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Estrogen receptor-beta sensitizes breast cancer cells to the anti-estrogenic actions of endoxifen

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Abstract

Introduction

We have previously demonstrated that endoxifen is the most important tamoxifen metabolite responsible for eliciting the anti-estrogenic effects of this drug in breast cancer cells expressing estrogen receptor-alpha ($ER\alpha$). However, the relevance of $ER\beta$ in mediating endoxifen action has yet to be explored. Here, we characterize the molecular actions of endoxifen in breast cancer cells expressing $ER\beta$ and examine its effectiveness as an anti-estrogenic agent in these cell lines.

Methods

MCF7, Hs578T and U2OS cells were stably transfected with full-length $ER\beta$. $ER\beta$ protein stability, dimer formation with $ER\alpha$ and expression of known ER target genes were characterized following endoxifen exposure. The ability of various endoxifen concentrations to block estrogen-induced proliferation of MCF7 parental and $ER\beta$ -expressing cells was determined. The global gene expression profiles of these two cell lines was monitored following estrogen and endoxifen exposure and biological pathway analysis of these data sets was conducted to identify altered cellular processes.

Results

Our data demonstrate that endoxifen stabilizes $ER\beta$ protein, unlike its targeted degradation of $ER\alpha$, and induces $ER\alpha/ER\beta$ heterodimerization in a concentration dependent manner. Endoxifen is also shown to be a more potent inhibitor of estrogen target genes when $ER\beta$ is expressed. Additionally, low concentrations of endoxifen observed in tamoxifen treated patients with deficient CYP2D6 activity (20-40 nM) markedly inhibit estrogen-induced cell proliferation rates in the presence of $ER\beta$, whereas

much higher endoxifen concentrations are needed when ER β is absent. Microarray analyses reveal substantial differences in the global gene expression profiles induced by endoxifen at low concentrations (40nM) when comparing MCF7 cells which express ER β to those that do not. These profiles implicate pathways related to cell proliferation and apoptosis in mediating endoxifen effectiveness at these lower concentrations.

Conclusions

Taken together, these data demonstrate that the presence of ER β enhances the sensitivity of breast cancer cells to the anti-estrogenic effects of endoxifen likely through the molecular actions of ER α/β heterodimers. These findings underscore the need to further elucidate the role of ER β in the biology and treatment of breast cancer and suggest that the importance of pharmacologic variation in endoxifen concentrations may differ according to ER β expression.

Introduction

Each year, nearly 1.3 million women are diagnosed with breast cancer worldwide and about two-thirds of these individuals are determined to have hormone sensitive tumors based on the expression of estrogen receptor-alpha ($ER\alpha$). Tamoxifen, a selective estrogen receptor modulator (SERM), remains an important therapeutic agent in the treatment of women with endocrine sensitive breast cancer as it is known to effectively inhibit the proliferation-inducing effects of 17β -estradiol (estrogen) in $ER\alpha$ positive breast tumor cells.

Like many drugs, tamoxifen is extensively metabolized in the body by the cytochrome P450 enzyme system resulting in the production of three primary metabolites; 4-hydroxytamoxifen (4HT), N-desmethyl-tamoxifen (NDT) and endoxifen [1-3]. Recent reports have demonstrated that steady state circulating levels of tamoxifen, 4HT, and NDT in women receiving the standard dose of tamoxifen therapy (20 mg/day) are 300 nM, 7nM, and 700 nM respectively [4]. However, plasma endoxifen concentrations are highly variable, ranging from 5-180 nM, due to the activity of the cytochrome P450 2D6 (CYP2D6) mediated oxidation of NDT [3]. Prospective studies have demonstrated that genetic CYP2D6 polymorphisms, and drugs, which reduce or abrogate CYP2D6 enzyme activity, significantly decrease endoxifen plasma concentrations [3-5]. These findings encouraged investigators to examine the hypothesis that CYP2D6 genotype status, and thus endoxifen concentrations, would affect clinical outcome in women treated with tamoxifen for their breast cancer. Although some controversy remains, the majority of the reports indicate a relationship between CYP2D6-related low levels of endoxifen and poor outcomes [6-15]. Past studies from this

laboratory support these clinical findings as we have demonstrated that endoxifen is the most potent tamoxifen metabolite responsible for inhibiting estrogen induced gene expression changes and proliferation rates in ER α positive breast cancer cells at clinically relevant concentrations [16]. At this time, the clinical development of endoxifen is ongoing, with NCI supported phase I studies of endoxifen hydrochloride set to commence in early 2011 at both the Mayo Clinic and NCI.

Tamoxifen and its metabolites are known to function by blocking the effects of estrogen, a steroid hormone that binds to, and activates, two main ER isoforms, ER α and ER β . The role of ER α in breast cancer has been studied extensively for years, and its protein expression remains the most important biomarker in the treatment of this disease. However, the potential functions of ER β in the progression and treatment of breast cancer have largely remained a mystery. *In vitro* studies have revealed that the actions of these two receptors are drastically different at the level of gene expression, both in response to estrogen and anti-estrogens [17-23]. Numerous reports have demonstrated that exposure of ER α expressing breast cancer cells to estrogen results in increased rates of proliferation while more recent studies have suggested that expression of ER β alone [21, 24, 25], or in combination with ER α [26-28], inhibits cell proliferation following estrogen exposure.

The summation of these *in vitro* studies suggests that ER β may function as a tumor suppressor. A number of clinical studies have revealed that the presence of ER β protein in breast tumors correlates with improved rates of recurrence, disease-free survival and overall survival [29-38] while others indicate little correlation [39-41] or even worse prognosis [42, 43]. Additional studies have suggested that the expression of

ER β in breast tumors increases the effectiveness of tamoxifen therapy [44-46] and one report found that 47% of breast tumors classified as ER α negative express ER β [33]. These observations highlight the need to further define the relevance of ER β in breast cancer progression and treatment.

Based on the fore-going, the objective of the present study was to determine the role of ER β in mediating endoxifen action in breast cancer cells. The results of this study demonstrate that ER β enhances the anti-estrogenic effects of endoxifen in breast cancer cells likely through the actions of ER α/β heterodimers, and suggest that the achievement of higher endoxifen concentration may not be necessary in patients whose tumors express ER β and that these same patients may benefit from tamoxifen therapy regardless of their CYP2D6 genotype.

Materials and methods

Cell culture, chemicals and reagents

MCF7 cells were generously provided by Dr. Robert Clarke (Georgetown University) and were cultured in phenol red-free Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium containing 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) antibiotic-antimycotic (AA) solution in a humidified 37°C incubator with 5% CO₂. MCF7 cells stably expressing ER β were generated using an S-tagged-Flag-tagged ER β expression construct (pIRES2-EGFP) developed in our laboratory. Individual MCF7-ER β clones were isolated following selection with 300 μ L/mL G418. Doxycycline inducible Hs578T-ER β , U2OS-ER β and U2OS-ER α/β cells lines were cultured as previously described [19-21]. All cell treatments were conducted in phenol

red-free DMEM/F12 medium containing 10% triple charcoal stripped FBS. 17 β -estradiol and doxycycline were purchased from Sigma Aldrich (St. Louis, MO). PPT and DPN, were purchased from Tocris Biosciences Inc. (Baldwin, MO). (Z)-endoxifen was synthesized by Dr. Abdul Fauq (Mayo Clinic, Jacksonville, FL).

