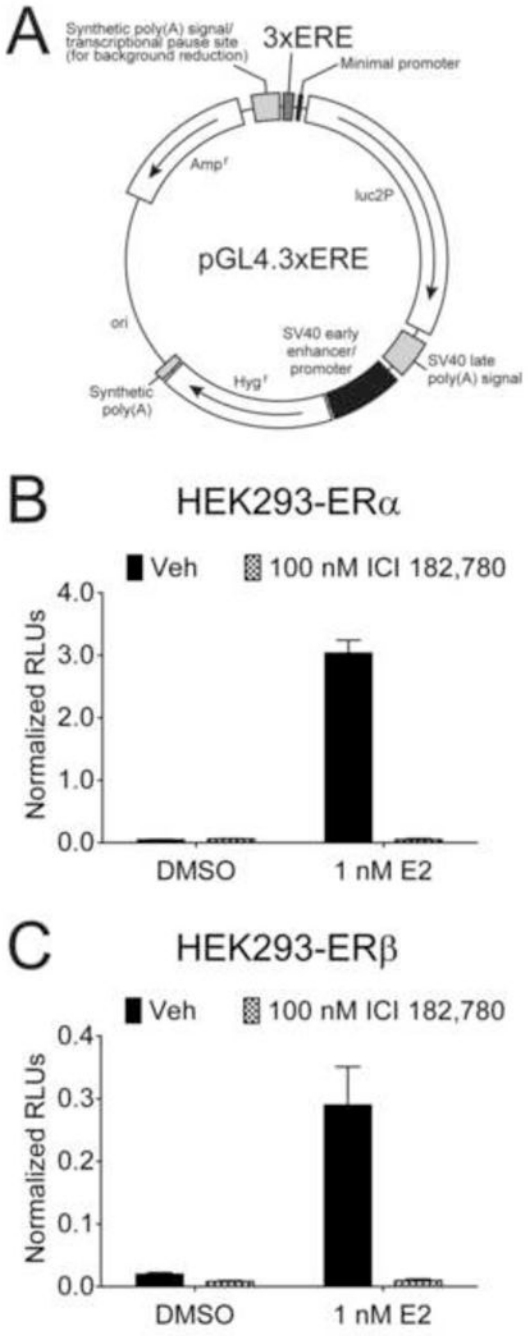


**Figure 1.**

The pGL4.3xERE reporter construct is estrogen responsive. (A) Three tandem EREs were inserted upstream of the *luc2P* gene in the pGL4.32 luciferase reporter construct. HEK293 cells were batch transfected with the pGL4.3xERE reporter construct, a β-galactosidase construct, and full length ERα (B) or ERβ (C). After allowing 24 hr for protein expression, cells were treated in triplicate with vehicle (DMSO) or 1 nM E2 and vehicle or 100 nM ICI 182,780 (0.15 % final DMSO concentration) for an additional 24 hr. Raw luciferase units (RLUs) were normalized to β-galactosidase to normalize for transfection efficiency. Error bars represent standard deviations.

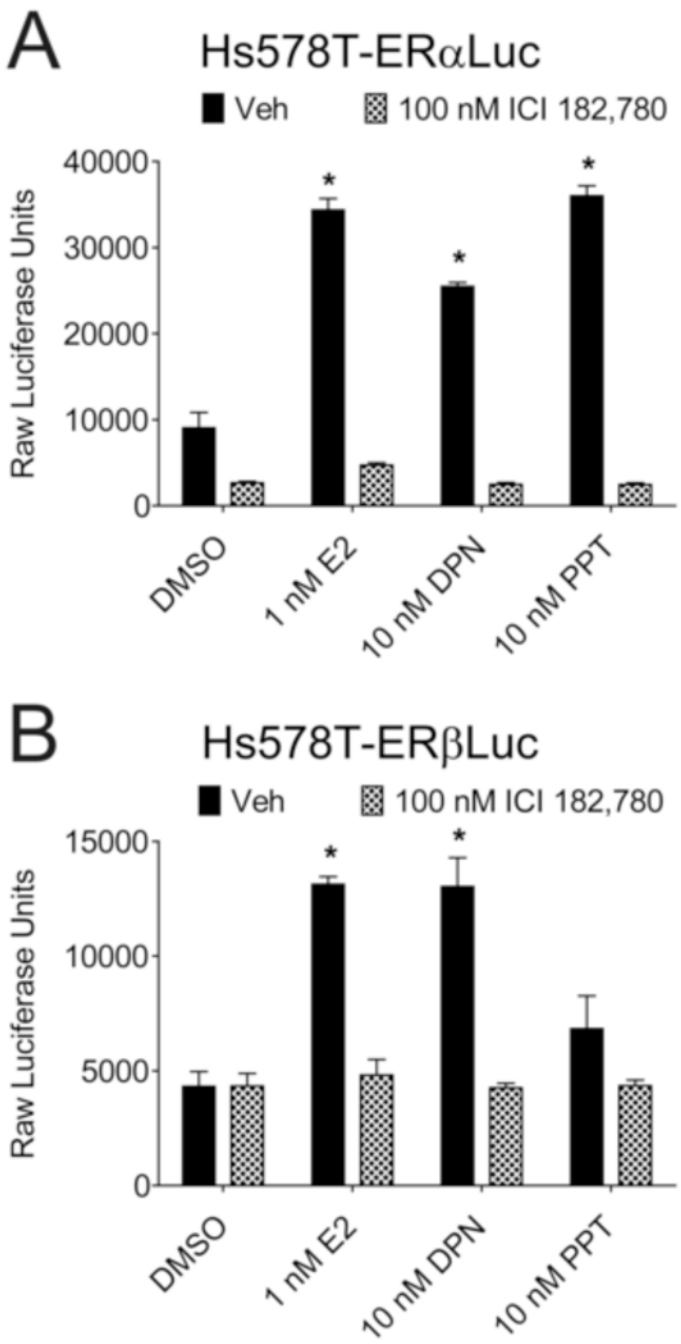


**Figure 2.**

ER subtype selective ligands selectively induce luciferase in Hs578T-ERαLuc and Hs578T-ERβLuc cells. Hs578T-ERαLuc (A) and Hs578T-ERβLuc (B) cells were seeded in triplicate on 96 well plates in the presence of 50 ng/mL Dox to induce ER expression. After 24 hr, cells were treated with vehicle (DMSO), 1 nM E2, 10 nM DPN, or 10 nM PPT in the presence or absence of 100 nM ICI 182,780 (0.15% final DMSO concentration). Cells were lysed 24 hr after ligand treatment and raw luciferase units were counted. Error bars represent standard deviations. * p values < 0.05

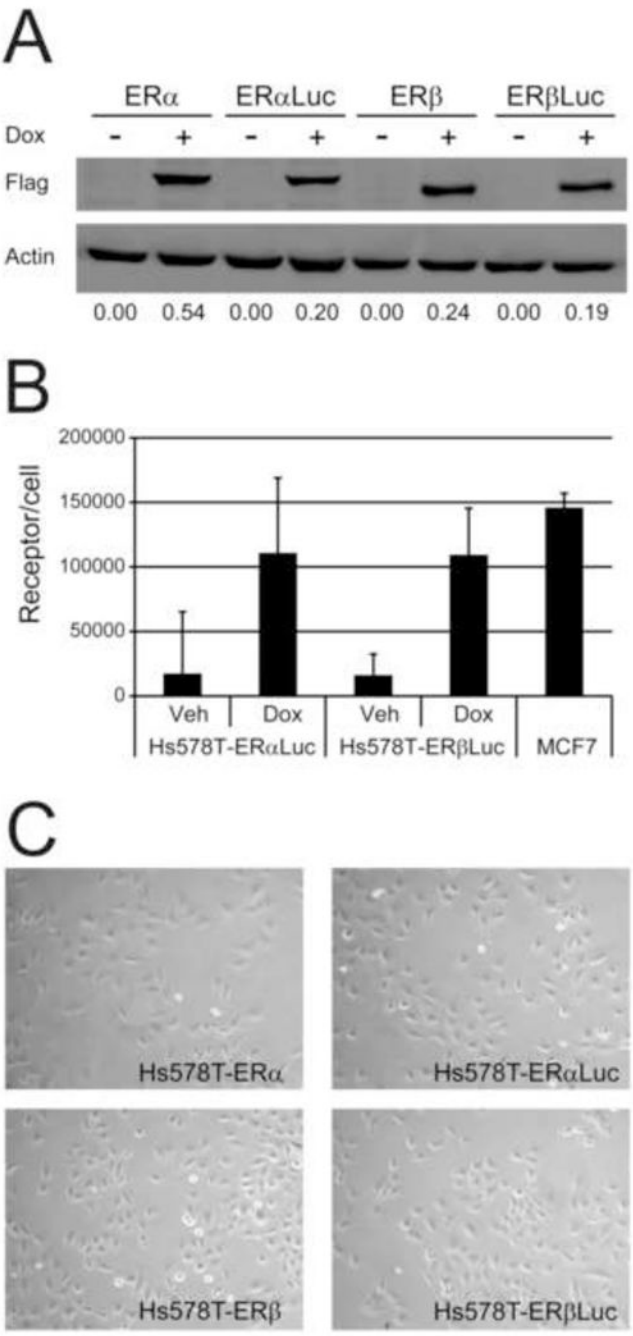


**Figure 3.**

Hs578T-ERαLuc and Hs578T-ERβLuc cells express similar levels of ER. (A) Quantitative western blot with Hs578T-ERα (ERα), Hs578T-ERαLuc (ERαLuc), Hs578T-ERβ (ERβ), and Hs578T-ERβLuc (ERβLuc) treated with vehicle (-Dox) or 50 ng/mL Dox (+Dox). ER expression was detected using FLAG antibody and quantified by normalizing to b-actin using the Licor Odyssey near-infrared gel reader. The normalized integrated intensity for the FLAG signal is shown below the images. (B) Ligand binding assays confirmed the quantitative western blots. Hs578T-ERαLuc and Hs578T-ERβLuc cells were seeded in triplicate and treated with vehicle or 50 ng/mL Dox for 24 hr. Cells were labeled with 20 nM [$^3$H]-E2 in the presence or absence of cold competitor for 2 hr, washed, and total cell lysate

was assessed for bound radioactivity as described in Materials and Methods. MCF7 cells were included for comparison. Two additional wells of each cell line and condition were used to determine the cell number and the numbers of receptors per cell were calculated based on a 1:1 molar ratio of ligand to receptor. The average and standard deviation of three independent experiments are shown. (C) The morphology of Hs578T-ERαLuc and Hs578T-ERβLuc was similar to that of the parent Hs578T-ERα and Hs578T-ERβ cell lines. Representative phase-contrast microscopy images of each cell line (100X magnification).

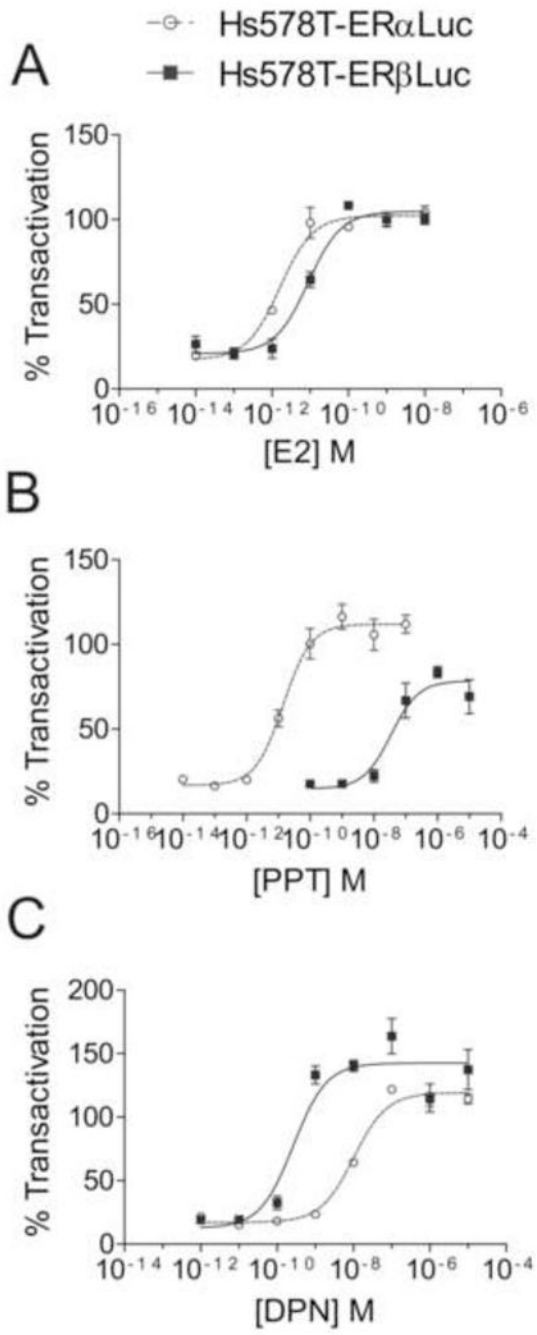


**Figure 4.**

Hs578T-ERαLuc and Hs578T-ERβLuc show subtype selective activation. Dose response curves of E2 (A), PPT (B), and DPN (C). Hs578T-ERαLuc and Hs578T-ERβLuc were seeded in triplicate and treated with 50 ng/mL Dox for 24 hr. Cells were then treated with a range of ligand concentrations (0.15% final DMSO concentration) for 24 hr. Each plate contained DMSO, 0.1 nM E2, and 100 nM ICI 182,780 for controls. Luciferase signal was normalized to total protein in each well and expressed as a percent transactivation relative to signal obtained from saturating E2 treatment (0.1 nM). Each dose response experiment was conducted at least 3 times; data shown are from one representative experiment. $EC_{50}$ values are shown in Table 2.

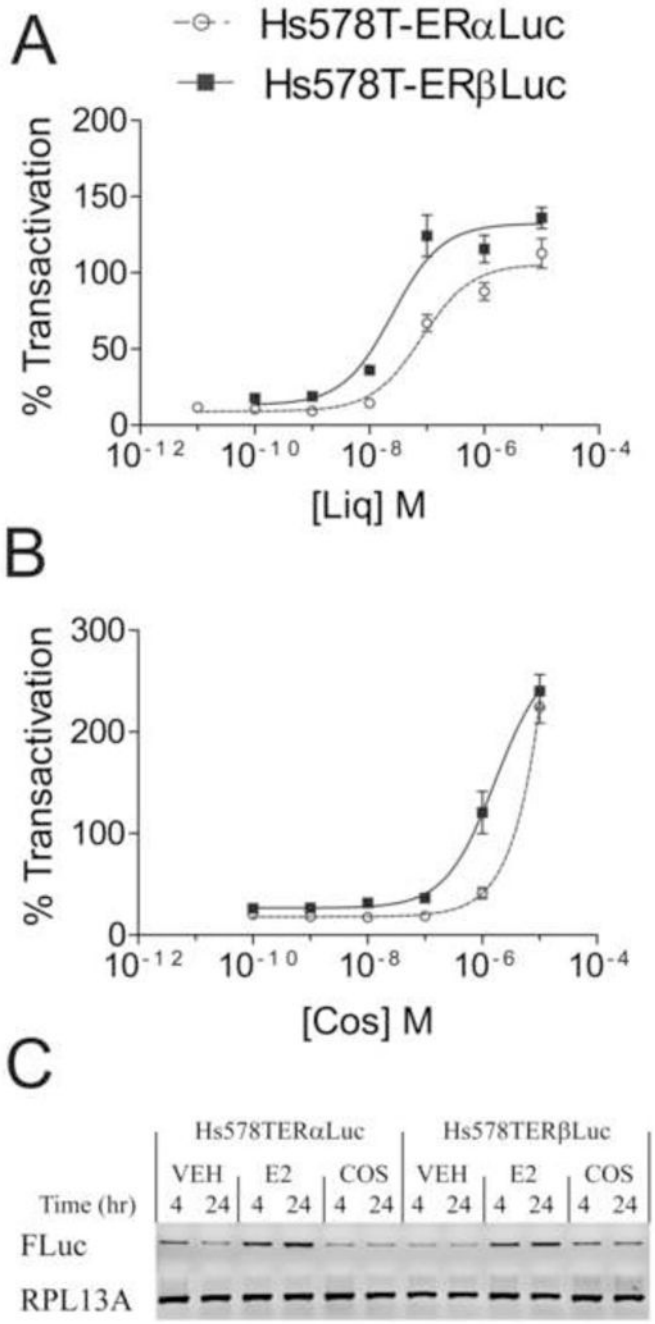


**Figure 5.**

Liquiritigenin (Liq) and cosmosiin (Cos) induce reporter expression in Hs578T-ERαLuc and Hs578T-ERβLuc. Dose response curves of liquiritigenin (A) and cosmosiin (B). Hs578T-ERαLuc and Hs578T-ERβLuc were seeded in triplicate and treated with 50 ng/mL Dox for 24 hr. Cells were then treated with a range of ligand concentrations (0.15% final DMSO concentration) for 24 hr. Each plate contained DMSO, 0.1 nM E2 and 100 nM ICI 182,780 for controls. Luciferase signal was normalized to total protein in each well and expressed as a percent transactivation relative to signal obtained from saturating E2 treatment (0.1 nM). Each dose response experiment was conducted at least 3 times; data shown are from one representative experiment. $EC_{50}$ values are shown in Table 2. $EC_{50}$ values for cosmosiin

could not be determined because of supramaximal reporter induction. The supramaximal induction by cosmosiin was not due to supramaximal transcription of the luciferase reporter (C). Hs578T-ERαLuc and Hs578T-ERβLuc cells were treated with 50 ng/mL Dox for 48 hr followed by treatment with DMSO (0.1%), 1 nM E2 or 1 μM cosmosiin for 4 or 24 hr. Firefly luciferase (FLuc) expression was determined by RT-PCR. RPL13A expression was used to ensure equal loading.

