Estrogen receptor prevents p53-dependent apoptosis in breast cancer

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More than two-thirds of breast cancers express the estrogen receptor (ER) and depend on estrogen for growth and survival. Therapies targeting ER function, including aromatase inhibitors that block the production of estrogens and ER antagonists that alter ER transcriptional activity, play a central role in the treatment of ER+ breast cancers of all stages. In contrast to ER− breast cancers, which frequently harbor mutations in the p53 tumor suppressor, ER+ breast cancers are predominantly wild type for p53. Despite harboring wild-type p53, ER+ breast cancer cells are resistant to chemotherapy-induced apoptosis in the presence of estrogen. Using genome-wide approaches, we have addressed the mechanism by which ER antagonizes the proapoptotic function of p53. Interestingly, both ER agonists such as estradiol and the selective ER modulator (SERM) tamoxifen promote p53 antagonism. In contrast, the full ER antagonist fulvestrant blocks the ability of ER to inhibit p53-mediated cell death. This inhibition works through a mechanism involving the modulation of a subset of p53 and ER target genes that can predict the relapse-free survival of patients with ER+ breast cancer. These findings suggest an improved strategy for the treatment of ER+ breast cancer using antagonists that completely block ER action together with drugs that activate p53-mediated cell death.

Although breast cancer is often curable when it is treated at an early stage, metastatic disease is almost uniformly fatal due to the development of therapeutic resistance (1). Approximately two-thirds of all breast cancer cases are attributed to the dysregulation of estrogen receptor (ER) signaling (2, 3). ER is also the most significant therapeutic target for ER+ breast cancer (4). Among drugs that are approved for the treatment of ER+ breast cancers are tamoxifen and fulvestrant (4). Tamoxifen, a selective ER modulator (SERM), competes with estradiol (E2) for ER binding and induces a conformation that favors corepressor binding rather than coactivator binding by ER, thus blocking the expression of most estrogen-responsive genes (5) and resulting in growth inhibition (6). Although treatment of women with ER+ metastatic breast cancer with tamoxifen is useful in controlling their disease, resistance almost invariably develops, leading to recurrence (6). Unlike tamoxifen, which is a partial antagonist, fulvestrant is considered a full antagonist (7). ER binding by fulvestrant blocks both the agonist effects of E2 and leads to degradation of the ER through an ubiquitin-mediated mechanism (8-10). Although fulvestrant has activity in some women with tamoxifen-resistant disease, resistance to fulvestrant also limits its utility (11).

Adjuvant treatment of early stage ER+ breast cancers most commonly includes chemotherapy followed by tamoxifen or, in postmenopausal women, an aromatase inhibitor that blocks the production of estrogen from androgens. Chemotherapy for breast cancer often includes drugs such as doxorubicin that induce DNA damage and subsequently the p53-mediated apoptotic response. p53 is a tumor suppressor gene that plays a role in cell cycle control and apoptosis, and is found to be mutated in >50% of cancers in which its loss facilitates transformation (12, 13). Curiously, despite its high mutation rate in other forms of cancer, p53 has been shown to be mutated in just 20–30% of all breast malignancies, and for the most part these are ER− (14).

Prior studies have suggested that ER interacts with p53 and that this association could lead to the suppression of p53-mediated repression (1, 15). Here, we explored the functional interplay between p53 and ER in ER+ breast cancers on a genome-wide scale through an integrative analysis of the gene expression programs mediated by each and their cistromes. We find that ER represses the p53-mediated apoptotic response induced by DNA damage through a mechanism that involves the recruitment of ER to a subset of proapoptotic p53 target genes at sites that are distinct from those bound by p53.