Antibodies

The polyclonal ER β specific antibody utilized in this study was developed by this laboratory. Briefly, a protein fragment of ER β spanning aminoacids 1-140 was cloned into the pGEX-5X-3 vector (Life Technologies, Carlsbad, CA) and expressed in DH5 α bacterial cells. Purified ER β protein was immunized in rabbits by Cocalico Biologicals Inc (Reamstown, PA). ER β specific antibodies were purified from serum using an affinity purification column containing the ER β protein fragment. We have extensively characterized this antibody through western blotting, immunoprecipitation, immunohistochemistry and immunofluorescence using multiple cell model systems which express either no ERs, ER α alone, ER β alone or a combination of ER α and ER β , to ensure its specificity for the β isoform. These data have revealed that this antibody is highly sensitive for the detection of ER β and exhibits no cross-reaction with ER α or other proteins (data not shown). ER α (H-20) antibody was purchased from Santa Cruz (Santa Cruz, CA). Flag (M2) and α -Tubulin (DM 1A) antibodies were purchased from Sigma Aldrich.

Western blotting

All cell lysates were harvested using NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40) and insoluble material was pelleted. Protein concentrations were determined using Bradford Reagent and equal amounts of cell lysate

were separated by SDS-PAGE. Proteins were transferred to PVDF membranes, probed with primary and secondary antibodies and visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Immunofluorescent staining analysis

MCF7-ER β cells were plated on cover slides at approximately 50% confluence and fixed with cold methanol for 1 hour followed by permeabilization with 0.1% Triton X-100 for 5 minutes on ice. Slides were pre-incubated in 5% goat serum for 1 hour followed by exposure to Flag antibody for another hour. Slides were washed 3 times with 1X PBS and subsequently incubated with Rhodamine-labeled anti-mouse secondary antibody (Sigma-Aldrich) for 30 minutes at room temperature. Nuclei were simultaneously stained with DAPI (Sigma-Aldrich). Immunofluorescent detection was conducted using a Zeiss Laser Scanning Microscope 510.

Luciferase assays

MCF7-ER β cells were plated in 12-well tissue culture plates at approximately 70% confluence and subsequently transfected in triplicate with 250 ng per well of the ERE-TK-luciferase reporter construct using Fugene 6 (Roche Applied Science). Following transfection, cells were treated as indicated for 24 h. Cells were lysed in 1X Passive Lysis Buffer (Promega, Madison, WI) and equal amounts of extract were assayed for luciferase activity.

Co-Immunoprecipitation assays

MCF7-ER β or U2OS-ER α/β cells were plated at a density of approximately 50% in 100 mm tissue culture plates. U2OS cells were treated with doxycycline as previously described to induce expression of ER α and ER β [20] and subsequently exposed to

indicated concentrations of endoxifen. Following 24 hours of incubation, cells were washed twice with PBS and lysed in NETN buffer. Equal amounts of cell lysates were immunoprecipitated at 4°C overnight using 1 µg of either Flag or ERβ antibody. Protein complexes were purified using protein G beads, separated by SDS-PAGE, transferred to PVDF membranes and blocked in 5% milk overnight. Western blotting was performed using indicated antibodies as described above.

Real-time reverse transcriptase polymerase chain reaction

MCF7 and MCF7-ERβ cells were plated in 12 well tissue-culture plates and treated in triplicate as indicated for 24 hours. Total RNA was isolated using Trizol reagent (Invitrogen) and 500 ng was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed in triplicate using a Bio-Rad iCycler (Hercules, CA) and a PerfeCTa™ SYBR Green Fast Mix™ for iQ real-time PCR kit (Quanta Biosciences, Gaithersburg, MD) as specified by the manufacturer.

Quantitation of the PCR results were calculated based on the threshold cycle (C_t) and were normalized using TATA Binding Protein as a control. All PCR primers were designed using Primer3 software [47] and were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences are provided in Additional file 1.

Cell proliferation assays

MCF7 and MCF7-ERβ cells were grown in 10% triple charcoal-stripped serum-containing medium for 3 days and subsequently plated at a density of 2,000 cells per well in 96-well tissue culture plates. Cells were treated with vehicle, 1nM estrogen or 1nM estrogen plus increasing concentrations of endoxifen (20-1000 nM) for 8 days. Culture

medium and treatments were replaced every other day. Proliferation rates were determined using a CellTiter-Glo Luminescent Cell Viability kit (Promega).

Illumina microarray analysis

Changes in the gene expression profiles of MCF7 and MCF7-ER β cells elicited by either 1 nM estrogen alone or estrogen plus 40nM endoxifen were determined using the Illumina HumanHT-12 expression BeadChip platform to screen more than 27,000 annotated genes represented by 48,804 probes by Mayo Clinic's Advanced Genomics Technology Center (Rochester, MN). Data was processed using BeadStudio Version 3.1 and normalized using the fastlo function [48] implemented in the statistical package R. Data were filtered to exclude probes (referred to as genes throughout) whose expression was at or below background levels as determined by detection P-values (≥ 0.05). Pair-wise comparisons were made to identify differentially expressed genes using LIMMA. Genes were determined to be significantly regulated if their differential P-value was < 0.05 between groups. Fold changes were calculated by raising 2 to the power of mean difference (log 2 scale) between the treatment groups and controls. The normalized and raw microarray data presented here is available in the Gene Expression Omnibus [49] under the accession number: [GEO:GSE27375].

Biological Pathway Analysis

Genes determined to be significantly regulated by the addition of 40 nM endoxifen relative to estrogen alone in both parental and ER β expressing MCF7 cells were further analyzed using MetaCore software to identify differences in biological pathways altered between the two cell lines. Genes with differential expression P-values < 0.05 from each comparison were used as focus genes and a hypergeometric test was

applied to each of over 600 canonical pathways. Enriched pathways with P-values <0.05 were suggested to be significantly regulated by the addition of 40 nM endoxifen within each cell line. Adjustment for multiple comparisons was conducted using a false discovery rate of 0.25.

Results

Development and characterization of MCF7-ER β cell lines

In order to determine the effects of ER β expression on the actions of endoxifen in breast cancer, we first developed MCF7 cell lines stably expressing this receptor. MCF7 cells were chosen for this study since they are the most well characterized ER α positive breast cancer cell line with regard to estrogen regulated gene expression and proliferation. As shown in Additional file 2, the parental MCF7 cell line used throughout this study was confirmed to be ER β negative by both real-time PCR and western blotting and these data are shown relative to the expression of ER β mRNA and protein in one of our over-expressing cell lines. Multiple MCF7-ER β clonal cell lines were developed by expressing S and Flag-tagged full-length ER β followed by selection with G418. All cell lines were screened for ER β protein expression by western blotting and three representative lines are shown in Figure 1A relative to parental cells. While all of the data presented in Figures 1, 2, 3, 4 were confirmed in multiple MCF7-ER β clones, the data collected from clone number 3 was chosen for representation in these Figures due to its robust expression of ER β . Immunofluorescent staining using a flag antibody was utilized to verify ER β positivity and cellular localization. The results demonstrate that ER β is expressed and localized in the nucleus of MCF7-ER β cells (Figure 1B). ER β

functionality was investigated using an estrogen response element (ERE) transcriptional assay involving an ERE-TK-luciferase reporter construct. The construct was transfected into parental and ER β expressing MCF7 cells followed by treatment with either estrogen, the ER α specific agonist PPT or the ER β specific agonist DPN. As shown in Figure 1C, estrogen significantly induced ERE activity in both MCF7 parental and ER β expressing cells. Interestingly, estrogen induction of the ERE was significantly lower in ER β expressing cells possibly due to the reported inhibitory actions of ER β on ER α transcriptional activity. The ER β specific agonist, DPN, resulted in significant activation of the ERE reporter construct in cells expressing ER β (Figure 1C). ERE activation in parental MCF7 cells by 10^{-8} M DPN is explained by its non-specific interactions with ER α at high concentrations [50]. As expected, all concentrations of the ER α specific ligand, PPT, resulted in identical ERE activation regardless of ER β expression (Figure 1C). These data demonstrate that our newly developed MCF7-ER β cell lines express intact and functional ER β protein.