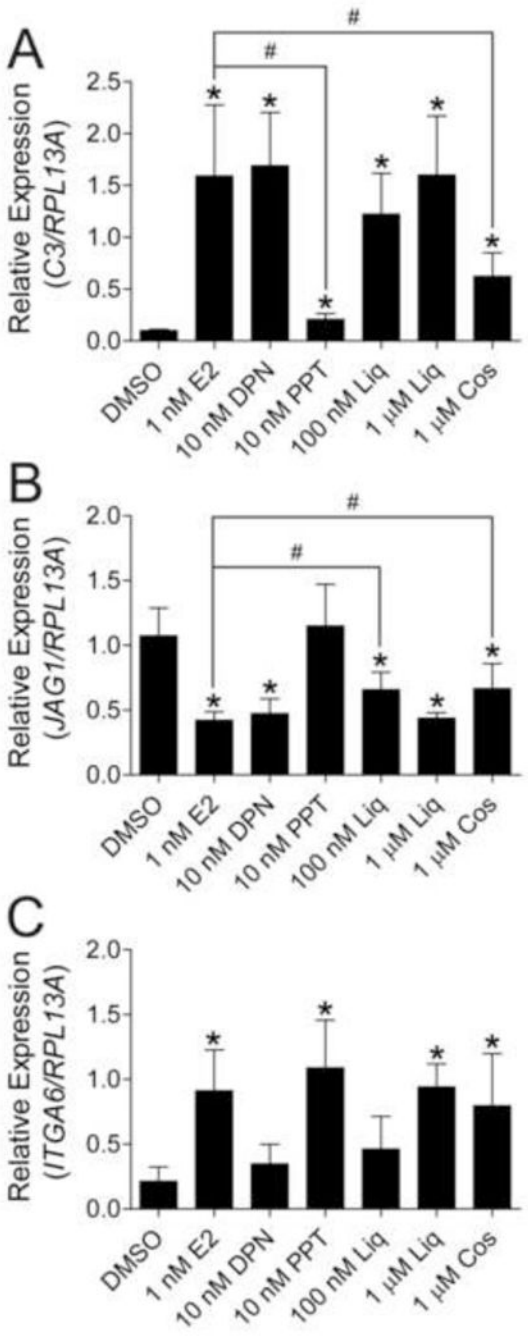


**Figure 6.**

ERβ selective ligands selectively regulate ER target genes. Hs578T-ERα and Hs578T-ERβ cells were treated with 50 ng/mL Dox for 48 hr to induce ER expression followed by treatment with the corresponding ligands for 24 hr. Total RNA was assayed for expression of the ERβ target genes *C3* and *JAG1* in Hs578T-ERβ cells (A, B respectively) and the ERα target gene *ITGA6* in Hs578T-ERα (C) cells by quantitative reverse-transcription polymerase chain reaction. Target gene expression was calculated using the ΔΔCq method by normalizing to the ribosomal protein *RPL13A*. Data represent the average and standard deviation of three biological replicates. * p values < 0.05 compared to DMSO control, # p values < 0.05 compared to E2 treatment

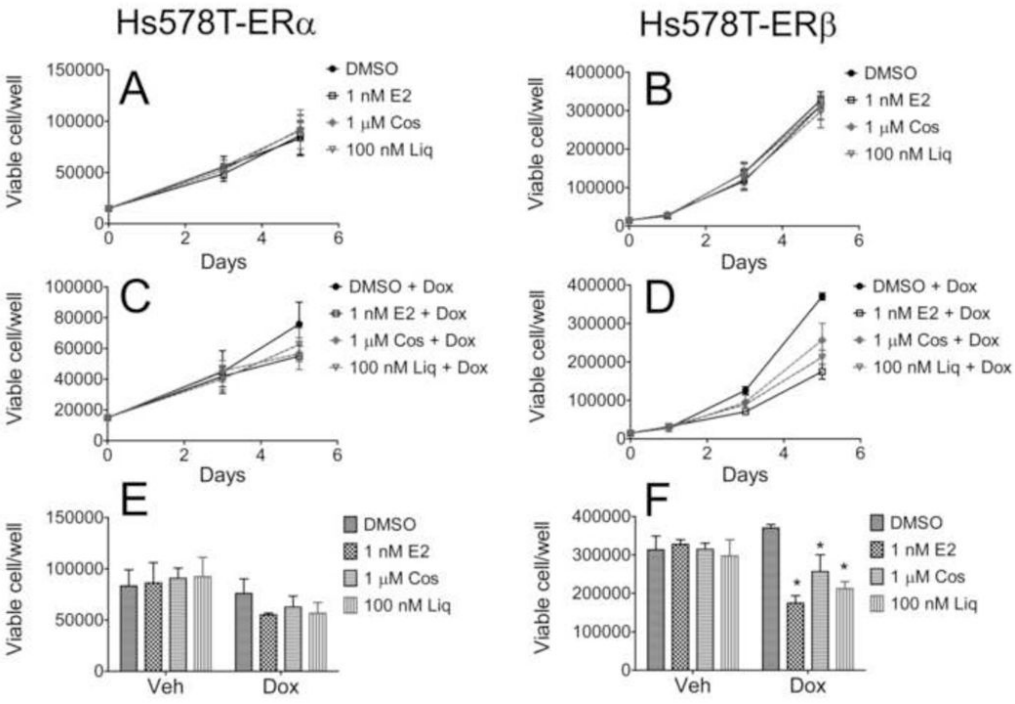


**Figure 7.**

Cosmosiin (Cos) and liquiritigenin (Liq) inhibit the growth of Hs578T-ERβ cells. Hs578T-ERα (A, C, E) and Hs578T-ERβ cells (B, D, F) were seeded in 6 well plates and treated with vehicle (A, B) or 50 ng/mL Dox (C, D). After 24 hr, the cells were treated with vehicle (0.1% DMSO) or the indicated ligands, and treatments were refreshed every 48 hr. Cells were counted at the times indicated using trypan blue exclusion. Comparisons of the cell number on day 5 are represented in panels E (Hs578T-ERα) and F (Hs578T-ERβ). Data represent two independent experiments. * p values < 0.05

**Table 1**

Primer and Probe Sequences

| | | |
|---|---|---|
| **RPL13A** | Primer 1 | 5′ - TGT TTG ACG GCA TCC CAC - 3′ |
| | Primer 2 | 5′ - CTG TCA CTG CCT GGT ACT TC - 3′ |
| | Probe | 5′ - CTT CAG ACG CAC GAC CTT GAG GG - 3′ |
| **C3** | Primer 1 | 5′ - AAC TAC ATC ACA GAG CTG CG - 3′ |
| | Primer 2 | 5′ - AAG TCC TCA ACG TTC CAC AG - 3′ |
| | Probe | 5′ - CGT TTC CCG AAG TGA GTT CCC AGA - 3′ |
| **JAG1** | Primer 1 | 5′ - GGA CTA TGA GGG CAA GAA CTG - 3′ |
| | Primer 2 | 5′ - AAA TAT ACC GCA CCC CTT CAG - 3′ |
| | Probe | 5′ - TCA CAC CTG AAA GAC CAC TGC CG - 3′ |
| **ITGA6** | Primer 1 | 5′ - ACC CGA GAA GGA AAT CAA GAC - 3′ |
| | Primer 2 | 5′ - CGC CAT CTT TTG TGG GAT TC - 3′ |
| | Probe | 5′ - TGG GTT GGA AGG GCT GTT TGT CA - 3′ |
| **FLuc** | Primer 1 | 5′ – GGC TGA ATA CAA ACC ATC GG – 3′ |
| | Primer 2 | 5′ – CTT TCT TGC TCA CGA ATA CGA – 3′ |

**Table 2**

Average $EC_{50}$ values for ER Ligands ($M \times 10^{-9}$)

| | Hs578T-ERαLuc | Hs578T-ERβLuc | α/β |
|---|---|---|---|
| **E2** | 0.001 (0.0005) | 0.0065 (0.008) | 0.15 |
| **DPN** | 8.5 (3) | 0.26 (0.02) | 33 |
| **PPT** | 0.016 (0.001) | 26 (21) | 0.001 |
| **Liquiritigenin** | 100 (40) | 28 (2) | 3.6 |

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# Differential Action of Monohydroxylated Polycyclic Aromatic Hydrocarbons with Estrogen Receptors α and β

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**Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of widespread environmental pollutants, some of which have been found to be estrogenic or antiestrogenic. Recent data have shown that hydroxylated PAH metabolites may be responsible for the estrogenic effects of some PAHs. The purpose of this study was to investigate the effects of several PAHs, as well as their monohydroxylated metabolites, on estrogen receptors (ERs), ERα and ERβ. Three parent PAHs and their monohydroxylated metabolites were each evaluated using transcriptional reporter assays in isogenic stable cell lines to measure receptor activation, competitive binding assays to determine ligand binding, and bioluminescence resonance energy transfer assays to assess dimerization. Finally, the estrogenic effects of the hydroxylated metabolites were confirmed by quantitative real-time PCR of estrogen-responsive target genes. Although the parent PAHs did not induce ERα or ERβ transcriptional activity, all of the monohydroxylated PAHs (1-OH naphthanol, 9-OH phenanthrene, 1-OH pyrene) selectively induced ERβ transcriptional activity at the concentrations tested, while not activating ERα. Additionally, the monohydroxylated PAHs were able to competively bind ERβ, induce ERβ homodimers, and regulate ERβ target genes. Although monohydroxylated PAHs appeared to have weak agonist activity to ERβ, our results showed that they can elicit a biologically active response from ERβ in human breast cancer cells and potentially interfere with ERβ signaling pathways.**

***Key Words:*** **polycyclic aromatic hydrocarbons; estrogen receptors; monohydroxylated metabolites; dimerization; transcription; ligand binding.**

Polycyclic aromatic hydrocarbons (PAHs) have been of increasing concern in the human health field due to their widespread dispersion in the environment and the adverse health effects associated with PAH exposure (Baird *et al*., 2005). Formed through the incomplete combustion of organic compounds, PAHs can be found in charbroiled foods, cigarette smoke, contaminated soil, vehicle exhaust, and in the atmosphere from the by-products of industrial processes. PAH exposure can have several adverse effects, including carcinogenesis and endocrine disruption.

Although PAHs are a diverse group of chemicals, most are metabolized by cytochrome P450s, a superfamily of enzymes that mediate the oxidation of lipophilic substrates (Anzenbacher, 2001; Bauer *et al*., 1995; Kim *et al*., 1998). The diol epoxide PAH metabolites are capable of inducing DNA damage (Baird *et al*., 2005), and many PAHs have been shown to be carcinogenic (Bauer *et al*., 1995; Kim *et al*., 1998). PAHs can also act as endocrine disrupting chemicals by interfering with normal estrogen signaling. Upon monohydroxylation, PAHs can induce estrogenic effects by directly interacting with estrogen receptors (ERs) (Arcaro *et al*., 1999; Fertuck *et al*., 2001a,b). These data suggest that the estrogenic effects of PAHs are primarily mediated by the monohydroxylated PAH metabolites.

ERs, members of the nuclear receptor superfamily of transcription factors, exist in two distinct isoforms, α and β. Encoded by separate genes on different chromosomes, ERα and ERβ have both overlapping and unique biological functions. The DNA-binding domains share 96% homology, and ERs bind similar estrogen response elements (EREs) to regulate transcription of target genes. The ligand-binding domains (LBDs), containing the hormone-dependent activation function (AF-2) (Tora *et al*., 1989), have 55% identity and have similar, but not identical, ligand-binding pockets (Pike *et al*., 1999). Upon ligand binding, the receptors dimerize and bind DNA to initiate transcription of target genes that mediate distinct biological effects. In the presence of estrogen, ERα is a known driver of cell proliferation, especially in breast cancer cells, whereas ERβ has been shown to inhibit ERα-mediated cell proliferation (Hartman *et al*., 2006; Paruthiyil *et al*., 2004; Treeck *et al*., 2010).

Given the critical roles ERs play in regulating cell growth in response to estrogens, there has been significant effort put forth to understand and predict the impacts of xenoestrogens on ER

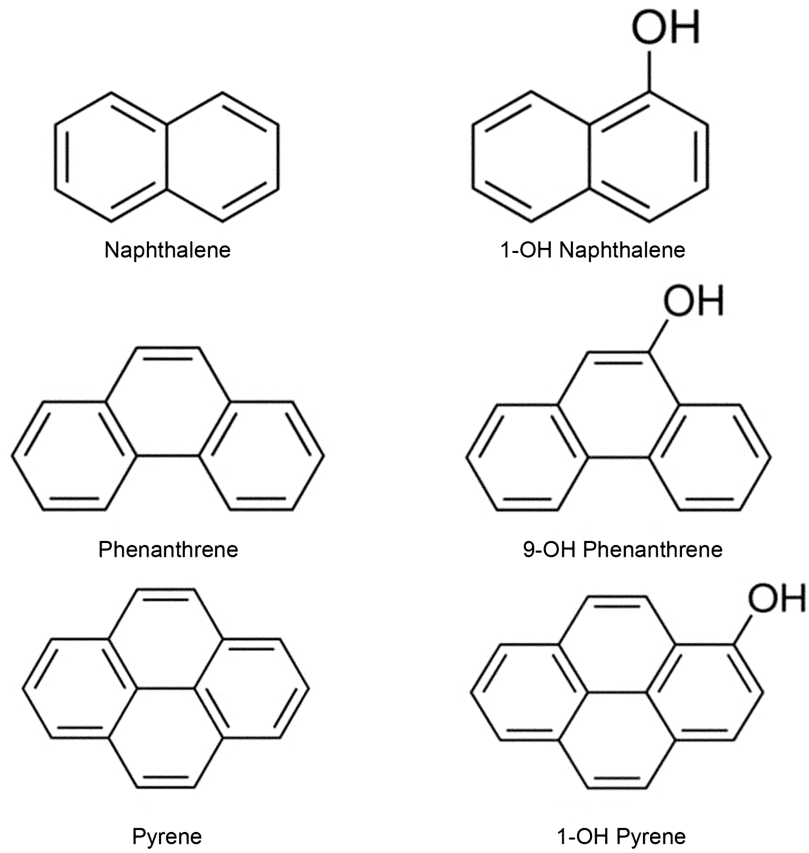



**FIG. 1.** Chemical structures of select polycyclic aromatic compounds and monohydroxylated metabolites studied.

singaling. However, most studies have been performed solely in the context of ERα, with a limited number of PAHs tested. Here we utilize several *in vitro* assays to assess the effects of three PAHs and their monohydroxylated metabolites, shown in Figure 1, on the transcriptional activation, ligand binding, and dimerization of both ERα and ERβ. Compounds were initially screened for transcriptional activation using a previously characterized pair of isogenic breast cancer cell lines with inducible expression of either ERα or ERβ and a stably integrated estrogen-responsive reporter (Shanle *et al*., 2011). These cell lines provide a sensitive tool to directly compare the transcriptional induction of ERα and ERβ. Next, bioluminescence resonance energy transfer (BRET) assays were performed to evaluate the dimerization status of ERs. BRET assays are able to monitor protein-protein interactions in a live, cell-based system (Powell and Xu, 2008; Tremblay *et al*., 1999). Fluorescence polarization experiments were utilized to generate competitive binding curves and determine half maximal inhibitory concentration ($IC_{50}$) values. This provided a simple, yet specific way to determine whether the tested compound can compete with estrogen for binding to ER. Finally, compounds were evaluated for their ability to upregulate ERβ target genes via quantitative real-time PCR (qPCR).

Naphthalene, phenanthrene, and pyrene were chosen as parent PAH compounds for study because they have been detected at high levels in contaminated environments (Arcaro *et al*., 1999), and they are considered by to be Priority Pollutants according to the U.S. Environmental Protection Agency. The hydroxylated metabolites were chosen due to their detection after metabolism of the parent compound (Cho *et al*., 2006; Rossbach *et al*., 2007). This is the first study to assess ER selective activity of these PAHs and their hydroxylated metabolites at the levels of transcriptional activity using isogenic reporter cell lines, ligand binding, and dimerization. The data demonstrate that monohydroxylated PAHs differentially interact with ERα and ERβ and exhibit stronger agonistic activity toward ERβ compared with ERα, suggesting that ERβ-mediated biological processes need to be evaluated to assess the outcomes of PAH exposure on humans.

## MATERIALS AND METHODS

***Chemicals.*** All PAH compounds were purchased from Sigma-Aldrich (St Louis, MO). Doxycycline (Dox) was obtained from Clontech (Mountain View, CA). ICI 182,780 was obtained from Tocris Bioscience (Ellisville, MO).