ER β protein levels are stabilized by endoxifen

Since we have previously demonstrated that endoxifen exposure results in rapid turnover of ER α protein in multiple cell types through proteasomal degradation [16], it was of interest to determine the effects of endoxifen on ER β protein levels. Unlike that of ER α , endoxifen exposure resulted in stabilization of ER β protein in MCF7-ER β cells in a concentration dependent manner (Figure 2A). These results were confirmed in Hs578T breast cancer cells and U2OS osteosarcoma cells stably expressing ER β (Figure 2 B and C).

Endoxifen induces ER α / β heterodimer formation

Given that ER β protein levels are stabilized by endoxifen and that ER β interacts with ER α , we next sought to determine if endoxifen exposure resulted in ER α/β heterodimer formation. Immunoprecipitation assays demonstrate that endoxifen induces ER α/β heterodimer formation in MCF7-ER β cells which results in the stabilization and accumulation of ER α protein levels (Figure 3A). These studies were conducted in U2OS cells stably expressing both ER isoforms and similar results were observed (Figure 3B). The results of these studies demonstrate that exposure of cells which express both ER isoforms to endoxifen results in stabilization and accumulation of both ER α and ER β protein likely due to its induction of heterodimer formation. It is speculated that ER α and ER β homodimer formation likely occurs to some degree as well.

Endoxifen's ability to inhibit estrogen induced gene expression and proliferation is enhanced by ER β

Our laboratory previously characterized the inhibition of estrogen induced gene expression and proliferation by endoxifen in MCF7 cells [16]. In order to determine the effects of ER β expression on the anti-estrogenic actions of endoxifen, we next compared the ability of endoxifen to inhibit estrogen induction of known ER α target genes in parental and ER β expressing MCF7 cells. The expression levels of cyclinD1, PS2, progesterone receptor and amphiregulin were monitored in both cell lines by real-time PCR following treatment with estrogen alone or estrogen plus increasing concentrations of endoxifen. In contrast to the ERE data presented in Figure 1, estrogen treatment further stimulated the expression of three of the four genes (PS2, PGR and AREG) in ER β expressing cells relative to the parental cell line (Figure 4 A-D). Interestingly, low concentrations of endoxifen (20 nM) significantly inhibited the estrogen induction of

three of the four genes (CCND1, PS2 and PGR) only in ER β expressing cells while higher endoxifen concentrations (100-1000 nM) resulted in similar patterns of gene expression between the two cell lines (Figure 4 A-D). These data suggest that expression of ER β , in ER α positive breast cancer cells, enhances the anti-estrogenic properties of endoxifen.

To confirm these data, and to determine if expression of ER β enhances the ability of endoxifen to suppress estrogen induced cell growth, proliferation assays were performed. Two independent ER β expressing cell lines are shown for these studies to ensure that the results are due to expression of ER β and not clonal variation. Induction of cell proliferation following estrogen treatment was identical between parental and ER β expressing MCF7 cells (Figure 5). Similar to the gene expression data presented in Figure 4, low concentrations of endoxifen (20-40 nM) significantly inhibited estrogen induced growth of MCF7-ER β cells but not parental MCF7 cells (Figure 5). In fact, 100-1000 nM concentrations of endoxifen were required to completely block estrogen induced growth of parental MCF7 cells while 20-40 nM endoxifen concentrations were essentially as effective in ER β positive cells (Figure 5). These studies confirm that ER β expression sensitizes breast cancer cells to the anti-estrogenic actions of endoxifen.

ER β expression results in unique gene expression patterns following estrogen and endoxifen exposure

In an effort to determine the mechanisms by which low concentrations of endoxifen effectively block estrogen induced growth of ER β expressing cells, but not parental MCF7 cells, the global gene expression profiles were examined in these two cell lines following treatment with estrogen alone or estrogen plus 40 nM endoxifen. The 40

nM endoxifen concentration was chosen since it resulted in the largest differences in proliferation rates between parental and MCF7-ER β cells. Microarray analysis revealed that estrogen treatment significantly altered the expression of 461 genes in parental MCF7 cells using a fold change cutoff of 1.5 (Figure 6A). Of these genes, 211 exhibited increased expression while 251 exhibited decreased expression. Nearly 2.5 times as many genes were determined to be significantly regulated in the MCF7-ER β cell line using the same statistical and fold change cutoff parameters. Specifically, 1137 genes were differentially expressed following estrogen treatment of which 604 were increased and 535 were decreased (Figure 6A). Comparison of these two data sets revealed that 381 (31%) were commonly regulated between the two cell lines while only 80 (7%) were unique to the parental cell line and 756 (62%) were unique to the ER β line (Figure 6A). A list of these genes and their detected fold changes is provided in Additional file 3. Two genes exhibiting increased expression and two genes exhibiting decreased expression in response to estrogen treatment were randomly selected for each cell line and confirmed by real-time PCR. The relative expression levels for these genes as determined by both microarray and real-time PCR are shown in Figure 6B.

We next compared the microarray results of parental and MCF7-ER β expressing cells treated with estrogen plus 40 nM endoxifen to estrogen treatment alone. Using the same selection criteria as above, significantly fewer genes were determined to be regulated by this dose of endoxifen. In the parental cell line, the expression levels of 75 genes were altered by at least 1.5 fold by the addition of endoxifen of which 44 were increased and 31 were decreased (Figure 6C). Similar results were observed in the MCF7-ER β cell line in that a total of 78 genes were differentially regulated in response to

endoxifen with 37 exhibiting increased expression and 41 exhibiting decreased expression (Figure 6C). Comparison of these two data sets indicated that 46 genes (43%) were commonly regulated in both cell lines while 29 (27%) were unique to the parental cells and 32 (30%) were unique to the ER β expressing cells (Figure 6C). A list of these genes and their detected fold changes is provided in Additional file 4. As above, two genes exhibiting increased expression and two genes exhibiting decreased expression in response to endoxifen treatment were randomly selected for each cell line and confirmed by real-time PCR. The relative expression levels of these genes as determined by both microarray and real-time PCR following estrogen treatment alone and estrogen plus endoxifen are shown in Figure 6D.

As with the proliferation data, to ensure that the detected gene expression differences in response to estrogen and endoxifen were truly due to the presence of ER β and not a result of clonal variation, the confirmation of gene expression studies were also carried out in a second ER β cell line (#1). These results revealed the same trends in gene expression elicited in response to both estrogen and endoxifen (Figure 7A and B) and suggest that these differences are in fact a result of ER β expression and not due to clonal variation between cell lines.

Pathway analysis identifies specific biological processes that are uniquely regulated by endoxifen in MCF7-ER β cells

Pathway analysis was performed on the gene lists generated from both the parental and MCF7-ER β cells treated with estrogen plus 40 nM endoxifen relative to estrogen alone. For this analysis, all genes whose expression levels were significantly altered ($P < 0.05$) by the addition of endoxifen and whose fold changes were > 2 standard

deviations away from all genes kept in the analysis (~1.2 fold) were utilized. This analysis identified 13 pathways in the parental cell line and 12 pathways in the MCF7-ER β cell line which had significant enrichment of genes based on a $P < 0.05$. While none of these pathways passed a false discovery rate threshold of 0.25 after adjusting for multiple comparisons likely due to relatively small numbers of genes, it is interesting to note that biological pathways involving ER α cell cycle regulation and cell migration were only affected by endoxifen in breast cancer cells expressing ER β (Table 1). As with the gene expression data, many of the identified pathways were unique to either the parental or ER β expressing cell lines (identified by an asterix) lending further support to the differential effects of endoxifen as a result of ER isoform specific expression (Table 1).

Discussion:

Endocrine sensitive breast cancer

The large majority of breast cancer patients display tumors that are estrogen dependent based on the expression of ER α . Deprivation of estrogen signaling, most commonly through the use of tamoxifen in the adjuvant setting, typically results in tumor regression. However, the use of ER α alone as an indicator of responsiveness to anti-estrogens is far from perfect as about one-half of ER α positive tumors do not respond to tamoxifen therapy and about 10% of ER α negative tumors do respond. These studies demonstrate that other estrogen and anti-estrogen receptors and/or signaling pathways may be involved in mediating the responsiveness of endocrine sensitive tumors to hormonal agents. Following the discovery of ER β , investigators have sought to uncover the role that this protein may play in mediating breast cancer progression and treatment. Here, we demonstrate that expression of ER β in ER α positive MCF7 cells significantly

enhances the anti-estrogenic effects of endoxifen. This study provides evidence that endoxifen stabilizes ER β protein levels and induces ER α/β heterodimer formation which results in differential gene expression patterns. Perhaps most importantly, our studies reveal that even low concentrations of endoxifen, mimicking that of poor and intermediate CYP2D6 metabolizers, results in repression of estrogen induced breast cancer proliferation in cells expressing ER β , but not in those that express only ER α .

Role of ER β in breast cancer

ER β is known to be expressed in normal breast epithelial cells and several studies have demonstrated that ER β expression levels are suppressed in many breast cancers [51-54]. However, re-expression of ER β in ER negative breast cancer cells has been shown to reduce both basal and estrogen induced proliferation rates [21, 24, 25]. Expression of ER β in ER α positive breast cancer cells also results in suppression of proliferation following estrogen exposure [26-28]. Furthermore, ER β expression has been shown to increase the effectiveness of high concentrations of SERMs such as 4HT, raloxifen and fulvestrant [28, 55] *in vitro*. While the latter studies did not analyze endoxifen, nor did they utilize clinically relevant concentrations of 4HT (plasma concentrations are less than 10 nM in patients receiving 20 mg/day), they do further implicate a role for ER β in mediating anti-estrogenic activities.

It is possible that the increased effectiveness of endoxifen in ER β expressing MCF7 cells is related to the molecular actions of ER α/β heterodimers since we demonstrate that heterodimer formation is induced in a concentration dependent manner following endoxifen exposure. Indeed, the global gene expression changes induced by estrogen and anti-estrogens are known to be different in cells expressing both estrogen

receptors relative to cells expressing only ER α or ER β [20, 21, 56]. The results of the present study also demonstrate that both estrogen and endoxifen regulate unique subsets of genes in MCF7 cells expressing both receptors relative to cells expressing ER α alone. Biological pathway analysis of endoxifen regulated genes revealed that the majority of altered pathways are unique to either the parental or ER β expressing cell lines. Pathways involving ER α mediated cell cycle regulation and cell migration were only affected in the presence of ER β suggesting that the increased effectiveness of endoxifen may be through the inhibition of ER α activity by ER β .

Compounds which induce ER heterodimer formation as therapeutic drugs

The ability of specific compounds to induce ER heterodimer formation is of significant importance since these two receptors are often expressed in the same cells of many different tissues, including breast tumors, and since different dimer pairs have distinct genomic targets [57]. Of particular interest is the observation that genistein, a compound originally thought to contribute to decreased breast cancer risk, specifically induces ER α homodimerization and transcriptional activity [58]. This observation correlates well with more recent studies demonstrating that genistein is not effective in the prevention of breast cancer [59, 60]. Conversely, liquiritigenin is a highly selective ER β agonist which does not stimulate ER α positive tumor formation [61] or ER α homodimerization [58] suggesting that it may serve as a suppressor of proliferation in ER β expressing cells. The summation of these studies indicates that the identification of compounds which can specifically induce and/or activate ER α/β heterodimers or ER β homodimers may have therapeutic potential. Endoxifen may serve as such a compound since it stabilizes ER β protein levels and induces heterodimer formation in cells

expressing both ER isoforms while simultaneously degrading ER α protein in ER β negative cells [16].

Impact of ER β positivity and increased endoxifen effectiveness

The identification of increased endoxifen effectiveness as an anti-estrogenic agent in the setting of ER β is of significant clinical importance due to the fact that ER β expression is reported to exist in approximately 75% of invasive breast cancers [33, 36, 37, 42, 62-64] and in a subset of tumors which are ER α negative [41, 45, 65]. The majority of reports suggest that the presence of ER β in breast tumors correlates with improved rates of recurrence, disease-free survival and overall survival [29-38], however, others indicate little correlation [39-41]. A few recent clinical studies have revealed that ER β increases the effectiveness of tamoxifen therapy for breast cancer [44-46]. Given that endoxifen is being developed for the treatment of ER positive breast cancer, future studies should evaluate the association between ER β expression and the activity of endoxifen in human breast tumors.

Conclusions

The present data indicate that ER β enhances the anti-estrogenic actions of endoxifen in breast cancer cells. These data correlate well with the clinical studies demonstrating increased benefit from tamoxifen therapy in those patients whose tumors are ER β positive and suggest that this benefit may be through the actions of endoxifen. The ability of low endoxifen concentrations to significantly inhibit estrogen induced gene expression and proliferation in ER β expressing breast cancer cells also suggests that benefit from tamoxifen therapy may still be observed in patients characterized as poor metabolizers based on CYP2D6 genotype if their tumors are ER β positive. Finally, these

studies highlight the need to further investigate the role of ER β in breast cancer, both as a prognostic and predictive factor, and lend additional support to the development of endoxifen as a novel therapeutic for the treatment of endocrine sensitive breast tumors.

Abbreviations

4HT: 4-hydroxy-tamoxifen; 17 β -estradiol: estrogen; AA: antibiotic/antimycotic; CYP2D6: Cytochrome P450 2D6; DMEM/F12: Dulbecco's Modified Eagle's Medium/F12; DPN: diarylpropionitrile; ER: estrogen receptor; ER α : estrogen receptor-alpha; ER β : estrogen receptor-beta; ERE: estrogen response element; FBS: fetal bovine serum; G418: geneticin; NCI: National Cancer Institute; NDT: N-desmethyl-tamoxifen; PPT: propyl pyrazole triol; RT-PCR: real-time polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SERM: selective estrogen receptor modulator.

Competing interests

XW, MS, SBG, ZS, VN, WLL, TCS and JRH declare that they have no competing interests. MPG and JNI are named inventors (along with Mayo Clinic) in regard to non-provisional patent applications regarding tamoxifen and *CYP2D6*; the technology is not licensed, and no royalties have accrued.

Authors' contributions

XW, MS, WLL, MPG, JNI, TCS and JRH conceived of the study, participated in its design and drafted the manuscript. XW, SBG, VN and JRH performed the laboratory

experiments and analyzed the data. ZS performed the microarray and biological pathway analysis. All authors read and approved the final manuscript.

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Figure legends:

Figure 1. Characterization of MCF7-ER β cell lines. (A) Western blot (WB) analysis demonstrating expression of ER β in three independent clonal MCF7 cell lines. (B) Immunofluorescence depicting nuclear localization of ER β protein in stably expressing MCF7 cell lines. (C) Luciferase assays demonstrating transcriptional activation of a transiently transfected ERE by either ER α or ER β in parental and MCF7-ER β cell lines using estrogen, the ER α specific agonist PPT or the ER β specific agonist DPN. * denotes significance at the $p < 0.05$ level (ANOVA) compared with vehicle controls while # denotes significance for a given treatment between the two cell lines.

Figure 2. Stabilization of ER β protein levels by endoxifen. Western blot (WB) analysis of ER β protein levels in MCF7-ER β (A), Hs578T-ER β (B) and U2OS-ER β (C) cells treated with indicated concentrations of endoxifen or vehicle for 24 hours. Tubulin levels are shown as protein loading controls.