***Cell culture and reporter assays.*** Cell culture media were obtained from Invitrogen (Carlsbad, CA). HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Gibco Fetal Bovine Serum (FBS; Invitrogen) at 37°C and 5% $CO_2$. Hs578T-ERαLuc and Hs578T-ERβLuc cells were previously created by Shanle *et al*. (2011) and were

**TABLE 1**
**Primer and Probe Sequences**

| | | |
|---|---|---|
| **RPL13A** | Primer 1 | 5′-TGT TTG ACG GCA TCC CAC-3′ |
| | Primer 2 | 5′-CTG TCA CTG CCT GGT ACT TC-3′ |
| | Probe | 5′-CTT CAG ACG CAC GAC CTT GAG GG-3′ |
| **C3** | Primer 1 | 5′-AAC TAC ATC ACA GAG CTG CG-3′ |
| | Primer 2 | 5′-AAG TCC TCA ACG TTC CAC AG-3′ |
| | Probe | 5′-CGT TTC CCG AAG TGA GTT CCC AGA-3′ |
| **JAG1** | Primer 1 | 5′-GGA CTA TGA GGG CAA GAA CTG-3′ |
| | Primer 2 | 5′-AAA TAT ACC GCA CCC CTT CAG-3′ |
| | Probe | 5′-TCA CAC CTG AAA GAC CAC TGC CG-3′ |
| **NRIP1** | Primer 1 | 5′-AGA TTC CCT GTC CTC CTT CA-3′ |
| | Primer 2 | 5′-GGA AGT GTT TGG ATT GTG AGC-3′ |
| | Probe | 5′-TGT GCA TCT TCT GGC TGT GTT TCT CC-3′ |

maintained in DMEM/F12 supplemented with L-glutamine and 10% Tet-system approved FBS (Clontech) at 37°C and 5% $CO_2$.

Reporter assays were performed as previously reported (Shanle *et al*., 2011). Briefly, cells were seeded in triplicate at $10^4$ cells/well on white 96-well tissue culture plates in phenol red-free DMEM/F12 supplemented with 5% charcoal-stripped FBS treated with 50 ng/ml Dox. After 24 h, media were removed and replaced with media treated with 50 ng/ml Dox and vehicle (0.15% dimethyl sulfoxide [DMSO]) or PAH compounds diluted in DMSO. After 24 h of treatment, the cells were washed with 30 µl of 1× PBS and lysed with 35 µl lysis buffer (100mM $K_2HPO_4$, 0.2% Triton X-100, pH 7.8). Thirty microliters of lysate were mixed 1:1 with luciferase substrate (Promega, Madison, WI), and luminescence was measured with a 700-nm filter on a Victor X5 microplate reader (PerkinElmer, Waltham, MA). Total protein was measured using the Bradford Method (Bio-Rad), and raw luciferase data were normalized to total protein. Approximate $EC_{50}$ values were calculated using GraphPad Prism Software (Version 5.04; Graph-Pad Software Inc., San Diego, CA) and a three-parameter log versus response nonlinear regression.

***BRET assays.*** The BRET assays were performed similarly to those previously reported (Powell and Xu, 2008). Briefly, HEK293T cells were transfected with BRET fusion plasmids (pCMX-ERα-RLuc and pCMX-ERα-YFP or pCMX-RLuc-ERβ and pCMX-YFP-ERβ). Twenty-four hours after transfection, cells were trypsinized and resuspended in triplicate in PBS at approximately 50,000 cells per well in a white 96-well plate. Cells were then incubated with vehicle (0.6% DMSO), 10nM E2, or monohydroxylated PAH compound for 1 h at room temperature. Coelenterazine h (Promega) was added to PBS at a final concentration of 5µM. Emission measurements at 460 nm and 535 nm were immediately taken on a Victor X5 microplate reader (PerkinElmer). BRET ratios were calculated as previously described (Koterba and Rowan, 2006; Powell and Xu, 2008).

***Competitive binding assays.*** Competitive binding assays were performed using the PolarScreen ERβ Competitive Binding Assay Kit, Green (Invitrogen) according to the manufacturer's protocol. Recombinant human ERβ (20nM) and fluorescein-labeled estradiol were incubated for 4 h with the monohydroxylated PAH compounds. Fluorescence polarization was measured using a Victor X5 microplate reader (PerkinElmer). Approximate $IC_{50}$ values were determined by GraphPad Prism Software (Graph-Pad Software Inc.) from competitive binding curves.

***Western blot analysis.*** Western blots were performed similarly to those previously reported (Shanle *et al*., 2011) with cells treated for 48 h with vehicle (DMSO) or 10µM monohydroxylated PAH compound. Total protein was quantified using Bio-Rad Protein Assay (Bio-Rad), 35 µg of protein was resolved by SDS-PAGE, and membranes were incubated with 1:1000 anti-FLAG-M2 antibody (Sigma) overnight at 4°C. Membranes were then incubated with goat anti-rabbit HRP secondary antibody (Licor Biosciences, Lincoln, NE) for 1 h at room temperature and visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, Waltham, MA) on autoradiography film. Membranes were then washed and incubated with 1:5000 anti-β-Actin (Sigma) for 1 h at room temperature, then incubated with goat anti-mouse HRP secondary antibody (Licor Biosciences) for 1 h at room temperature and visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) on autoradiography film.

***qPCR analysis.*** Hs578T-ERβLuc cells were cultured in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped FBS for 3 days prior to experiment to remove any residual estrogens. Cells were seeded into 10-cm tissue culture plates in phenol red-free DMEM/F12 supplemented with 5% stripped serum and treated with 50 ng/ml of Dox 24 h prior to PAH treatment. Cells were then treated with 50 ng/ml Dox plus 0.1% DMSO control, 10nM E2, 10µM 1-OH-naphthalene, 5µM 9-OH phenanthrene, or 5µM 1-OH pyrene for 24 h. Total RNA was extracted using HP Total RNA Kit (VWR Scientific, West Chester, PA) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed using Superscript II RT according to the manufacturer's protocol (Invitrogen), and qPCR was performed using TaqMan Prime Time custom designed assays (IDT, Coralville, IA), FastStart Universal Probe Master Mix (Roche Scientific, Basel, Switzerland), and a CFX96 instrument (Bio-Rad). Primer and probe sequences are shown in Table 1.

***Statistical analyses.*** Two-tailed Student's *t*-tests were performed using GraphPad Prism version 5.04 for Windows, GraphPad Software (www.graphpad.com).

## RESULTS

### *Monohydroxylated PAHs Selectively Activate ERβ in Reporter Cell Lines*

In order to test the hypothesis that hydroxylated PAHs may have estrogenic activity with differential effects on ERα and ERβ, we first utilized Hs578T-ERαLuc and Hs578T-ERβLuc reporter cells (Shanle *et al*., 2011). These cell lines have inducible expression of ERα or ERβ, respectively, and a stably integrated luciferase reporter just downstream of three tandem EREs. Previous work has shown that these cell lines are highly sensitive to estrogenic ligands and can be used to distinguish ER subtype selective ligands (Shanle *et al*., 2011). In this system, cells are first treated with Dox to induce expression of the receptor, followed by treatment with the corresponding compounds. In our initial experiments comparing the activation of ERα and ERβ, we observed that only hydroxylated PAHs conferred estrogenic activity at 10µM (Fig. 2). The monohydroxylated PAH

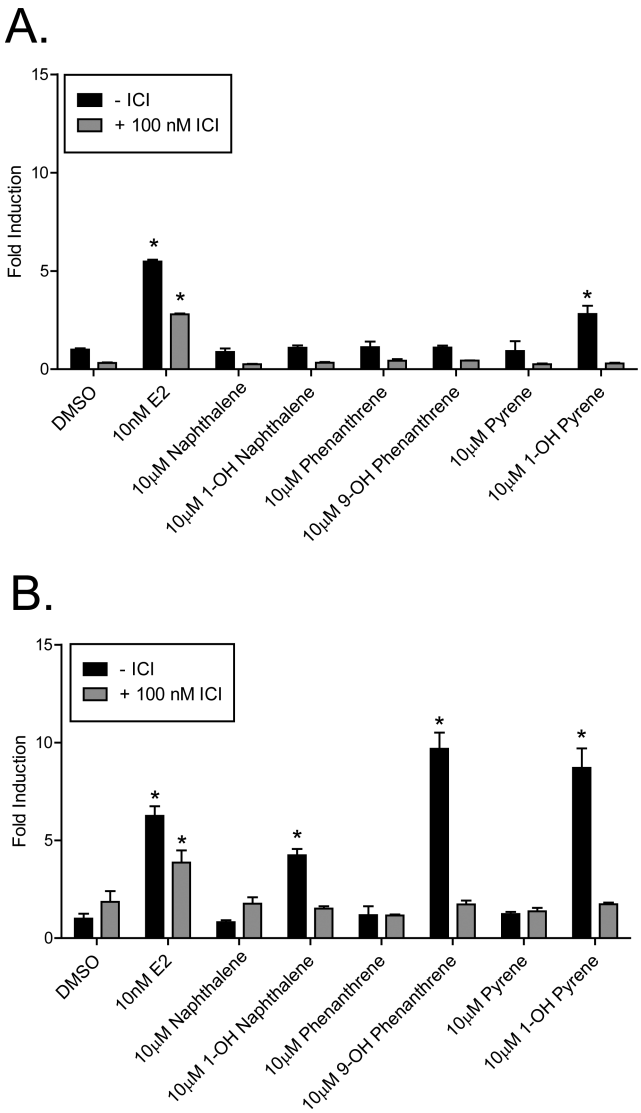



**FIG. 2.** Differential activation of ERα and ERβ by select monohydroxylated PAH compounds. (A) Hs578T-ERαLuc and (B) Hs578T-ERβLuc stable cell lines were treated in triplicate with 10μM of PAH compound in the presence or absence of 100nM ICI 182,780 for 24h. Data are expressed as fold induction of raw luciferase units per mg protein over the DMSO control ± SD. Experiments were repeated at least twice. *$p < 0.01$ compared with DMSO control.

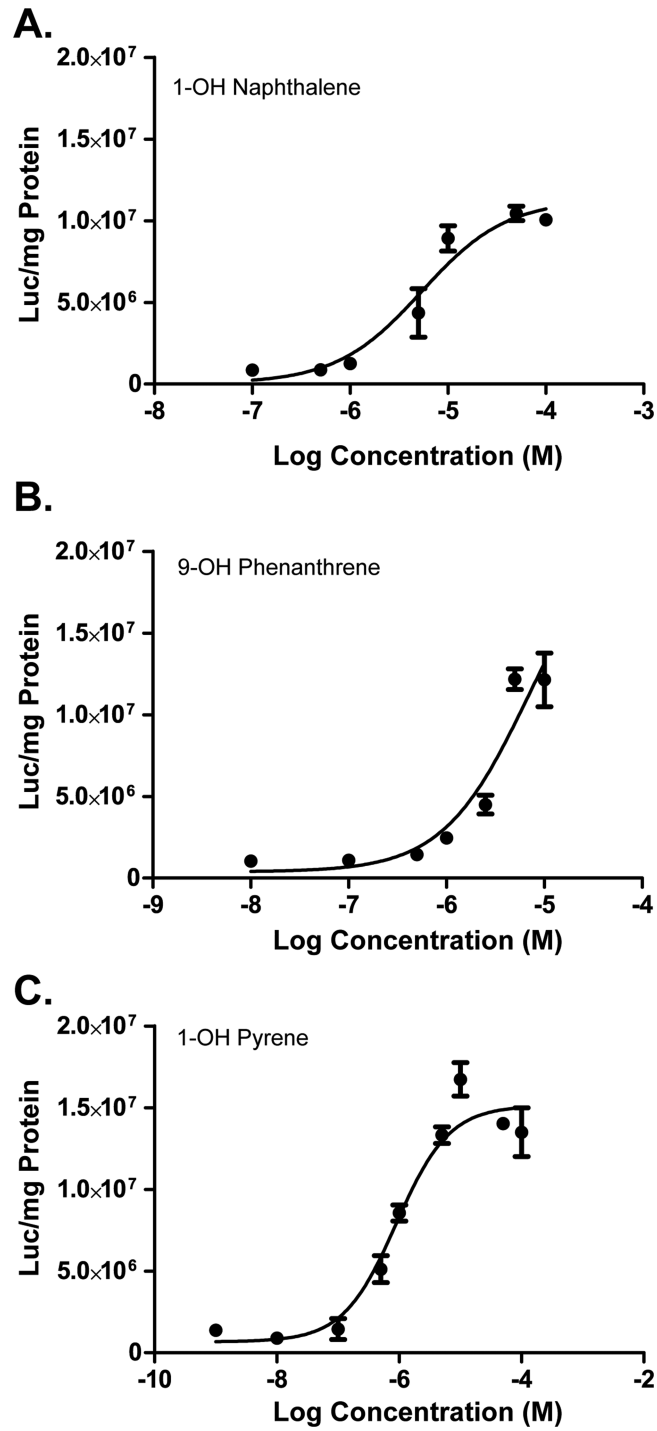



**FIG. 3.** Monohydroxylated PAHs activate ERβ in a dose-dependent manner. Hs578T-ERβLuc cells were treated with Dox for 24h followed by treatment with a range of concentrations of (A) 1-OH naphthalene, (B) 9-OH phenanthrene, or (C) 1-OH pyrene. The mean and SD shown are from triplicates of one representative experiment repeated twice.

compounds were able to induce the ERE-luciferase reporter activity primarily in the Hs578T-ERβLuc cells (Fig. 2B). In these cells, 1-OH naphthalene, 9-OH phenanthrene, and 1-OH pyrene induced a 4.2-, 9.7-, and 8.7-fold change over DMSO vehicle control, respectively ($p < 0.01$ in all cases). In contrast, only 1-OH pyrene induced the ERE-luciferase reporter activity in the Hs578T-ERαLuc cell line ($p < 0.01$), but not nearly to the same degree as that of 17β-estradiol (E2) (Fig. 2A). The ER antagonist ICI 182,780 blocked PAH-induced expression in all cases, reducing the luciferase signal to that of vehicle-treated cells. Reporter expression induced by 10nM E2 was not fully blocked by ICI 182,780 cotreatment because of the high concentration and potency of E2. No induction of reporter gene activity was seen in control experiments in which cells were not treated with Dox (Supplementary fig. 1), further confirming ER-mediated induction of the luciferase reporter.

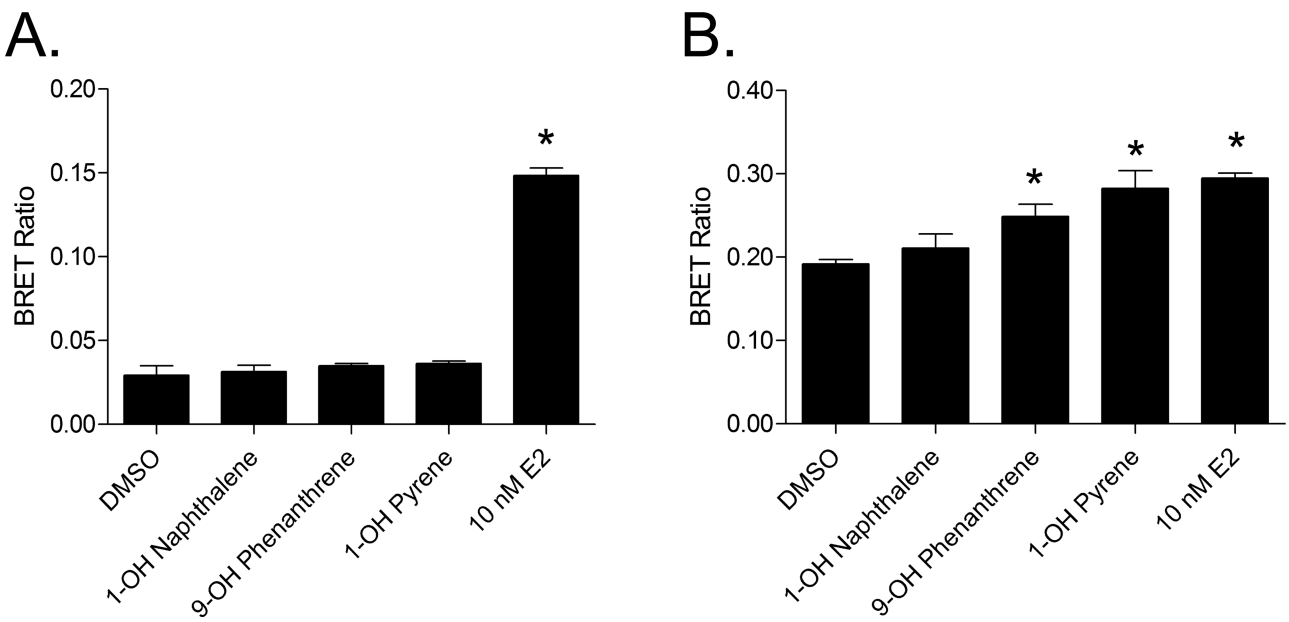



**FIG. 4.** Monohydroxylated PAH compounds selectively induce ERβ/β homodimers. (A) BRET data for 293T cells transfected with CMX-ERα-RLuc and CMX-ERα-YFP, showing no ERα/α dimerization upon treatment with PAH compounds in triplicate. (B) BRET data for 293T cells transfected with CMX-RLuc-ERβ and CMX-YFP-ERβ, showing ERβ/β dimerization when treated with PAH compounds in triplicate. The experiment was performed three times. Error bars represent SEM. *$p < 0.05$ compared with DMSO control.