Figure 3. Endoxifen induces ER α/β heterodimer formation. MCF7-ER β (A) or U2OS-ER α/β (B) cells were treated with indicated concentrations of endoxifen or vehicle for 24 hours. Equal amounts of cell lysates were immunoprecipitated with an ER β specific antibody. Immunoprecipitated protein (IP) complexes were separated by SDS-PAGE and western blotting (WB) was performed using an ER α specific antibody. Non-immunoprecipitated ER α and ER β protein levels were also determined by western

blotting in whole cell extracts (WCE) following endoxifen treatment. Tubulin levels are shown as protein loading controls.

Figure 4. Repression of known ER target genes by endoxifen is enhanced in ER β expressing cells. Parental MCF7 and MCF7-ER β cells were treated as indicated for 24 hours. Real-time RT-PCR analysis was performed to detect expression levels of (A) cyclin D1 (CCND1), (B) PS2, (C) progesterone receptor (PR) and (D) amphiregulin (AREG). * denotes significance at the p<0.05 level (ANOVA) compared to vehicle, # compared to estrogen treatment and δ for a given treatment between the two cell lines.

Figure 5. Low concentrations of endoxifen inhibit estrogen induced proliferation of MCF7-ER β cells. Parental and MCF7-ER β cells (cell lines #1 and #3) were treated as indicated for 8 days and cell proliferation rates were analyzed. Graphs depict fold change from vehicle treated cells. * denotes significance at the p<0.05 level (ANOVA) compared to vehicle controls, # compared to estrogen treated cells and δ for a given treatment between the two cell lines.

Figure 6. Microarray analysis of estrogen and estrogen plus endoxifen treatment in parental and MCF7-ER β cells. (A and C) Venn diagrams indicating the number of genes whose expression levels were significantly altered by at least 1.5 fold in response to 24 hour treatments of 1 nM estrogen in MCF7 or MCF7-ER β cells (A) or 1 nM estrogen + 40 nM endoxifen relative to estrogen treatment alone (C). (B and D) Real-time PCR confirmation of selected genes whose expression levels were either increased or decreased by the addition of estrogen in MCF7 or MCF7-ER β cells (B) or 1 nM estrogen + 40 nM endoxifen relative to estrogen treatment alone (D). The fold changes

of each gene as detected by microarray analysis are shown for comparison purposes and all data have been normalized to vehicle controls (dotted line).

Figure 7. Confirmation of microarray data in a second ER β expressing MCF7 cell line. (A and B) Real-time PCR confirmations were also carried out in a second ER β cell line (#1) to ensure that the detected gene expression changes were due to the presence of ER β and were not a result of potential clonal variation between cell lines. Relative fold changes of genes determined to be regulated by 1nM estrogen alone (**A**) or by 1 nM estrogen + 40 nM endoxifen relative to estrogen treatment alone (**B**) are shown following normalization to vehicle controls (dotted line). The same trends in gene expression were detected in response to both estrogen and endoxifen in this second ER β expressing cell line.

Additional files

Additional file 1

Table indicating the sequences of primers used throughout this manuscript.

Additional file 2

Confirmation of ER β negativity in parental MCF7 cells.

(**A**) Real-time PCR and (**B**) western blot analysis demonstrating that ER β expression at both the mRNA and protein level is undetectable in parental MCF7 cells. These data are shown in comparison to one of the ER β -expressing clonal cell lines (cell line #3).

Additional file 3

Table indicating the genes determined to be significantly regulated by 1nM estrogen in parental and ER β expressing MCF7 cells.

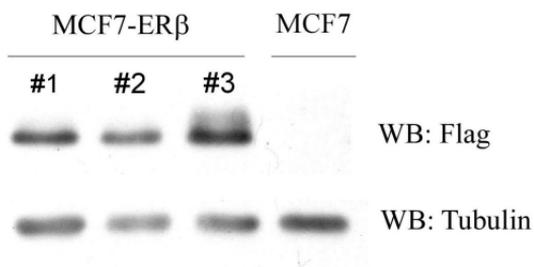
Additional file 4

Table indicating the genes determined to be significantly regulated by 1nM estrogen + 40 nM Endoxifen in parental and ER β expressing MCF7 cells.

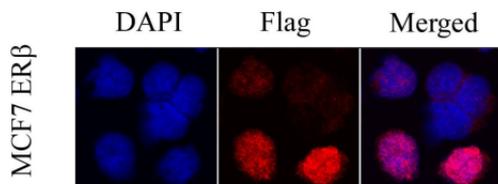
Table 1: Biological pathways detected to be significantly regulated by estrogen plus 40nM endoxifen relative to estrogen treatment alone

MCF7 Parental Cells			P-Value	# Genes
Pathway #		Pathway Name		
1	*	Immune response_Antiviral actions of Interferons	0.0008	6/20
2	*	Immune response_IFN gamma signaling pathway	0.0177	6/36
3		Regulation of lipid metabolism_Regulation of fatty acid synthase activity	0.0182	3/10
4		Neurodisease_Parkin disorder under Parkinson disease	0.0191	4/18
5	*	Regulation of lipid metabolism_Regulation of acetyl-CoA carboxylase 1	0.0195	2/4
6	*	Cholesterol Biosynthesis	0.0276	4/20
7	*	Niacin-HDL metabolism	0.0305	3/12
8	*	Cytoskeleton remodeling_Thyroliberin in cytoskeleton remodeling	0.0305	3/12
9		CFTR-dependent regulation of ion channels in Airway Epithelium	0.0326	4/21
10		Immune response_IL-27 signaling pathway	0.0380	3/13
11	*	Development_A1 receptor signaling	0.0440	4/23
12	*	Neurophysiological process_PGE2-induced pain processing	0.0450	2/6
13	*	Immune response_Th1 and Th2 cell differentiation	0.0463	3/14
MCF7-ERβ Cells				
1	*	wtCFTR and deltaF508 traffic / Membrane expression (norm and CF)	0.0026	5/16
2		Regulation of lipid metabolism_Regulation of fatty acid synthase activity	0.0026	4/10
3		Immune response_IL-27 signaling pathway	0.0077	4/13
4	*	Blood coagulation_Blood coagulation	0.0132	4/15
5	*	Cell cycle_ER α regulation of G1/S transition	0.0167	5/24
6	*	Globo-(isoglobo) series GSL Metabolism	0.0228	3/10
7		Neurodisease_Parkin disorder under Parkinson disease	0.0254	4/18
8	*	ER α action on cytoskeleton remodeling and cell migration	0.0299	3/11
9	*	Transcription_Ligand-Dependent Transcription of Retinoid-Target genes	0.0306	4/19
10	*	ENaC regulation in airways (normal and CF)	0.0364	4/20
11		CFTR-dependent regulation of ion channels in Airway Epithelium	0.0427	4/21
12	*	wtCFTR and delta508-CFTR traffic / Generic schema (norm and CF)	0.0463	5/31

A



B



C

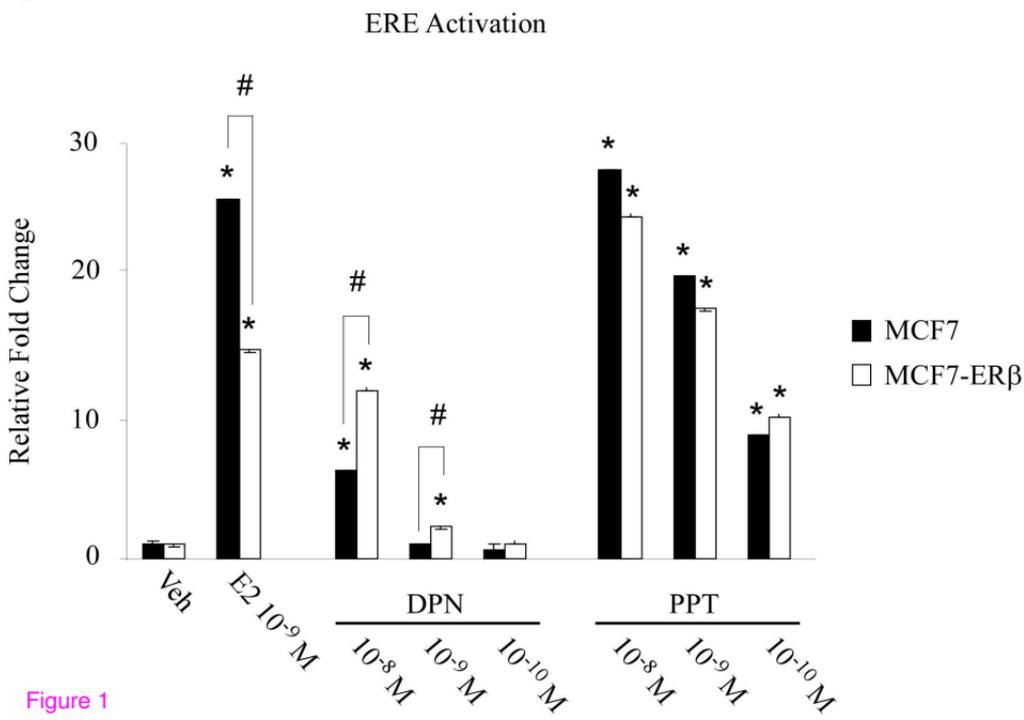


Figure 1

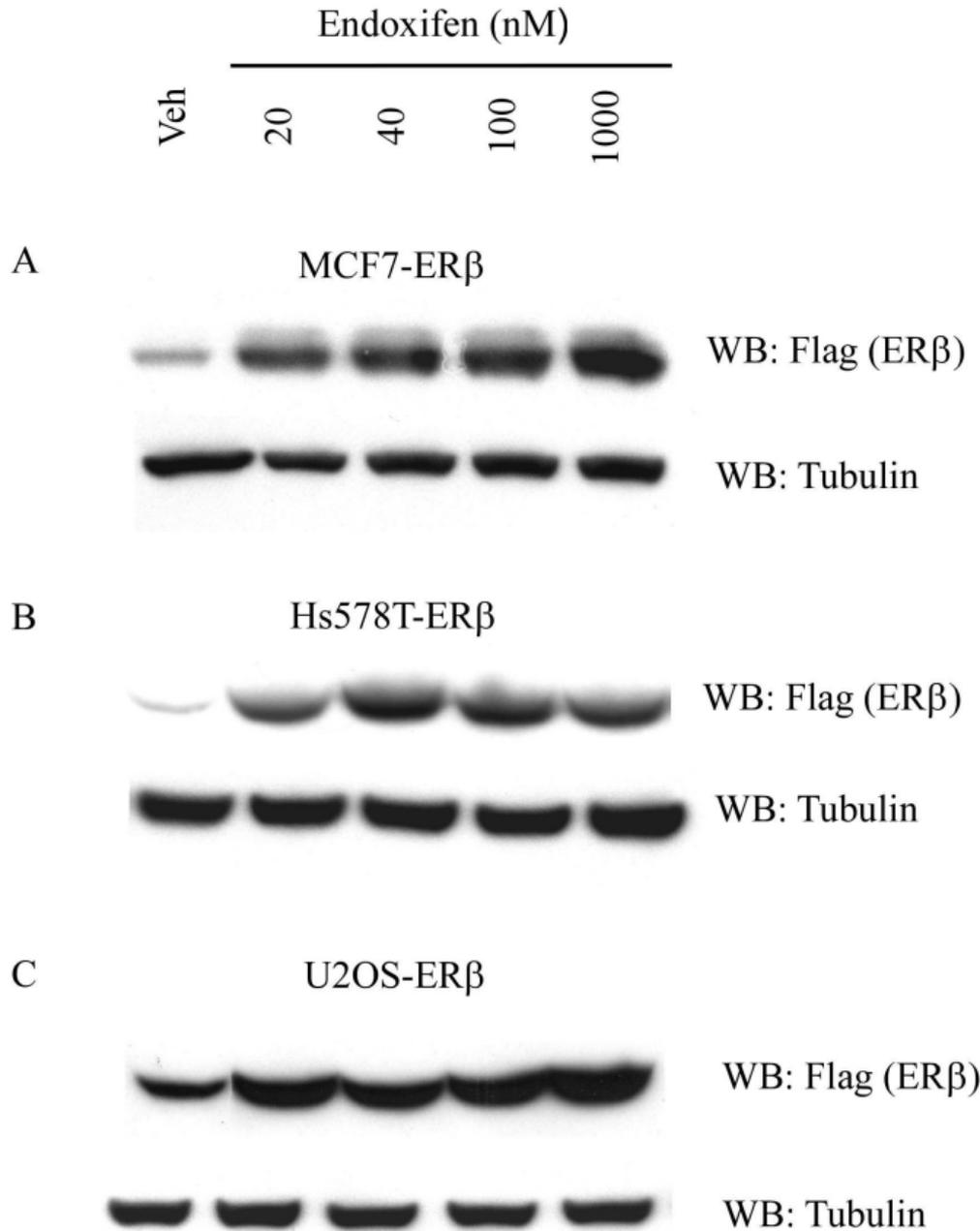
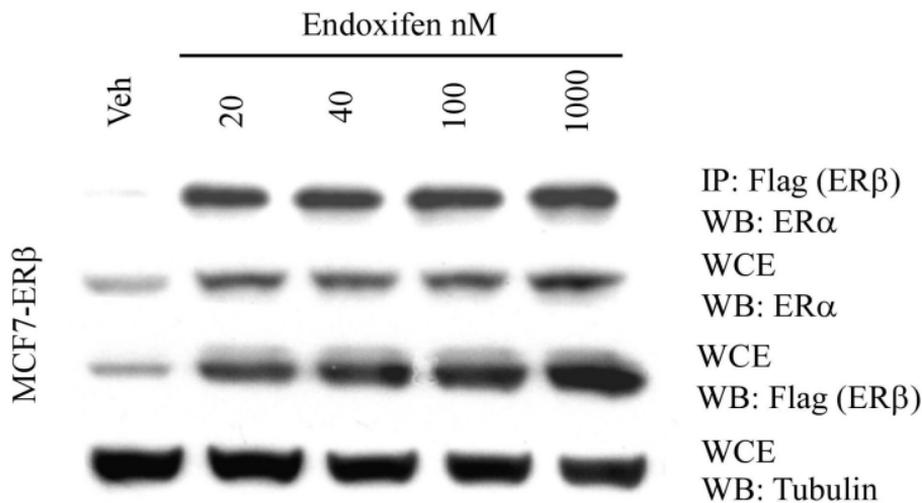