We next determined the dose-dependent effects of the hydroxylated PAHs in the Hs578T-ERβLuc cells (Fig. 3). The half maximal effective concentration ($EC_{50}$) values for 1-OH naphthalene and 1-OH pyrene were found to be approximately 5.38 and 0.89μM, respectively. 9-OH Phenanthrene proved to be cytotoxic at concentrations greater than 10μM, and the dose-response curve did not adequately saturate; however, an approximate $EC_{50}$ value was estimated to be ≥ 6.8μM.

### *Monohydroxylated PAHs Induce ERβ Dimers and Directly Bind the Receptor*

To further dissect the mechanism through which the monohydroxylated PAHs activate ERβ and confirm the selectivity of the compounds, ER dimerization induced by the compounds was assessed using BRET assays. BRET assays allow the determination of dimer formation in live cells by transfecting cells with an energy donor (ER fused to Renilla luciferase) and acceptor (ER fused to yellow fluorescent protein) (see Powell and Xu, 2008). Upon transfecting the cells with the fusion constructs for ERα or ERβ, 9-OH phenanthrene and 1-OH pyrene were shown to significantly induce ERβ homodimerization ($p = 0.02$ and 0.01, respectively) (Fig. 4B). In contrast, 1-OH naphthalene did not significantly induce ERβ dimerization as determined by the BRET assay ($p = 0.35$). Following the trend seen in the ERα ERE-reporter assay, the monohydroxylated PAH compounds were unable to induce ERα homodimers (Fig. 4A).

In order to confirm that ERβ dimerization and ERE-luciferase activity were directly induced by ligand binding, the ability of the monohydroxylated PAH compounds to displace fluorescein-labeled estradiol from human ERβ was assessed in a competitive binding assay (Fig. 5). The competition with E2 indicates that compounds directly bind to ERβ in the same ligand-binding pocket as E2. These competitive binding data yielded half maximal inhibitory concentration ($IC_{50}$) values for 9-OH phenanthrene and 1-OH pyrene at 9.75 and 0.69μM, respectively. In support of the BRET results, 1-OH naphthalene showed a much lower affinity for ERβ as evidenced by Figure 5A, but it was still able to displace E2 at higher concentrations. The approximate $IC_{50}$ value for 1-OH naphthalene was estimated at or greater than 0.48μM.

After determining that monohydroxylated PAHs bind ERβ, Western blots with FLAG antibody were used to determine the degradation status of the receptor (Supplementary fig. 2), as some ER ligands cause degradation of the receptor upon binding. These Western blots confirmed that ERβ was not degraded by the monohydroxylated PAHs within 48 h of treatment.

### *Monohydroxylated PAHs Exhibit Estrogenic Activity on ERβ Target Genes*

To further validate the reporter assay and BRET assay results, the regulation of endogenous ERβ target genes was assessed. Estrogen responsive target genes of ERβ were previously identified in Hs578T-ERβ cells (Secreto *et al.*, 2007). Two upregulated target genes (*CC3* and *NRIP1*) and one downregulated target gene (*JAG1*) were selected for analysis by qPCR (Fig. 6). At 10μM, 1-OH naphthalene was able to induce *CC3* and *NRIP1* expression 2.1- and 2.2-fold over DMSO, respectively. Although the increased expression of *CC3* did not reach statistical significance ($p = 0.06$),

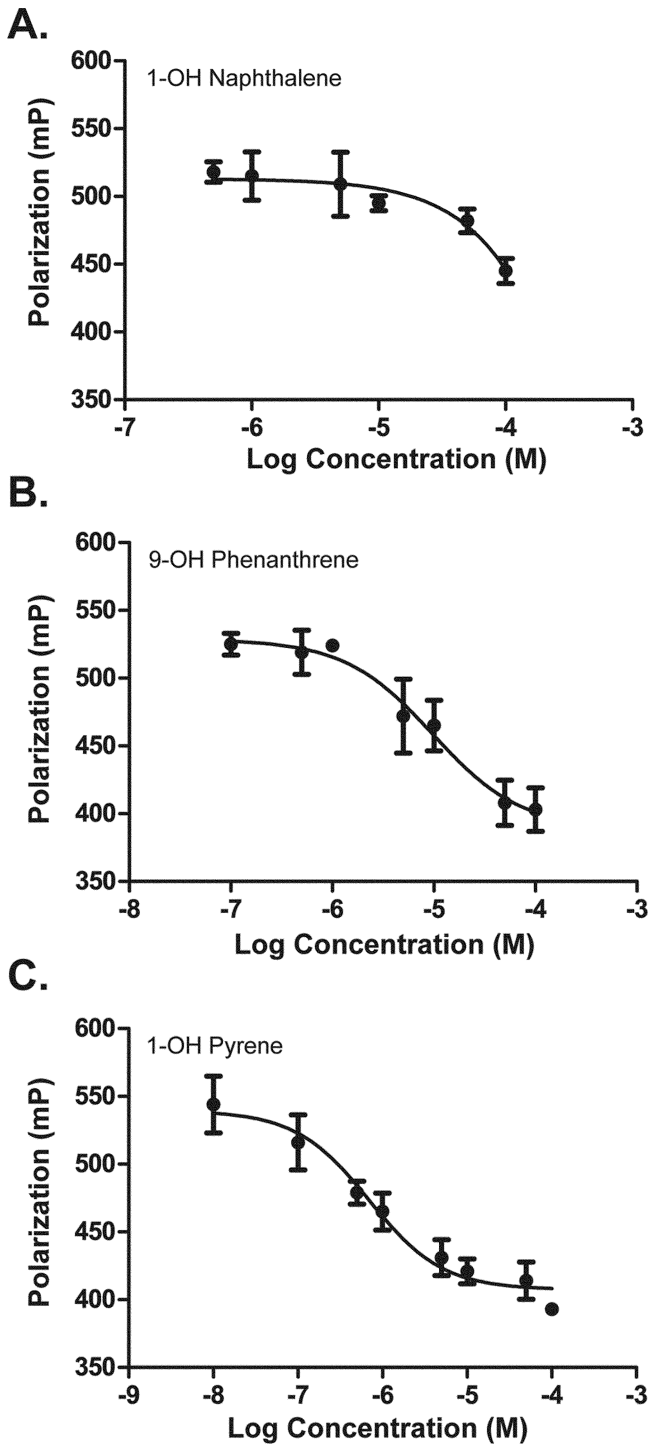



**FIG. 5.** Monohydroxylated PAHs can bind ERβ *in vitro*. Competitive binding curves for monohydroxylated PAH compounds displacing fluorescein-labeled estradiol from human ERβ. Purified hERβ and fluorescein-labeled estradiol were incubated for 4 h with serial dilutions in triplicate of (A) 1-OH naphthanol, (B) 9-OH phenanthrene, and (C) 1-OH pyrene. Error bars represent SD.

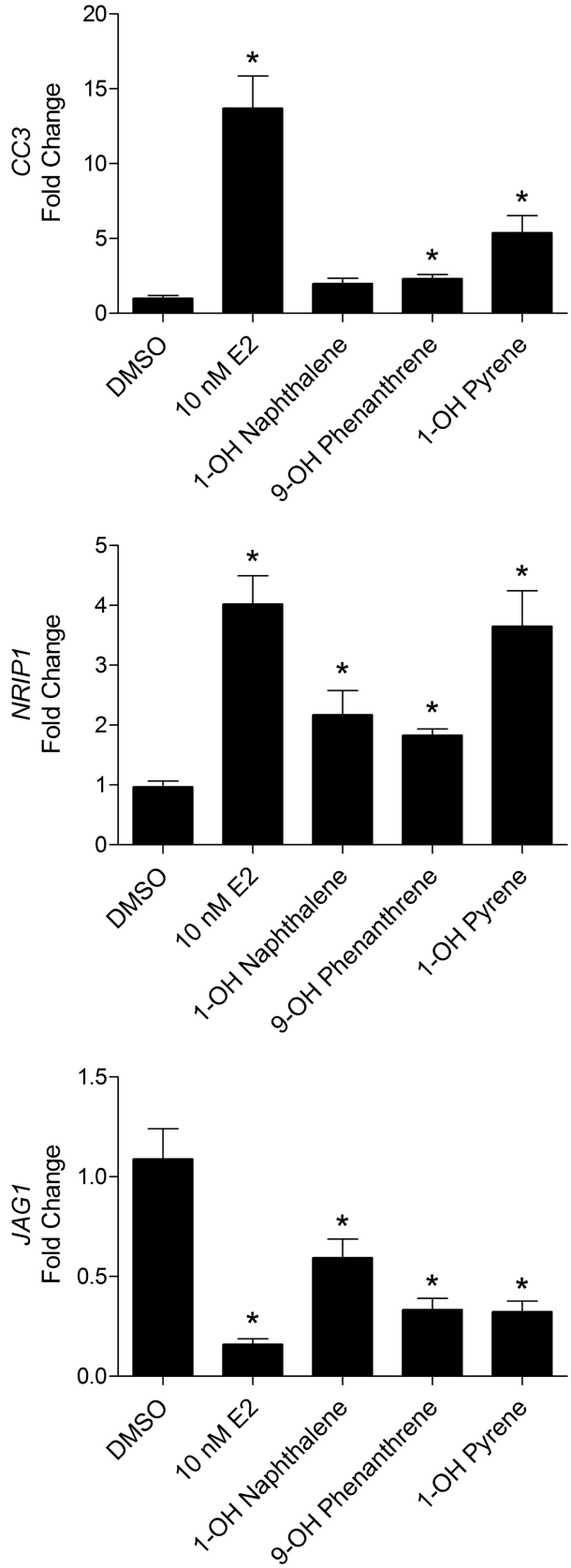
*NRIP1* was significantly upregulated by 1-OH naphthalene ($p = 0.02$). Treatment with 5μM 9-OH phenanthrene was able to significantly induce *CC3* and *NRIP1* expression 2.4-fold ($p = 0.02$) and 1.9-fold ($p < 0.01$) over DMSO, respectively. Similarly, 5μM 1-OH pyrene was able to significantly induce *CC3* and *NRIP1* expression 5.6-fold ($p = 0.02$) and 3.8-fold ($p < 0.01$) over DMSO, respectively. Additionally, all three monohydroxylated PAH compounds were able to downregulate the expression of *JAG1*, generating mean fold changes of 0.64 ($p = 0.02$), 0.36 ($p < 0.01$), and 0.32 ($p < 0.01$) over the DMSO control. It is important to note that although all compounds displayed some estrogenic activity on the target genes tested, the estrogenic response was not as robust as that of E2.

## DISCUSSION

Numerous studies have investigated the relationship between PAHs, their hydroxylated metabolites, and potential interactions with the ERs, yet most have focused on ERα (reviewed by Santodonato, 1997). Hayakawa *et al.* (2007) reported estrogenic and antiestrogenic activity for multiple monohydroxylated derivatives of common PAHs in a yeast two-hybrid assay expressing ERα. Similar to our findings, they also reported that the parent PAH compounds lacked any estrogenic or antiestrogenic activity. Charles *et al.* (2000) also reported estrogenic activity for hydroxylated metabolites of the carcinogen benzo[a]pyrene (B[a]P) in MCF-7 cells, which primarily express ERα. Despite these previous findings, there have been relatively few studies comparing the effects of monohydroxylated PAHs on the differential activation and dimerization of ERα and ERβ.

Our results, consistent with prior studies, indicate that hydroxylated PAHs are the active estrogenic species and can differentially activate either ERα or ERβ. Although the compounds we tested exhibited no interaction with ERα, the interaction with ERβ is novel and significant. Inhibition of luciferase signal by the ER antagonist ICI 182,780, as well as the lack of luciferase signal in the absence of Dox, demonstrates that the results of the reporter assay are ERβ mediated. Competition with fluorescein-labeled estradiol indicates that these monohydroxylated PAH compounds directly bind to ERβ at the same ligand-binding pocket as E2. Fertuck *et al.* (2001a) investigated different parental PAH and metabolite compounds, and they similarly reported that hydroxylated PAHs were able to compete with estrogen and bind ERs with a slight preference for ERβ. Their data, consistent with our findings, suggest that hydroxylated PAHs may preferentially affect ERβ signaling. Given ERβ's role in normal development and function in reproductive tissues as well as in the lungs, colon, prostate, and cardiovascular system, disruption of and interference with ERβ signaling could have implications in normal development, as well as in cancers and malfunctions of these tissues.

In addition to the reporter assay and competitive binding data, the BRET and qPCR data confirm that 9-OH phenanthrene and 1-OH pyrene induce a biologically active ERβ response in this




system. Given our data, 1-OH naphthalene may not necessarily induce ERβ homodimers even at the high concentration tested (10μM). In support of these data, ligand-binding assays with 1-OH naphthalene demonstrate a relatively low binding affinity for ERβ. Despite the negative BRET data, qPCR for endogenous ERβ target genes suggest that 1-OH naphthalene is capable of inducing a slight biologically active ERβ response for some ERβ target genes although not to the same extent as E2. Collectively, the data obtained for 1-OH naphthalene demonstrate an important consideration of the *in vitro* assays used in this study: different assays have different sensitivities for detecting estrogenic activity and ER subtype selectivity. The ERβ homodimerization BRET assay typically shows a 1.5- to 2-fold induction with E2 treatment because of high ligand-independent dimerization (Powell and Xu, 2008). In addition, the BRET ratios ultimately depend on the conformational changes within the receptor fusion proteins, which allow for efficient energy transfer, and different ligands will induce different conformational changes, thereby affecting the BRET ratio output. Despite the lower fold changes for the ERβ homodimerization assay, BRET has been successfully used in a high-throughput manner to identify ER dimer selective ligands (Powell *et al*., 2010) and, in this study, demonstrated a significant induction of ERβ homodimerization by two other monohydroxylated PAHs, 1-OH pyrene and 9-OH phenanthrene.

Although each monohydroxylated PAH tested gave a similar pattern of results, the relative activity of each compound is quite different. Our data indicate that 1-OH naphthalene is the weakest ERβ agonist among the tested metabolites, as demonstrated by low reporter gene output, a lack of saturation in the dose-response reporter assays, low induction of ERβ dimerization, and a lower binding affinity for ERβ. In contrast, 1-OH pyrene and 9-OH phenanthrene appear to be fairly efficient ERβ agonists. Both ligands induced ERE-reporter gene activity similar to E2 and effectively displaced E2 from the ERβ ligand-binding pocket. Both compounds also significantly elicited ERβ homodimerization. 9-OH Phenanthrene generated data similar to 1-OH pyrene with the exception that it proved to be cytotoxic at concentrations greater than 10μM, resulting in difficulty to obtain accurate $EC_{50}$ values. Despite the cytotoxicity of 9-OH phenanthrene at high concentrations, treatment with lower concentrations of 9-OH phenanthrene (5μM) stimulated the regulation of endogenous ERβ target genes in Hs578T-ERβLuc cells. These data suggest that some monohydroxylated PAHs can affect ERβ-mediated signaling prior to inducing general cytotoxicity.

**FIG. 6.** Monohydroxylated PAHs can regulate ERβ target genes similar to estradiol. Expression of ERβ target genes (*CC3*, *NRIP1*, and *JAG1*) was determined by measuring relative mRNA levels using qPCR. RNA was collected following treatment with 0.1% DMSO, 10nM E2, 10μM 1-OH naphthalene, 5μM 9-OH phenanthrene, or 5μM 1-OH pyrene for 24-h and 48-h treatment with 50 ng/ml Dox. Data are expressed as fold induction compared with DMSO control. Error bars represent SEM. *$p < 0.05$ compared with DMSO control.

Although our data did not indicate that any of the monohydroxylated PAHs tested had an effect on ERα, others have reported ERα estrogenic effects for these compounds. Hayakawa *et al.* (2007) reported that all three monohydroxylated PAHs exhibited little to no ERα estrogenic activity, but that 1-OH pyrene was able to compete with E2 for ERα binding. Additionally, Wiele *et al.* (2004) reported that 1-OH pyrene showed ERα estrogenic activity in colon extracts from a simulator of the human intestinal microbial ecosystem. Discrepancies across these studies may be due to the use of different assays and cell lines to assess the estrogenic activity.

Overall, these data suggest that common monohydroxylated PAHs can interact, positively or negatively, with ER signaling. We can conclude from our results and from other studies that hydroxylated PAHs are the active estrogenic species and can differentially bind ERα or ERβ, likely in a cell- and tissue-specific manner. Few studies assessing the physiological serum concentrations of monohydroxylated PAHs have been published, although monohydroxylated PAHs may be used as urine biomarkers to assess exposure to PAHs (Elovaara *et al.*, 2006). It is therefore difficult to predict the concentrations of monohydroxylated PAHs that reach tissues such as the mammary gland, and the concentrations shown to be estrogenic in these studies may or may not be reached in the serum. Some estrogenic compounds in the diet, such as genistein found in soy products, can reach serum concentrations near the micromolar range (Cassidy *et al.*, 2006). Ultimately, the physiological exposure to monohydroxylated PAHs will be a function of both exposure and metabolic activity, which will greatly vary among individuals. These *in vitro* studies, however, demonstrate the potential for monohydroxylated PAHs to impact ERβ-mediated signaling and provide a framework for assessing the impacts of other environmental chemicals on the dimerization and transcriptional activities of ERα and ERβ.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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There are no conflicts of interest.