Figure 2

A



B

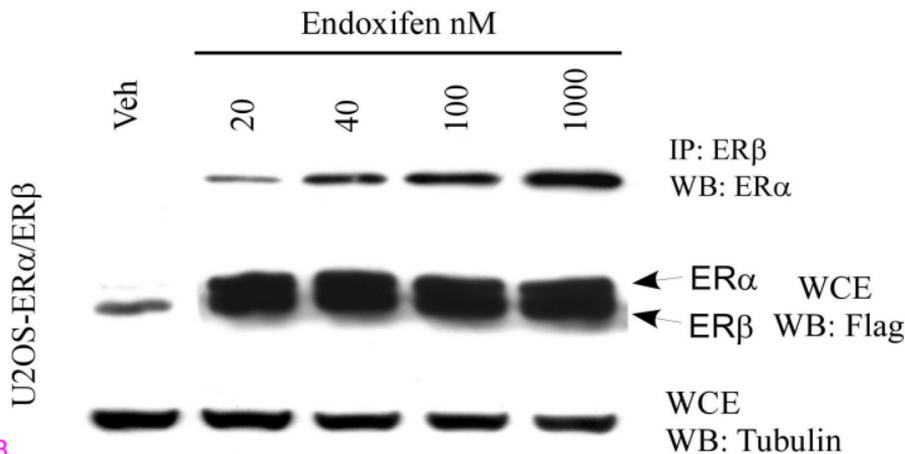
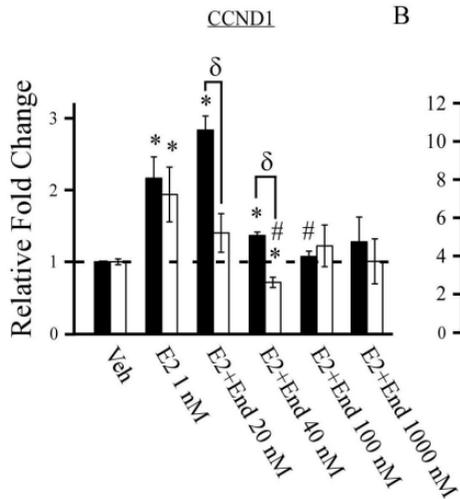
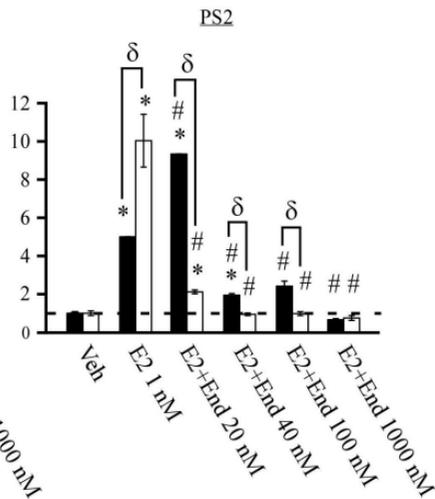