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# Research Resource: Global Identification of Estrogen Receptor β Target Genes in Triple Negative Breast Cancer Cells

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Breast cancers that are negative for estrogen receptor α (ERα), progesterone receptor, and human epidermal growth factor receptor 2 are known as triple-negative breast cancers (TNBC). TNBCs are associated with an overall poor prognosis because they lack expression of therapeutic targets like ERα and are biologically more aggressive. A second estrogen receptor, ERβ, has been found to be expressed in 50% to 90% of ERα-negative breast cancers, and ERβ expression in TNBCs has been shown to correlate with improved disease-free survival and good prognosis. To elucidate the role of ERβ in regulating gene expression and cell proliferation in TNBC cells, the TNBC cell line MDA-MB-468 was engineered with inducible expression of full-length ERβ. In culture, ERβ expression inhibited cell growth by inducing a G1 cell cycle arrest, which was further enhanced by 17β-estradiol treatment. In xenografts, ERβ expression also inhibited tumor formation and growth, and 17β-estradiol treatment resulted in rapid tumor regression. Furthermore, genomic RNA sequencing identified both ligand-dependent and -independent ERβ target genes, some of which were also regulated by ERβ in other TNBC cell lines and correlated with ERβ expression in a cohort of TNBCs from the Cancer Genome Atlas Network. ERβ target genes were enriched in genes that regulate cell death and survival, cell movement, cell development, and growth and proliferation, as well as genes involved in the Wnt/β-catenin and the G1/S cell cycle phase checkpoint pathways. In addition to confirming the anti-proliferative effects of ERβ in TNBC cells, these data provide a comprehensive resource of ERβ target genes and suggest that ERβ may be targeted with ligands that can stimulate its growth inhibitory effects. **(*Molecular Endocrinology* 27: 1762–1775, 2013)**

Estrogen signaling is primarily mediated by two estrogen receptors (ERs): ERα and ERβ. ERα is expressed in approximately 70% of breast cancers, and many of these cancers respond to endocrine therapies that block the proliferative action of ERα. However, approximately 15% to 20% of all breast cancers lack expression of ERα, its target gene progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) and are clinically defined as triple-negative breast cancers (TNBCs). Full-length ERβ protein has been detected in 50% to 90% of ERα-negative breast cancers (1–4), and ERβ expression has been shown to correlate with improved disease-free survival and good prognosis in TNBC (2). Unlike ERα, for which PR expression is indicative of receptor

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Abbreviations: Dox, doxycycline; E2, 17β-estradiol; ERα, estrogen receptor α; ERβ, estrogen receptor β; FBS, fetal bovine serum; GFP, green fluorescent protein; IPA, Ingenuity pathway analysis; HER2, human epidermal growth factor receptor 2; 4-OH Tam, 4-hydroxytamoxifen; PR, progesterone receptor; RSEM, RNA sequencing expectation maximization; RNA-seq, RNA sequencing; SFS, charcoal stripped FBS; TCGA, the Cancer Genome Atlas; Tet, tetracycline; TNBC, triple-negative breast cancers.

expression and function, a target gene or gene set indicating ERβ functionality has yet to be identified.

Like ERα, ERβ is a nuclear receptor that regulates target gene expression in estrogen responsive tissues, such as the mammary gland. Multiple isoforms of ERβ may be expressed in the mammary gland, but the full-length receptor is the only isoform able to bind ligand with high affinity and regulate target gene expression (5, 6). Several studies have assessed the effects of full-length ERβ expression on the growth of ERα-positive breast cancer cells (7–11). The results of these studies demonstrate that ERβ expression inhibits the proliferative response mediated by ERα. Fewer reports have assessed the growth effects of ERβ expression in breast cancer cells that lack ERα, but ERβ expression in ERα-negative breast cancer cells has been shown to inhibit growth in ligand-independent and -dependent manners (12–15). This leads to the hypothesis that some ERα-negative breast cancers, including TNBCs, may benefit from therapies that target ERβ (16).

In regard to gene expression, even fewer studies have aimed to identify ERβ target genes in the absence of ERα. Microarray analyses comparing ERα and ERβ target genes in U2OS osteosarcoma cells (17) and Hs578T breast cancer cells (15) demonstrated that the two receptors have both overlapping and distinct target genes. However, only ligand-dependent ERβ target genes have been identified in ERα-negative breast cancer cells, and a comprehensive assessment of both ligand-independent and -dependent ERβ target genes in TNBC cells has not yet been completed.

In an effort to identify ERβ target genes globally in TNBC cells and assess the growth inhibition of ERβ expression in vitro and in vivo, we generated a TNBC cell line with inducible expression of full-length ERβ. We confirmed ERβ-mediated growth inhibition in vitro and in vivo and identified ERβ target genes using RNA sequencing. We further show that some of the ERβ target genes are associated with ERβ expression in a cohort of TNBCs. These data demonstrate the growth inhibitory properties of ERβ and provide a global view of ligand-independent and -dependent ERβ target genes in the absence of ERα expression. This study also provides a foundation to identify target genes further that may indicate ERβ functionality in TNBCs.

## Materials and Methods

### Cell culture and reagents

Cell culture media were obtained from Invitrogen. MDA-MB-468 cells were cultured in DMEM + 10% fetal bovine serum (FBS; Invitrogen), and BT549 and HCC1143 cells were cultured in RPMI 1640 + 10% FBS. HCC1143 breast cancer cells were obtained from the Leibniz Institute DSMZ, German Institute for Microorgansims and Cell Culture. All cells were cultured at 37°C and 5% $CO_2$. MDA-MB-468-ERβ inducible cells were cultured in DMEM + 10% Tet-system approved FBS (Clontech Mountain View), 500 mg/L Zeocin, and 5 mg/L Blasticidin S (Research Products International). Before all experiments, MDA-MB-468-ERβ cells were cultured in phenol red free DMEM + 10% charcoal stripped FBS (SFS) for at least 3 days to remove residual estrogens. Doxycycline (Dox) was obtained from Clontech. The Flag and β-actin antibodies were obtained from Sigma-Aldrich, and the Hsp90 antibody was obtained from Santa Cruz Biotechnology. ICI 182,780 and ERB-041 were purchased from Tocris. All other reagents were purchased from Sigma Aldrich unless otherwise noted.

### Generation of MDA-MB-468-ERβ inducible cells

MDA-MB-468-ERβ inducible cells were created as previously described using the TRex system (Invitrogen) (15). Briefly, MDA-MB-468 cells were transfected with pcDNA6/TR, and a stable clone was selected after selection with 5 μg/mL Blasticidin S. The MDA-MB-468-Tet cells were then transfected with pcDNA4/TO-ERβ, which encodes the 530 amino acids. full-length ERβ isoform, and selected with 500 μg/mL Zeocin. Stable clones were selected and screened for inducible expression of ERβ using Western blotting with Flag antibody.

### Ligand binding assays and Western blots

Ligand binding assays were performed as previously described with slight modification (18). Briefly, $2 \times 10^5$ MDA-MB-468-ERβ cells were seeded in a 24-well plate and treated with vehicle or 50 ng/mL Dox. After 48 hours, cells were incubated with 20 nM radiolabeled [$^3$H]-E2 (89.2 Ci/mmol specific activity; Perkin Elmer) in the presence or absence of 450 nM diethylstilbestrol cold competitor. After a 2-hour incubation, cells were washed and lysed and the retained radioactivity was determined using a liquid scintillation counter.

To assess ERβ expression using Western blots, MDA-MB-468-ERβ cells were treated with the corresponding Dox concentrations for the given amount of time. Cells were then washed in PBS and lysed as previously described (18). After separation by SDS-PAGE and transfer to a nitrocellulose membrane, the membrane was incubated with the corresponding antibody and visualized by enhanced chemiluminescence on a Chemidoc XRS system (BioRad) or by fluorescence detection on a Licor Odyssey gel reader (Licor Biosciences) as previously described (18).

### Phalloidin staining

MDA-MB-468 ERβ#18 cells were maintained in DMEM supplemented with 10% stripped FBS for 3 days. The cells were then cultured in DMEM + 5% SFS with or without Dox to induce ERβ expression. After 24 hours, the cells were treated with or without 10 nM E2. Forty-eight hours before immunostaining, the cells were seeded onto coverslips in a 6-well plate. After 5 days of treatment, the cells were fixed in 4% formaldehyde for 15 minutes, and the coverslips were washed three times in PBS. Alexa Fluor 555 Phalloidin (Cell Signaling Technology) was diluted 1:20 (6.6 μM stock concentration in methanol) in PBS and added to the cells. After 15 minutes incubation at room temperature, coverslips were washed once with PBS. The cov-

erslips were mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). Fluorescence was detected using a Leica DM5000B microscope (Buffalo Grove) with the appropriate wavelengths.

## Cell proliferation and cell cycle assays

For cell proliferation assays, $1 \times 10^5$ MDA-MB-468-ERβ cells were seeded in triplicate onto 6-well plates in DMEM + 5% SFS. After 24 hours, cells were treated with vehicle or 50 ng/mL Dox for 24 hours. Cells were then treated with the corresponding ligands or 0.1% DMSO for the given amount of time. Media were refreshed every 48 hours. Cells were trypsinized and counted after trypan blue exclusion using an automated cell counter (BioRad) according to the manufacturer's protocol.

To assess the cell cycle distribution, $3 \times 10^5$ MDA-MB-468-ERβ cells were seeded in triplicate onto 6-cm plates in DMEM + 5% SFS. After 24 hours, the cells were treated with vehicle or 50 ng/mL Dox for 24 hours followed by treatment with 0.15% DMSO or the corresponding ligands for 72 hours. Cells were collected by trypsinization, fixed in cold 95% ethanol, and washed in PBS. The fixed cells were then resuspended in propidium iodide staining solution (200 μg/mL RNase A, 50 μg/mL propidium iodide, 0.1% [v/v] Triton X-100 in PBS + 1% BSA) and incubated overnight at 4°C. Samples were analyzed by flow cytometry at the University of Wisconsin Flow Cytometry Laboratory.

## Xenograft experiments

All animal work was performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. 468-ERβ#18 cells were infected with retrovirus generated from a pLNCX-FLuc (firefly luciferase) vector. Cells were then cultured in regular media containing 400 μg/mL G418 (Invitrogen) for at least eight passages to generate luciferase-labeled 468-ERβ#18 cells. To assess the effects of ERβ expression on tumorigenicity, $2 \times 10^6$ luciferase-labeled 468-ERβ#18 cells were injected bilaterally into the inguinal mammary fat pads of 5- to 6-week-old ovariectomized athymic nude-Foxn1nu mice (n = 4 per group). Mice were given control diets or Dox-containing diets (2000 ppm) obtained from Harlan Laboratories immediately after the injections. To assess tumor regression in response to E2, $2 \times 10^6$ luciferase-labeled 468-ERβ#18 cells were injected as described above, and the tumors were allowed to form in the absence of Dox for 10 weeks. On day 0, a 60-day release 0.05 mg E2 pellet (Innovative Research of America) was implanted into each mouse. Mice (n = 3 per group) were then separated randomly and given a control or Dox-containing diet. Luciferase-based noninvasive bioluminescent imaging and analysis were performed as previously described with an IVIS Imaging System (Caliper Life Sciences) (19). Briefly, mice were anesthetized and injected ip with 2 mg D-luciferin (10 mg/mL in PBS) (Gold Biotechnology). Imaging was completed between 15 and 20 minutes after injection. For bioluminescence plots, total photon flux was calculated for each mouse by using a circular region of interest.

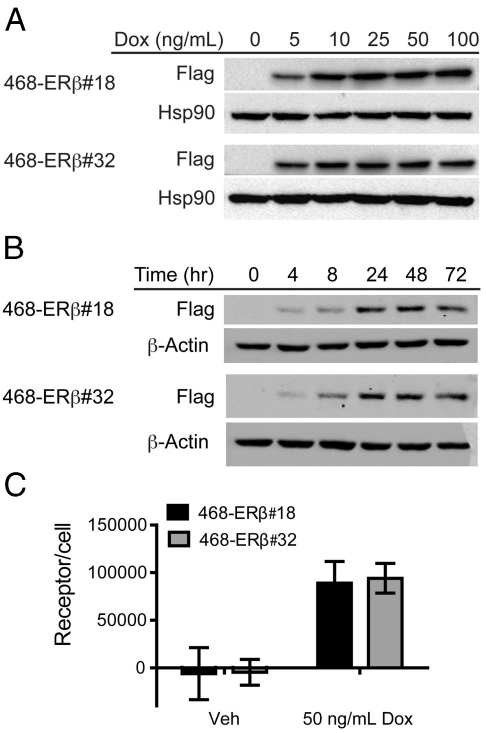


**Figure 1.** MDA-MB-468-ERβ cells express ERβ after Dox treatment. Dox-dependent expression of ERβ was initially characterized in two MDA-MB-468-ERβ clones (468-ERβ#18 and 468-ERβ#32). (A) A Western blot using Flag antibody shows that both clones express detectable levels of ERβ after treatment with as little as 5 ng/mL Dox for 48 hours. Further in vitro studies were performed with 50 ng/mL Dox. (B) A Western blot using Flag antibody shows that treatment with 50 ng/mL Dox for as little as 4 hours induces expression of ERβ. (C) Ligand binding assays performed with radiolabeled E2 demonstrate that both clones express similar levels of ERβ corresponding to approximately 90 000 receptors/cell after 24 hours of Dox treatment. The data represent the mean and SD.

## RNA sequencing (RNA-seq) of ERβ target genes and pathway analysis

To identify ERβ target genes globally, 468-ERβ#18 cells were treated with vehicle or 50 ng/mL Dox for 48 hours followed by treatment with 0.1% DMSO or 10 nM E2 for 4 hours. Total RNA was extracted using a RNEasy Plus Kit according to manufacturer protocol (Qiagen), and three independent experiments were performed. The total RNA was submitted to the University of Wisconsin Biotechnology Center for RNA quality analysis, sequencing library generation, and sequencing. An Illumina HiSeq 2000 was used to generate 100-bp single-end reads. Reads were trimmed for quality (15 bp from the 5′ end and 2 bp from the 3′ end) and aligned to the University of California, Santa Cruz build hg19 genome using RNA sequencing expectation maximization (RSEM). It has now been well recognized that discarding RNA-seq reads that align to multiple genes introduces bias in expression estimates of gene families and genes containing or inside of repetitive elements (20–23). RSEM implements a principled and biologically motivated probabilistic model for this problem (20, 24). It is based on a generative model of the RNA-seq protocol that takes into account aspects such as sequencing error, fragment length distributions, and nonuniformities in the distribution of read loca-

tions along transcripts. The RSEM alignment results are presented in Supplemental Table 2, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. For the identification of differentially expressed genes, the fractional counts were rounded to the nearest integer, and DESeq (25) was used to assess differentially expressed genes at a 1.5-fold change cutoff and a false discovery rate <0.01. The filtered gene lists for each comparison are provided in Supplemental Materials and Methods. The raw data will also be submitted to the Gene Expression Omnibus. Pathway enrichments and functional analyses of the gene lists were determined using ingenuity pathway analysis (IPA) software (Ingenuity Systems, www.ingenuity.com). The DESeq and IPA results for all comparisons are available in the Supplemental Materials and Methods.

## Quantitative PCR

468-ERβ#18 cells were treated with DMSO, 10 nM E2, or 100 nM ERB-041 as described for the initial RNA-seq experiment. For validation in HCC1143 cells, the cells were infected with retrovirus generated from pLNCX-GFP or pLPCX-ERβ vectors. Twenty-four hours after infection, the media were replaced with phenol red free RPMI 1640 + 5% SFS. After an additional 24 hours, cells were treated with 0.1% DMSO or 10 nM E2 for 4 hours. Total RNA was extracted as described above

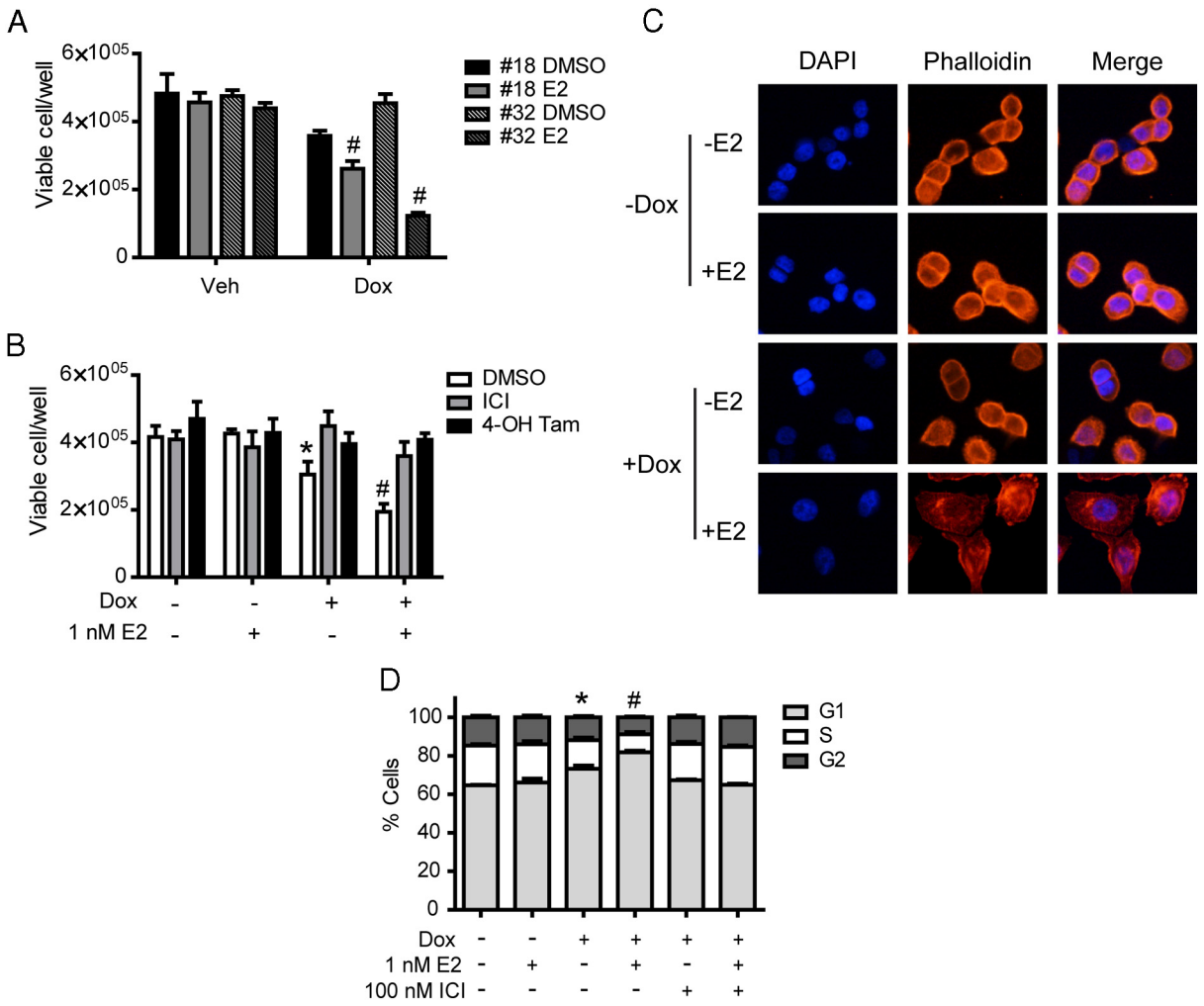


**Figure 2.** ERβ expression and E2 treatment inhibit the growth of MDA-MB-468-ERβ cells. (A) Two MDA-MB-468-ERβ clones (#18 and #32) were treated in triplicate with vehicle or Dox for 24 hours followed by treatment with DMSO or 1 nM E2. The total number of viable cells was determined using trypan blue exclusion after the given number of days. *, $P < .01$ compared to DMSO control; #, $P < .01$ compared to DMSO+Dox control. (B) Treatment with ER antagonists rescue the growth inhibition mediated by ERβ. 468-ERβ#18 cells were treated in triplicate with vehicle or Dox for 24 hours followed by treatment with DMSO or E2 in the presence or absence of 100 nM ICI or 4-OH Tam. The total number of viable cells was determined by trypan blue exclusion after 5 days. *, $P < .01$ compared to DMSO control; #, $P < .01$ compared to DMSO+Dox control. (C) ERβ expression and E2 treatment alter the morphology of the 468-ERβ#18 cells. Cells were treated with vehicle or Dox for 24 hours followed by treatment with DMSO or E2 for 5 days and subsequently stained with Alexa Fluor 555 Phalloidin (*red*), which stains actin filaments to highlight the cytoplasm, and 4′,6-diamidino-2-phenylindole (*blue*) for nuclear staining. (D) ERβ expression and E2 treatment induce a G1 cell cycle arrest. 468-ERβ#18 cells were treated in triplicate with vehicle or Dox for 24 hours followed by treatment with DMSO or E2 in the presence or absence of 100 nM ICI for 72 hours. The proportion of cells in each phase of the cell cycle was determined using flow cytometry as described in the *Materials and Methods*. For the proportion of cells in G1; *, $P < .01$ compared to DMSO control; #, $P < .01$ compared to DMSO+Dox control. The data represent the mean and SD.

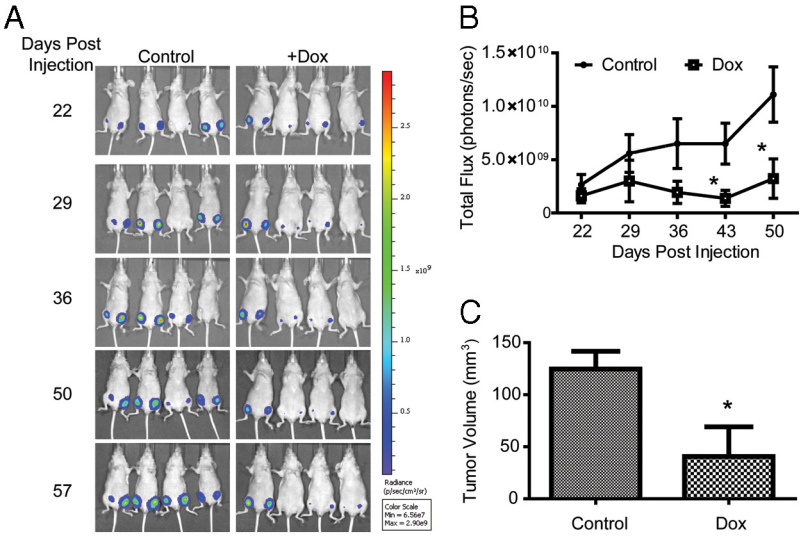


**Figure 3.** ERβ expression inhibits tumor growth in xenografts. (A) 468-ERβ#18 cells were labeled with luciferase and injected into the mammary fat pads of nude mice (n = 4 per group). Mice were then provided a control or Dox-containing diet. The tumor growth was monitored using bioluminescence imaging as described in the *Materials and Methods*. (B) Quantitative plot of bioluminescence for each group over time. (C) Final tumor volumes for each group. The data represent the mean and SEM of the tumors from each group. *, $P < .05$ compared to control.

and reverse-transcribed using Superscript II RT according to the manufacturer's protocol (Invitrogen). Quantitative PCR was performed using TaqMan Prime Time custom designed assays (IDT), FastStart Universal Probe Master Mix (Roche Scientific), and a CFX96 instrument (BioRad). Primer and probe sequences are shown in Supplemental Table 1. Data were analyzed using the ΔΔCq method calculated by CFX Manager Software (Bio-Rad). Student *t* tests were performed with GraphPad Prism Software (Version 5.04; GraphPad Software Inc).

### Correlation analysis of ERβ target genes and ERβ expression in human tumor samples

To compare the RNA sequencing results further with previous studies of TNBC, the Agilent mRNA expression microarray data from the Cancer Genome Atlas (TCGA) database, among which n = 43 samples were identified as TNBC, was examined to assess any correlations between the expression of ERβ and its target genes (26). Using all of the genes identified in the comparison of 468-ERβ#18 cells treated with E2 versus E2+Dox (comparison IV, Figure 5A) with false discovery rate <0.05, a total of 2472 genes were present in both the microarray data and our gene list. The correlation coefficient was calculated for the expression of each of the genes and that of ERβ based on the microarray data. To assess the strength of the correlation, the *P* values were calculated for a two-sided alternative that these correlation coefficients are significantly different from zero (meaning no correlation). To account for multiple testing, these *P* values were then converted to the so-called *q* values (27, 28). For those genes with $q < .05$ for the correlation and a fold change >1.5 in the RNA sequencing results, the correlation coefficient determined for the array data was graphed against the fold change observed for the gene in the 468-ERβ#18 cells treated with E2 versus E2+Dox.

## Results

### Inducible expression of ERβ and E2 treatment induces a G1 cell cycle arrest and inhibits the growth of MDA-MB-468 cells in vitro and in vivo

To assess the growth and gene expression effects of ERβ expression and activation in TNBC cells, MDA-MB-468 cells were engineered with inducible expression of full-length ERβ using the TRex system from Invitrogen. After creating MDA-MB-468-Tet cells, which express the tetracycline (Tet) repressor, these cells were transfected with a construct encoding Tet-operated full-length Flag-tagged ERβ. Two MDA-MB-468-ERβ clones with inducible ERβ expression were selected for further characterization (486-ERβ#18 and 468-ERβ#32). First, ERβ expression was assessed in response to increasing Dox concentrations (Figure 1A). As little as 5 ng/mL Dox treatment for 48 hours induced expression of the receptor, and subsequent in vitro experiments were performed with 50 ng/mL Dox. Next, a time course was established for the Dox-inducible expression of ERβ (Figure 1B). ERβ was maximally expressed after 24 hours, and this expression level was sustained after 48 hour and 72 hour treatments. Finally, the expression level was quantified using radiolabeled ligand binding assays (Figure 1C). After Dox treatment, both clones expressed approximately 90 000 receptors per cell, which is comparable to MCF7 breast cancer cells that express ~150 000 ERα molecules per cell (18).

After confirming Dox-inducible expression of ERβ in 486-ERβ#18 and 468-ERβ#32 cells, we assessed the growth effects of ERβ expression and activation by 17β-estradiol (E2) (Figure 2A). The number of viable cells was significantly inhibited by ERβ expression and E2 treatment for both clones. 468-ERβ#18 showed slight growth inhibition when ERβ was expressed in the absence of ligand. Dox and E2 treatment had no effect on the growth of MDA-MB-468-Tet cells (Supplemental Figure 1). To confirm that the growth inhibitory effects observed in MDA-MB-468-ERβ cells were mediated by ERβ, 468-ERβ#18 cells were treated with vehicle or Dox and DMSO or E2 in the presence or absence of the antagonist ICI 182,780 (ICI) or the selective estrogen receptor modulator 4-hydroxytamoxifen (4-OH Tam) (Figure 2B).

Dox treatment alone significantly inhibited the growth of 468-ERβ#18 cells, and this effect was rescued by ICI or 4-OH Tam co-treatment. Treatment with E2 further inhibited the growth of the cells, and this inhibition was also rescued by ICI or 4-OH Tam co-treatment. The ERβ-selective ligand, ERB-041 (29), also inhibited cell growth similar to E2 (Supplemental Figure 2). Interestingly, the morphology of the cells was altered after 5 days of treatment with Dox and E2 (Figure 2C), suggesting that the cells may have undergone some type of cell cycle arrest. Indeed, an analysis of the cell cycle phase distribution by flow cytometry revealed an increase in the proportion of cells in the G1 phase after Dox treatment alone, which was further increased by E2 treatment (Figure 2D). ERβ expression and ligand treatment did not induce detectable levels of apoptosis (data not shown).

Next, we aimed to assess the effects of ERβ expression and activation on tumor growth of MDA-MB-468 cells. First, soft agar colony formation assays revealed that ERβ expression alone completely blocked the formation of colonies (Supplemental Figure 3). Xenograft experiments were then performed using 468-ERβ#18 cells retrovirally labeled with luciferase to monitor tumor growth by bioluminescence imaging (Figure 3). After injecting cells into the mammary fat pads, the mice were given a control diet or a Dox-containing diet, and the tumor size was monitored using bioluminescence imaging (Figure 3A). In the presence of Dox, the growth of the tumors was reduced and one mouse did not develop tumors (Figure 3, A and B). The final tumor volume was significantly smaller when ERβ was expressed (Figure 3C), and Dox treatment had no effect on the growth of control MDA-MB-468-Tet tumors (Supplemental Figure 4). To assess the effects of ERβ expression and activation on tumor growth, luciferase-labeled 468-ERβ#18 cells were allowed to form tumors for 10 weeks. Mice were then implanted with a slow release E2 pellet and given a control or Dox-containing diet. The tumors rapidly regressed only when ERβ was expressed (Figure 4). These data demonstrate the growth inhibitory effects of ERβ expression and activation in TNBC cells in vivo.

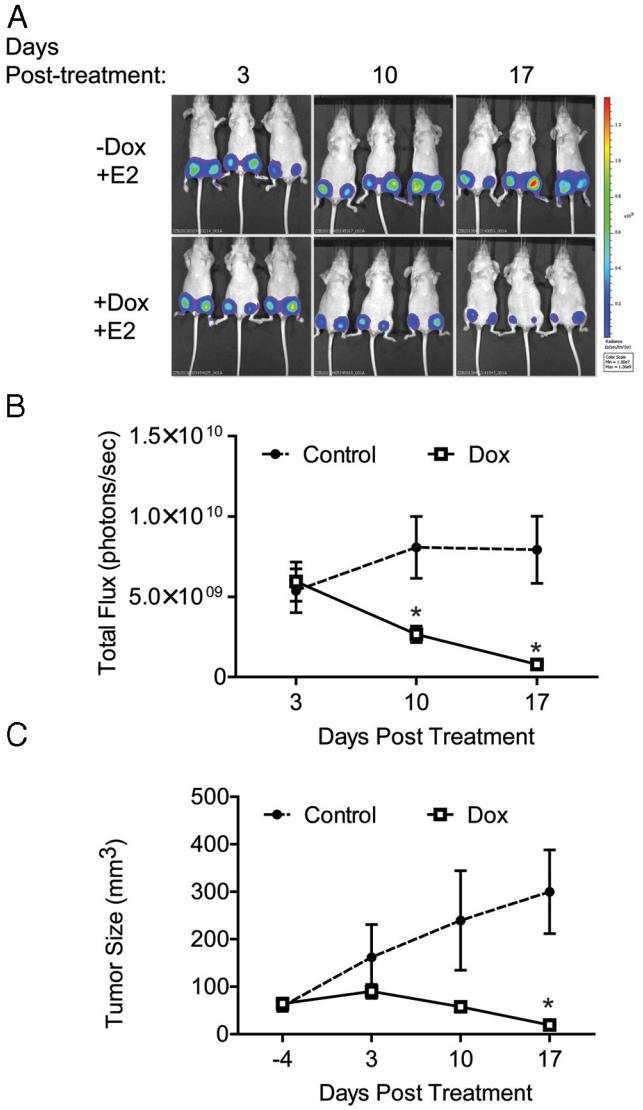


**Figure 4.** Activation of ERβ in xenografts causes tumor regression. (A) 468-ERβ#18 cells were labeled with luciferase and injected into the mammary fat pads of nude mice. After allowing tumors to form for 10 weeks, slow release E2 pellets were implanted into each mouse. Mice were separated into two groups (n = 3 per group) and provided a control or Dox-containing diet. The tumor growth was monitored using bioluminescence imaging and caliper measurements as described in the *Materials and Methods*. (B) Quantitative plot of bioluminescence for each group over time. The data represent the mean and SEM of the tumors from each group. (C) Quantitative plot of tumor size as determined by caliper measurements for each group over time. The data represent the mean and SEM of the tumors from each group. *, $P < .05$ compared to control.