Figure 3

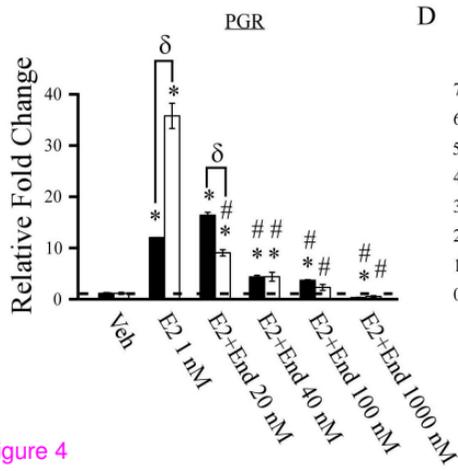
A



B



C



D

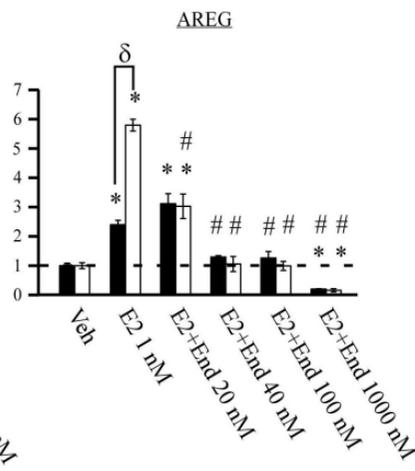


Figure 4

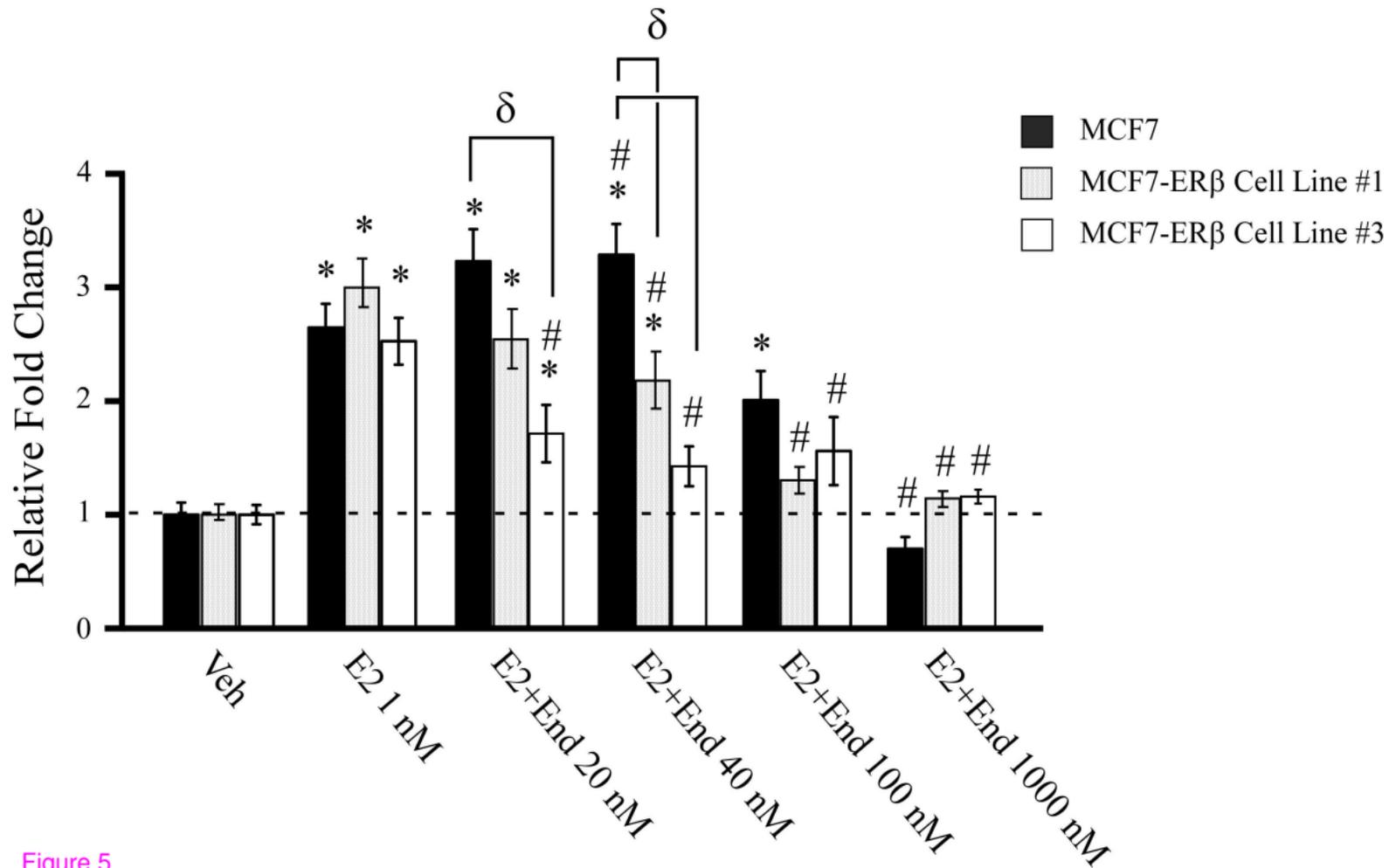


Figure 5

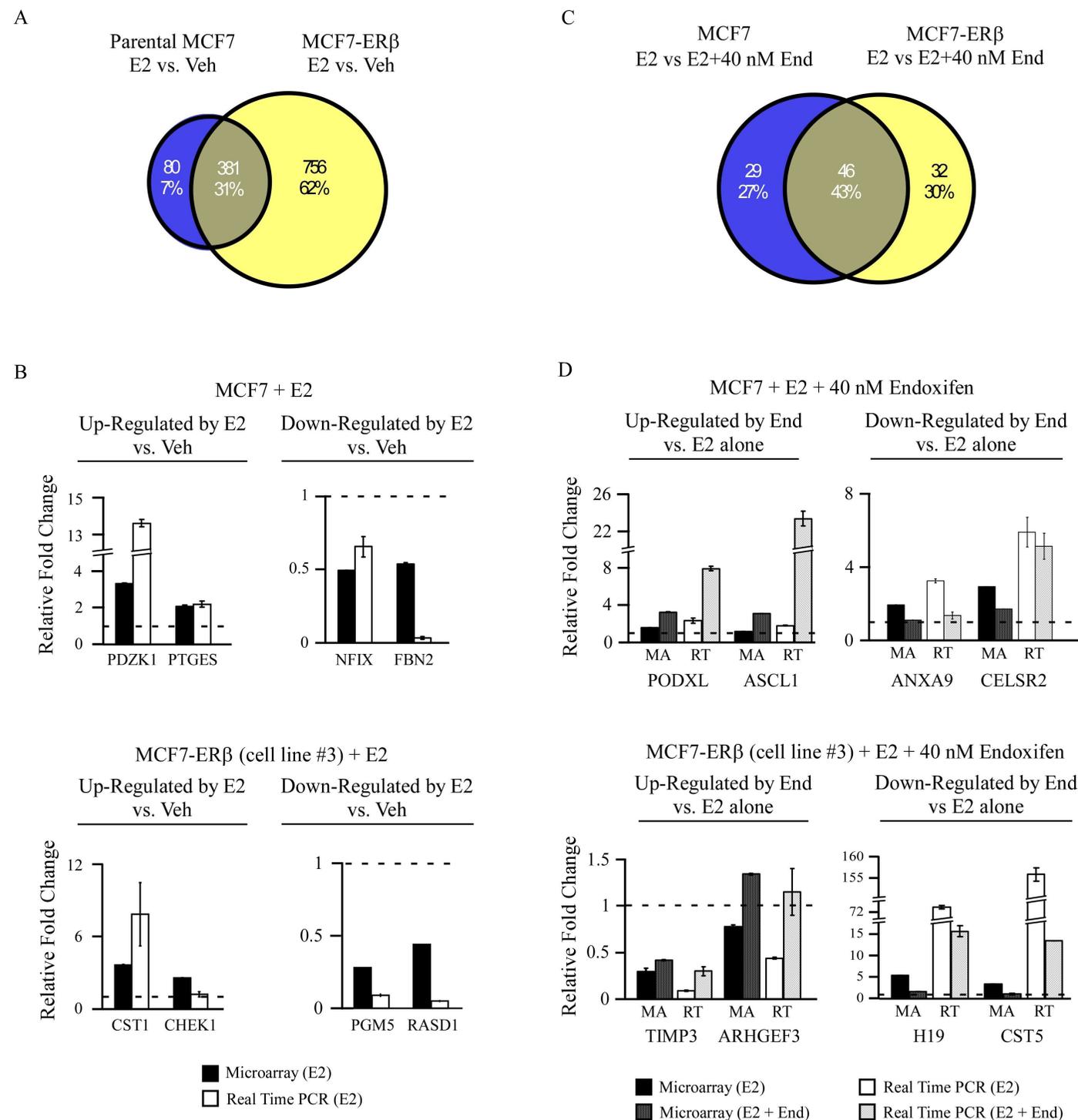
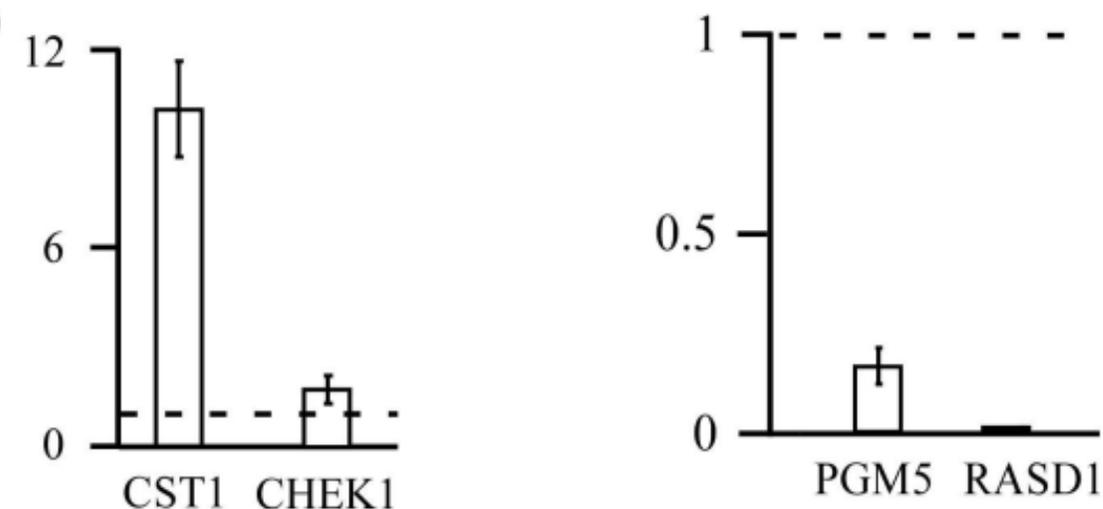


Figure 6

A

MCF7-ER β (cell line #1) + E2Up-Regulated by E2
vs. VehDown-Regulated by E2
vs. Veh

Relative Fold Change

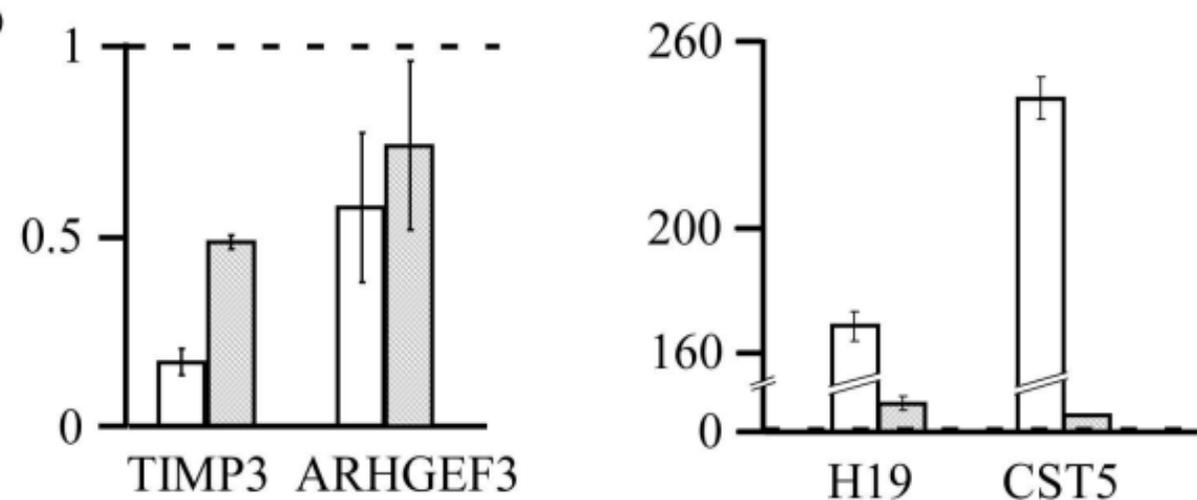


□ Real Time PCR (E2)

B

MCF7-ER β (cell line #1) + E2 + 40 nM EndoxifenUp-Regulated by End
vs. E2 aloneDown-Regulated by End
vs E2 alone

Relative Fold Change



□ Real Time PCR (E2)

■ Real Time PCR (E2 + End)

Figure 7

Additional files provided with this submission:

Additional file 1: Additional File 1.xls, 17K

<http://breast-cancer-research.com/imedia/1698970015267880/supp1.xls>

Additional file 2: Additional File 2.jpg, 132K

<http://breast-cancer-research.com/imedia/1653893342526788/supp2.jpeg>

Additional file 3: Additional File 3.xls, 168K

<http://breast-cancer-research.com/imedia/1641491965526789/supp3.xls>

Additional file 4: Additional File 4.xls, 29K

<http://breast-cancer-research.com/imedia/6808770352678933/supp4.xls>