## ERβ target gene identification using RNA-seq

The MDA-MB-468-ERβ inducible cells are useful for identifying the ligand-independent and ligand-dependent target genes of ERβ in the absence of ERα. To identify direct ERβ target genes, 468-ERβ#18 cells were treated with or without Dox followed by treatment with E2 for 4 hours. RNA-seq was subsequently performed as described in the *Materials and Methods*. Reads were aligned using RSEM (20), and the results of the alignment are shown in Supplemental Table 2. Differentially expressed genes were determined for the four conditions by DESeq (25) (Figure 5A, 1.5-fold change cutoff). No genes were differentially expressed after E2 treatment in the absence of ERβ (DMSO vs E2, comparison I), confirming that MDA-MB-468 cells are not estrogen responsive. Only 109 genes were differentially expressed after Dox treatment (DMSO vs DMSO+ERβ, comparison II), whereas 481 genes were differentially expressed after E2 treatment

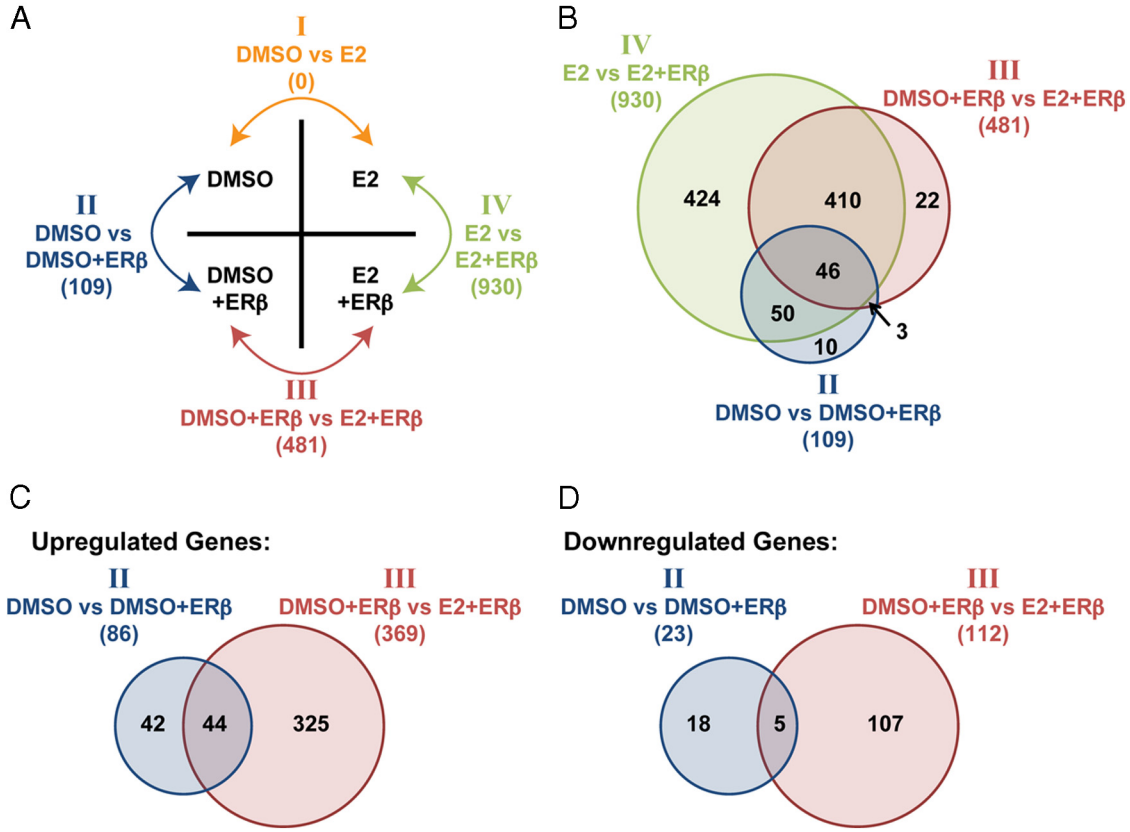


**Figure 5.** Global identification of ERβ target genes using RNA-seq. 468-ERβ#18 cells were treated with vehicle or Dox for 48 hours followed by treatment with DMSO or 10 nM E2 for 4 hours. Total RNA was extracted and sequenced as described in the *Materials and Methods*. Differentially expressed genes were identified using DESeq with a 1.5-fold change cutoff and a false discovery rate <0.01. (A) A matrix describing the four contrasts performed to identify differentially expressed genes. No genes were differentially expressed in the DMSO vs E2 group (I), 109 were differentially expressed in the comparison between DMSO vs DMSO+ERβ (II), 481 genes were differentially expressed between DMSO+ERβ vs E2+ERβ (III), and 930 genes were differentially expressed between E2 vs E2+ERβ (IV). (B) A Venn diagram showing the overlap among genes identified in the three groups. (C) A Venn diagram showing the overlap between the up-regulated genes identified in DMSO vs DMSO+ERβ and DMSO+ERβ vs E2+ERβ. Most ERβ target genes were up-regulated, and approximately half of the DMSO vs DMSO+ERβ genes were identified in DMSO+ERβ vs E2+ERβ. (D) A Venn diagram showing the overlap between the down-regulated genes identified in DMSO vs DMSO+ERβ and DMSO+ERβ vs E2+ERβ. Fewer ERβ target genes were down-regulated, and only five genes were identified in both groups.

in the presence of ERβ (DMSO+ERβ vs E2+ERβ, comparison III), suggesting that most ERβ target genes are ligand-dependent in these cells. The top genes regulated in comparisons II and III are shown in Tables 1 and 2, respectively. A comparison of E2 treatment with or without Dox (E2 vs E2+ERβ, comparison IV) revealed 930 differentially expressed genes, potentially capturing the total change in gene expression in response to both ERβ expression and activation by E2. The top genes in this comparison are shown in Table 3.

Most of the genes in comparisons II and III were captured in comparison IV (Figure 5B). In addition, most genes identified in comparisons II and III were up-regulated, and about half of the potentially ligand-independent genes (comparison II) were also regulated in a ligand-dependent manner (Figure 5C). Fewer genes were found to be down-regulated (Figure 5D). Several genes were validated by quantitative PCR in a new set of biological replicates of 468-ERβ#18 cells treated with vehicle or Dox followed by DMSO or E2 for 4 hours. Two ligand-independent genes were confirmed (*CTGF* and *C3*; Figure 6A) and three ligand-dependent genes were confirmed (*SDC1*, *CDH1*, and *DKK1*; Figure 6B). Four genes regulated in both a ligand-dependent and a ligand-independent manner were also validated (*WNT4*, *THRSP*, *CABLES1*, and *S100A7*; Figure 6C). Importantly, the expression of several ERβ target genes was assessed in MDA-MB-468-Tet cells treated with vehicle or Dox, and none of the genes were regulated by Dox in the absence of ERβ expression (Supplemental Figure 5). In addition, the ERβ-selective ligand ERB-041 was found to regulate ERβ target genes similar to E2 (Figure 7, A and B).

## ERβ target genes are enriched in pathways regulating cell proliferation, differentiation, and cell cycle

After globally identifying the ERβ target genes in ERα-negative TNBC cells, we conducted a pathway analysis of

**Table 1.** Most Highly Up-regulated and Down-regulated Genes in the DMSO vs DMSO+ERβ Comparison

| Up-regulated Genes | | Down-regulated Genes | |
|---|---|---|---|
| Gene Symbol | Fold Change | Gene Symbol | Fold Change |
| *ESR2* | 92.93 | *TMEM189-UBE2V1* | 0.25 |
| *FGF19* | 44.42 | *ACER1* | 0.26 |
| *PDZK1* | 13.76 | *NOV* | 0.32 |
| *SCGB2A2* | 10.70 | *ADAMTS5* | 0.41 |
| *THRSP* | 9.25 | *CTGF* | 0.43 |
| *EPHA8* | 8.11 | *NDRG1* | 0.49 |
| *RBP3* | 7.55 | *KDR* | 0.52 |
| *CA12* | 6.28 | *KRT6B* | 0.54 |
| *CYP2A13* | 5.60 | *NTRK2* | 0.55 |
| *MAPK4* | 5.04 | *KRT14* | 0.57 |
| *CD34* | 4.77 | *IGFBP3* | 0.57 |
| *KCNK15* | 4.73 | *CLIC5* | 0.58 |
| *CYP2B7P1* | 4.10 | *MAOA* | 0.59 |
| *C3* | 3.69 | *FOXQ1* | 0.59 |
| *ST8SIA6* | 3.57 | *SYTL4* | 0.59 |
| *S100A7A* | 3.49 | *KRT16* | 0.61 |
| *MGAT3* | 3.42 | *CASP14* | 0.61 |
| *S100A7* | 3.39 | *PPP1R3C* | 0.62 |
| *ANPEP* | 3.31 | *SMOC1* | 0.63 |
| *TMEM229B* | 3.04 | *KRT17* | 0.63 |

the total ERβ target genes identified in comparison IV (Figure 5A) using IPA. These ERβ target genes were enriched in several molecular and cellular functions, including cell movement, cell death and necrosis, and cell differentiation (Table 4). In addition, genes involved in cell proliferation and cell morphology were enriched in the ERβ target genes. Similar molecular and cellular functions were enriched in genes identified in comparisons II and III (Figure 5A), representing potential ligand-independent and ligand-dependent targets, respectively (Supplemental Materials and Methods). The top pathways enriched in the total ERβ target gene set included the Wnt/β-catenin pathway and the G1/S cell cycle checkpoint regulation pathway (Table 5). Indeed, several genes involved in Wnt signaling were verified as ERβ target genes, including *DKK1*, *WNT4*, and *CDH1* (Figure 6, B and C). In addition, the gene encoding the cyclin-dependent kinase inhibitor p21, *CDKN1A*, was found to be up-regulated by ERβ expression and E2 treatment (Figure 7A). The pathway analysis results support the potential for ERβ to regulate key cellular process that may ultimately play a role in growth inhibition.

**Table 2.** Most Highly Up-regulated and Down-regulated Genes in the DMSO+ERβ vs E2+ERβ Comparison

| Up-regulated Genes | | Down-regulated Genes | |
|---|---|---|---|
| Gene Symbol | Fold Change | Gene Symbol | Fold Change |
| *EGR3* | 43.43 | *ADAMTS5* | 0.13 |
| *OTOF* | 34.18 | *CHRM1* | 0.25 |
| *SHISA2* | 26.24 | *ADAMTS1* | 0.27 |
| *ASB2* | 18.22 | *LOC152225* | 0.29 |
| *PTH1R* | 17.93 | *STON1* | 0.33 |
| *CYTH4* | 15.16 | *SOX11* | 0.34 |
| *LOC100507584* | 14.34 | *RASSF10* | 0.36 |
| *BSND* | 14.19 | *KLHL38* | 0.39 |
| *TMIE* | 10.74 | *ARL4A* | 0.40 |
| *ENPP2* | 10.51 | *ARID5B* | 0.41 |
| *LOXL4* | 9.85 | *ABCA1* | 0.42 |
| *IP6K3* | 8.89 | *JAG1* | 0.42 |
| *PDK4* | 8.80 | *VAV3* | 0.42 |
| *FAM25A* | 8.65 | *FAM84A* | 0.44 |
| *HAND1* | 8.13 | *SERTAD4* | 0.44 |
| *A4GALT* | 8.13 | *PPP1R3C* | 0.44 |
| *WISP2* | 7.88 | *RDH10* | 0.44 |
| *VWF* | 7.67 | *PPP1R3C* | 0.62 |
| *THRSP* | 7.65 | *SMOC1* | 0.63 |
| *CABP7* | 6.75 | *KRT17* | 0.63 |

**Table 3.** Most Highly Up-regulated and Down-regulated Genes in the E2 vs E2+ERβ Comparison

| Up-regulated Genes | | Down-regulated Genes | |
|---|---|---|---|
| Gene Symbol | Fold Change | Gene Symbol | Fold Change |
| *ISM1* | 99.57 | *ADAMTS5* | 0.06 |
| *FGF19* | 95.53 | *STON1* | 0.15 |
| *EGR3* | 85.29 | *DKK1* | 0.16 |
| *WISP2* | 69.21 | *ADAMTS1* | 0.16 |
| *ESR2* | 65.01 | *CHRM1* | 0.20 |
| *THRSP* | 54.88 | *HRCT1* | 0.23 |
| *CYTH4* | 53.59 | *GRIN2A* | 0.23 |
| *OTOF* | 51.63 | *KLHL38* | 0.24 |
| *ENPP2* | 39.12 | *NOV* | 0.24 |
| *SCGB3A1* | 36.32 | *PPP1R3C* | 0.25 |
| *CD34* | 29.79 | *SOX11* | 0.25 |
| *PDLIM4* | 27.72 | *IL8* | 0.26 |
| *ANGPTL2* | 26.90 | *FGF1* | 0.26 |
| *PGLYRP2* | 26.83 | *RASSF10* | 0.27 |
| *LOC727710* | 25.48 | *FOXQ1* | 0.28 |
| *SHISA2* | 25.25 | *SHROOM2* | 0.28 |
| *CRB2* | 25.05 | *ARL4A* | 0.28 |
| *TMIE* | 24.75 | *ACER1* | 0.28 |
| *FOXN1* | 24.04 | *FAM105A* | 0.29 |
| *MGAT3* | 23.58 | *IGFBP3* | 0.29 |

## ERβ target genes are regulated in additional breast cancer cell lines and correlate with ERβ expression in TNBCs

To confirm that a common set of ERβ target genes are shared among TNBC cells, the genes identified in MDA-MB-468-ERβ cells were compared to those identified in other TNBC cell lines. First, the gene list was compared to that obtained by Secreto and coworkers (15) in which Hs578T breast cancer cells with inducible ERβ expression were used to identify ligand-dependent ERβ target

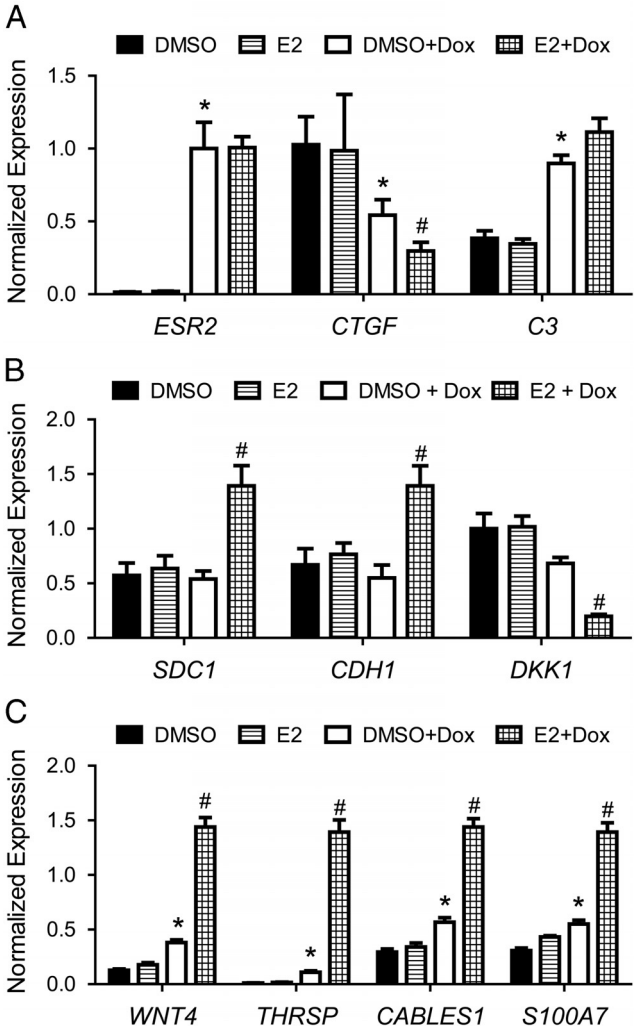


**Figure 6.** Validation of ERβ target genes in 468-ERβ#18 cells. 468-ERβ#18 cells were treated with vehicle or Dox for 48 hours followed by treatment with DMSO or 10 nM E2 for 4 hours. Quantitative PCR was performed as described in the *Materials and Methods*. (A) Expression of *ESR2* and 2 ligand independent target genes: *CTGF* and *C3*. (B) Expression of three ligand-dependent target genes: *SDC1*, *CDH1*, and *DKK1*. (C) Expression of four genes regulated in both ligand-independent and ligand-dependent manners: *WNT4*, *THRSP*, *CABLES1*, and *S100A7*. Data represent the mean and SEM of three independent replicates. *, $P < .05$ compared to DMSO control; #, $P < .05$ compared to DMSO+Dox control.

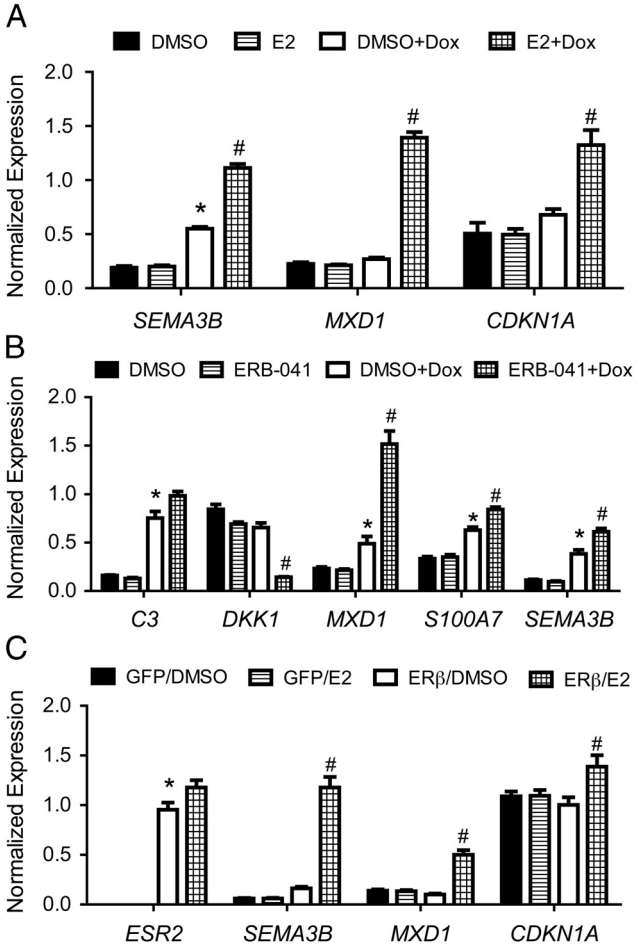


**Figure 7.** ERβ expression regulates common target genes in HCC1143 TNBC cells. (A) Quantitative PCR validation of *SEMA3B*, *MXD1*, and *CDKN1A* in 468-ERβ#18 cells treated with vehicle or Dox for 24 hours followed by treatment with DMSO or 10 nM E2 for 4 hours. Data represent the mean and SEM of three independent replicates. *, $P < .05$ compared to DMSO control; #, $P < .05$ compared to DMSO+Dox control. (B) Quantitative PCR validation of *ESR2*, *SEMA3B*, *MXD1*, and *CDKN1A* in HCC1143 TNBC cells infected with retrovirus for GFP or ERβ expression followed by treatment with DMSO or 10 nM E2 for 4 hours. The data represent the mean and SEM of three replicates. *, $P < .05$ compared to GFP/DMSO control; #, $P < .05$ compared to ERβ/DMSO control. (C) Quantitative PCR validation of *SEMA3B*, *MXD1*, and *CDKN1A* in 468-ERβ#18 cells treated with vehicle or Dox for 24 hours followed by treatment with DMSO or 100 nM ERB-041 for 4 hours. Data represent the mean and SEM of three independent replicates. *, $P < .05$ compared to DMSO control; #, $P < .05$ compared to DMSO+Dox control.

genes after 24 hours of E2 treatment. Although the authors used a targeted microarray to assess changes in gene expression, 27 genes were found to be commonly regulated in both MDA-MB-468-ERβ and Hs578T-ERβ cells (Table 6). Most of these genes were regulated in the same direction, with the exceptions of *BMP5*, *CEBPD*, and *PMAIP1*. Four ERβ target genes were then verified in another TNBC cell line, HCC1143, infected with retrovirus encoding green fluorescent protein (GFP) as a control or full-length ERβ followed by treatment with DMSO or E2 for 4 hours (Figure 7C). Indeed, all four genes including the cell cycle regulator *CDKN1A* were regulated in a similar manner in HCC1143 when ERβ was expressed and activated by E2 treatment. These data suggest that ERβ regulates multiple common targets across TNBC cell lines.

The ERβ target genes identified in MDA-MB-468-ERβ cells were next compared to ERβ target genes identified by Grober and colleagues (30) in ERα-positive MCF7 cells engineered to express full-length ERβ. Using chromatin precipitation followed by deep sequencing for ERβ binding sites and gene expression microarrays, they found

**Table 4.** Top Biological Functions in the E2 vs E2+ERβ Gene Set

| Top Biological Functions, Molecular and Cellular Functions | *P* Value | No. of Molecules |
|---|---|---|
| Cell movement | | |
| Cell movement | 1.21E-18 | 166 |
| Migration of cells | 2.73E-18 | 154 |
| Cell death and survival | | |
| Necrosis | 2.19E-17 | 201 |
| Cell death | 2.66E-16 | 236 |
| Cellular development | | |
| Differentiation of cells | 6.66E-17 | 129 |
| Proliferation of tumor cell lines | 1.54E-12 | 143 |
| Cellular growth and proliferation | | |
| Proliferation of tumor cell lines | 1.54E-12 | 143 |
| Proliferation of cells | 1.70E-12 | 249 |
| Cellular morphology | | |
| Formation of lamellipodia | 4.56E-09 | 22 |
| Collapse of growth cone | 2.31E-07 | 12 |

424 "primary" ERβ target genes that were differentially expressed when ERβ was expressed and contained ERβ binding sites within 10 kb of the gene. Of the 930 ERβ target genes identified in MDA-MB-468-ERβ cells (comparison IV, Figure 5A), 99 genes (10.6%) were also identified as primary ERβ targets. Fifty-two of these genes were differentially regulated in cells expressing ERβ and not in parental MCF7 cells (Supplemental Materials and Methods). These results indicate that despite the differences in cell lines (ie, ERα-negative basal-like cells versus ERα-positive luminal cells), ERβ can regulate common target genes. Most of these genes were also regulated in the absence of ERβ or contained ERα binding sites, but three genes were found to contain only ERβ binding sites and were only regulated when ERβ was expressed: *CCDC103*, *IGSF9*, and *P2RY6*. Although *CCDC103* was only identified in comparison IV (Figure 5A), *IGSF9* was found to be a ligand-dependent target (comparison III) and *P2RY6* was a ligand-dependent target (comparison II) in MDA-MB-468-ERβ cells.

**Table 5.** Top Pathways in the E2 vs E2+ERβ Gene Set

| Top Canonical Pathways | *P* Value | Ratio |
|---|---|---|
| Hepatic fibrosis/hepatic stellate cell activation | 7.24E-07 | 0.16 |
| Axonal guidance signaling | 2.57E-05 | 0.09 |
| Wnt/β-catenin signaling | 1.91E-04 | 0.12 |
| Role of IL-17A in psoriasis | 3.63E-04 | 0.39 |
| Aryl hydrocarbon receptor signaling | 6.17E-04 | 0.11 |
| Molecular mechanisms of cancer | 8.51E-04 | 0.09 |
| Glioblastoma multiforme signaling | 1.66E-03 | 0.10 |
| Human embryonic stem cell pluripotency | 1.82E-03 | 0.10 |
| Coagulation system | 2.04E-03 | 0.18 |
| Glutamate receptor signaling | 2.45E-03 | 0.13 |
| Cell cycle: G1/S checkpoint regulation | 3.55E-03 | 0.14 |

To validate the ERβ target genes in TNBCs, a correlation analysis was performed using gene expression data from 43 TNBCs available through TCGA (26). Of the 930 ERβ target genes identified in MDA-MB-468-ERβ cells (comparison IV, Figure 5A), 109 genes were significantly correlated with *ESR2* expression ($q < .05$, denoted by points in Figure 8; a complete list is provided in Supplemental File 2). Fifteen of these were found to be ligand-independent in MDA-MB-468-ERβ (identified comparison II, Figure 5A), and 58 of them were regulated in a ligand-dependent manner (identified in comparison III, Figure 5A). Six of these genes were also identified in Hs578T-ERβ cells (highlighted in red in Figure 8), thereby validating that these genes are likely ERβ target genes in TNBCs. However, two genes (*ADAMTS1* and *ADAMTS5*) showed a positive correlation with ERβ expression but were down-regulated in both cell lines, suggesting that ERβ target gene regulation in breast cancer samples is not completely recapitulated by the cell culture models. In addition, two of the genes identified as primary ERβ target genes by Grober et al (30) were significantly correlated with *ESR2* expression in the TCGA cohort (highlighted in green in Figure 8), although the correlation coefficients were quite low. This could be due to the difference in breast cancer subtypes: Grober and colleagues used an ERα-positive cell line to identify ERβ target genes, while only TNBCs were included in the TCGA analysis. Overall, these results validate that a set of ERβ target genes identified in cell culture models are correlated with ERβ expression in TNBCs.

## Discussion

Because ERβ is expressed in 50% to 90% of ERα-negative breast cancers (1–4) and its expression has been associated with a better prognosis in TNBCs (2), ERβ may be a therapeutic target for a subset of TNBCs. The results of this study support this hypothesis and confirm the growth inhibitory effects of ERβ expression and activation in TNBC cells. However, the requirement of ligand to stimulate ERβ's effects is likely cell context specific. Previously, ERβ expression was found to inhibit ERα-negative breast cancer cell growth independent of ligand in MDA-MB-231 and SKBR3 cells (12, 13). In Hs578T TNBC cells, ERβ expression inhibited cell growth in a ligand-dependent manner through a G1 cell cycle arrest (15). In culture, ERβ expression also induced a G1 arrest in MDA-MB-468 cells, but this effect was both E2-independent and -dependent. One way that ERβ may regulate the cell cycle is through up-regulation of the cyclin-dependent kinase inhibitor p21 (encoded by *CDKN1A*), which

**Table 6.** Common ERβ Target Genes Identified in Hs578T-ERβ (15) and 468-ERβ#18

| Gene Symbol | Gene Name | Fold Change MDA468-ERβ | Fold Change Hs578T-ERβ |
|---|---|---|---|
| *ADAMTS1* | ADAM metallopeptidase with thrombospondin type 1 motif, 1 | 0.2 | 0.4 |
| *ADAMTS5* | ADAM metallopeptidase with thrombospondin type 1 motif, 5 | 0.1 | 0.4 |
| *BMP5* | Bone morphogenetic protein 5 | 0.5 | 2.6 |
| *C3* | Complement component 3 | 4.4 | 12.8 |
| *CA12* | Carbonic anhydrase XII | 10.5 | 2.9 |
| *CD34* | CD34 molecule | 29.8 | 2.1 |
| *CEBPD* | CCAAT/enhancer binding protein (C/EBP), δ | 0.5 | 2.7 |
| *CTGF* | Connective tissue growth factor | 0.3 | 0.4 |
| *DKK1* | Dickkopf homolog 1 (*Xenopus laevis*) | 0.2 | 0.3 |
| *EHD2* | EH-domain containing 2 | 2.9 | 2.3 |
| *ENPP2* | Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin) | 39.1 | 2.0 |
| *FUT8* | Fucosyltransferase8 (α [1,6] fucosyltransferase) | 2.1 | 2.1 |
| *IGFBP3* | Insulin-like growth factor binding protein 3 | 0.3 | 0.4 |
| *IGFBP5* | Insulin-like growth factor binding protein 5 | 4.6 | 2.5 |
| *JAG1* | Jagged 1 (Alagille syndrome) | 0.3 | 0.1 |
| *LMO2* | LIM domain only2 (rhombotin-like 1) | 1.5 | 3.3 |
| *PDE4B* | Phosphodiesterase 4B, cAMP-specific | 2.0 | 2.3 |
| *PFKFB3* | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 | 4.5 | 2.1 |
| *PIM1* | Pim-1 oncogene | 2.2 | 2.3 |
| *PLAC1* | Placenta-specific 1 | 2.2 | 3.1 |
| *PLAU* | Plasminogen activator, urokinase | 0.5 | 0.4 |
| *PMAIP1* | Phorbol-12-myristate-13-acetate-induced protein 1 | 2.1 | 0.5 |
| *SEMA3B* | Sema domain, immunoglobulin domain (Ig), (semaphorin) 3B | 7.2 | 3.6 |
| *SFRP1* | Secreted frizzled-related protein 1 | 1.8 | 2.1 |
| *TH* | Tyrosine hydroxylase | 17.7 | 2.2 |
| *TPD52L1* | Tumor protein D52-like 1 | 1.6 | 2.0 |
| *WISP2* | WNT1 inducible signaling pathway protein 2 | 69.2 | 3.0 |

regulates the progression from G1 to S phase. Indeed, one of the top pathways identified in ERβ target genes was the G1/S checkpoint regulation pathway (Table 5), which includes *CDKN1A*. In MDA-MB-231 TNBC cells, ERβ increased the expression of p21 via interaction with another transcription factor, inhibitor of differentiation-1 (14). Future studies are warranted to determine if inhibitor of differentiation-1 plays a role in the regulation of *CDKN1A* in MDA-MB-468 cells.

ERβ-mediated growth inhibition may also depend on the cellular environment. ERβ was found to elicit growth inhibitory effects in vitro and in vivo in MDA-MB-468-ERβ cells. In contrast, in mammary epithelial and breast cancer cells that endogenously coexpress ERα and ERβ, ERβ-selective ligands were found to induce apoptosis in vitro while promoting tumor growth in vivo (31) by coincubation with a basement membrane extract, suggesting that the cellular environment can modulate ERβ activity. Inhibition of extracellular signal-regulated kinase 1 and 2 (ERK1/2) could restore ERβ growth inhibitory effects (31). It appears that cellular context modulates ERβ growth inhibitory effects. Either the coexpression of ERα or the activation of kinase pathways in the mammary epithelial cell model may account for the differential in vivo effects of ERβ in the MDA-MB-468 orthotropic xenograft model.

Like ERα, ERβ has two activation functions (AF-1 and AF-2) that confer transcriptional activity. The AF-1 can mediate transcription independent of ligand, and the AF-2 functions in a ligand-dependent manner (32). Although the AF-1 of ERβ was found to have negligible activity in a Gal4 assay (33), ERβ was found to interact with DNA in a ligand-independent manner (34). In addition, MAP kinase has been shown to phosphorylate the N-terminal activation function (AF-1) of ERβ, thereby promoting the recruitment of steroid receptor coactivator 1, which confers ligand-independent transcriptional activity (35). In MDA-MB-468 cells, ERβ appears to have both ligand-independent and -dependent activity in regard to target gene expression. Interestingly, most target genes were regulated in response to E2 treatment, suggesting strong ligand-dependent activity in these cells. In contrast, most ERβ target genes were regulated independent of ligand in U2OS osteosarcoma cells (36). Therefore, the ligand-independent activity of ERβ is likely the result of the availability of cofactors, the activation of signaling pathways, and the promoter context.

Both the ligand-dependent and -independent ERβ target genes identified in MDA-468-ERβ cells were enriched in genes that regulate cell death, proliferation, movement, and morphology. ERβ target genes identified after 24

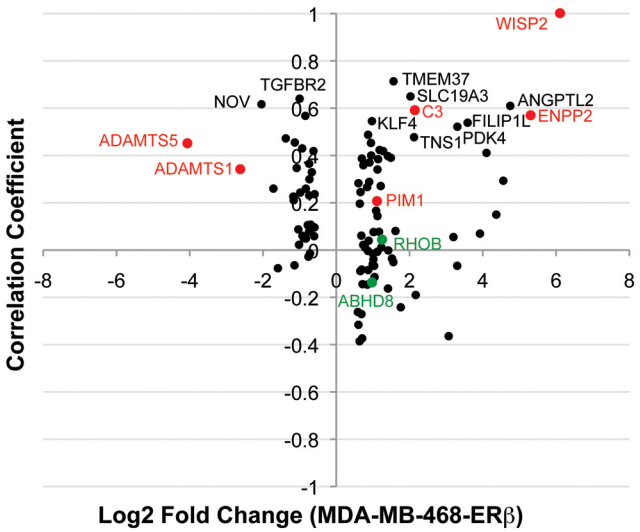


**Figure 8.** ERβ target genes are correlated with ERβ expression in a cohort of TNBCs. Gene expression data from the TCGA (26) was used to perform a correlation analysis of ERβ target genes and ERβ expression. Of the 930 genes identified 468-ERβ#18 cells (comparison IV, Figure 5A), 109 genes were significantly correlated with ERβ expression ($q < .05$). The correlation coefficient versus the fold change observed in the RNA-seq results for each gene is shown in the scatterplot. The gene symbols of those genes with the highest correlation coefficients are shown on the plot. Genes in red are those that were also identified in Hs578T-ERβ cells (15), and genes in green are those that were identified in MCF7-ERβ cells (30).

hours of E2 treatment in HEK293 cells were found to be enriched in many of the same pathways (37). A pathway analysis of total ERβ target genes revealed significant enrichment of several pathways, including the Wnt/β-catenin pathway. Of the genes involved in the Wnt/β-catenin pathway, ERβ expression and E2 treatment resulted in the up-regulation of *WNT4* (a noncanonical Wnt ligand) and *CDH1* (a β-catenin interacting protein), as well as the down-regulation of *DKK1* (an inhibitor of Wnt signaling). Down-regulation of *DKK1* by ERβ expression and estrogen treatment was also observed in Hs578T breast cancer cells (15) and 293T kidney cells (37). Collectively, the gene expression data suggest that ERβ expression may affect the Wnt signaling pathway in breast cancer cells. Although activation of Wnt signaling can transform human mammary epithelial cells (38), a recent study has shown that paracrine Wnt signaling in fibroblasts can either promote or inhibit breast cancer cell growth in mice (39). ERβ has been detected in both mammary epithelial and stromal cells (40) and may therefore affect Wnt signaling in a cell type- and cell context-dependent manner.

Given the heterogeneous nature of TNBCs (41), identification of common ERβ target genes across all TNBCs would be challenging. Recently TNBC cell lines were classified into basal-like, mesenchymal-like, and luminal-androgen receptor subtypes by correlation with expression profiles of large breast cancer data sets (42). Although the RNA-seq was performed in basal-like MDA-MB-468 cells, we verified ERβ target gene expression in Hs578T, a mesenchymal-like cell line, and HCC1143, another basal-like cell line. Despite differences in the cell line and experimental design, 27 genes identified in MDA-MB-468-ERβ cells were found to be ERβ target genes in Hs578T-ERβ cells treated with E2 for 24 hours (15). Four ERβ target genes selected for verification showed the similar regulation by ERβ and E2 in HCC1143 and MDA-MB-468 cells (Figure 7C). In addition, an ERβ target gene identified in MDA-MB-468-ERβ cells, *S100A7*, was found to be an ERβ-specific target gene in MCF7 ERα-positive breast cancer cells with inducible expression of ERβ and was associated with ERβ in ERα-negative breast tumors (43). Several ERβ targets were also found to be primary ERβ target genes in MCF7 cells (30) and are likely direct targets of the receptor in MDA-MB-468-ERβ cells. Most importantly, 109 ERβ target genes were correlated with ERβ expression in a cohort of 43 TNBCs, including six genes that were also identified in Hs578T TNBC cells. Thus, the genes identified in this study provide the foundation for identifying common target genes of ERβ functionality across ERα-negative breast cancers, including TNBCs.

Overall, this study confirms the growth inhibitory effects of ERβ in TNBC cells, both in vitro and in vivo, and provides the most comprehensive identification of ERβ target genes in TNBC to date. These data also suggest that ERβ selective ligands may be useful for targeting ERβ in a subset of ERα-negative breast cancers, including TNBCs. ERβ-selective ligands could promote the inhibitory effects of ERβ while avoiding the proliferative effects mediated by ERα. ERβ selective ligands, such as ERB-041, have been found to be well-tolerated (44), supporting the use of these compounds clinically. In addition, the identification of ERβ target genes provides a foundation for identifying biomarkers of ERβ functionality in TNBCs. Collectively, these data support the hypothesis that ERβ may be a therapeutic target in a subset of TNBCs and suggest that ERβ may be targeted with ERβ-selective ligands that can stimulate its growth inhibitory effects.

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