A

Results

E2 Protects Cells Against p53-Mediated Cell Death. Because estrogen is required for the growth and survival of MCF7 ER+ breast cancer cells, we explored whether E2 had an effect on MCF7 cell survival in the presence of an apoptotic stimulus. To test this hypothesis, MCF7 cells were treated with the proapoptotic chemotherapeutic drug doxorubicin, and cell viability was determined in the presence or absence of estrogen after 3 d (Fig. 1A). In cells treated with doxorubicin under vehicle (i.e., charcoal-dextran-treated; CDT) conditions, only ~50% survived, whereas the addition of E2 increased survival to ~75%. Interestingly, tamoxifen was also able to block cell death induced by doxorubicin to the same extent as E2. In contrast, treatment with fulvestrant led to a similar loss in survival as with doxorubicin alone. We further examined the effects of E2, fulvestrant, and tamoxifen on cells treated with nutilin (16), a drug that stabilizes p53 by inhibiting the interaction between p53 and the E3 ubiquitin ligase mdm2 (Fig. 1B). Treatment with nutilin in the absence of E2 led to cell death, albeit to a lesser extent than doxorubicin. This cell death was significantly augmented in the presence of fulvestrant. In contrast, in the presence of E2 or tamoxifen, cell death was completely blocked, and there was increased cell number compared with baseline. These data demonstrate that E2 is protective against both doxorubicin- and nutilin-stimulated p53-mediated apoptosis in breast cancer cells.

E2 and Doxorubicin Regulate a Set of Common Genes. To begin to elucidate the mechanisms involved in the ER-mediated protection of doxorubicin-induced cell death, we first examined the genes differentially expressed in response to both stimuli. We compared the expression of genes altered in response to 12-h doxorubicin treatment using Affymetrix Human Genome U133A...
We found that a number of established p53 targets, including *apaf1*, *p21*, *mdm2*, and *fas*, contain p53-binding sites (Fig. S4A). Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed an enrichment for the p53 pathway (Fig. S4B), and GO analysis of genes near p53 binding sites (<30 kb) revealed a considerable enrichment for apoptosis biological process categories with significant *P* values (Fig. S5A). The distribution profile of the p53 binding sites was similar to other transcription factors where a majority of sites are found at distal intergenic regions (56%) and a minority at promoters (4%) (Fig. S5B). There was also a significant enrichment for p53 binding sites within introns (34%) and a small number of sites in the 5′ and 3′ UTRs (1% each) and immediately downstream of genes (2%) (Fig. S5B).

2.0 arrays and compared them to an E2-regulated MCF7 dataset (17). We found that there were 452 genes significantly regulated (*P < 0.01*) by doxorubicin treatment and 2,444 genes regulated by E2 treatment (Fig. 2). Of these genes, 179 were differentially regulated by both stimuli. These commonly regulated genes were associated with Gene Ontology (GO) categories, including those associated with cell growth and the DNA damage response (Fig. S1). When we clustered the 179 genes commonly regulated by both stimuli, we observed two major clusters that displayed disparate regulation: genes that were up-regulated by doxorubicin and down-regulated by E2 (cluster 1, 49 genes) and genes that were down-regulated by doxorubicin and up-regulated by E2 (cluster 2, 97 genes; Fig. 2B). GO analysis of these subsets revealed that the genes in cluster 1 were primarily associated with the apoptotic categories, and genes in cluster 2 were mainly associated with cell growth process categories (Fig. S2). These data suggest that doxorubicin and estrogen have opposing effects on a common set of genes that regulate cell growth and apoptosis.

To determine whether the 179 genes were clinically relevant in patients with breast cancer, we used Oncomine Concepts Map (18) to determine whether these same genes were differentially regulated in samples from patients with breast cancer. We found that the genes regulated by both stimuli were significantly associated with poor outcome, including metastasis, death, and recurrence within 5 y, in several independent ER+ breast cancer datasets (Fig. 2C).

**Determination of the p53 Cistrome.** To explore whether the commonly regulated genes are directly regulated by both p53 and ER, we determined the p53 cistrome in MCF7 cells by chromatin immunoprecipitation (ChIP) coupled with next-generation sequencing (ChIP-seq) following doxorubicin treatment and compared these data with an updated E2-regulated MCF7 dataset. A number of established p53 targets, including *apaf1*, *p21*, *mdm2*, and *fas*, contain p53-binding sites (Fig. S4A). Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed an enrichment for the p53 pathway (Fig. S4B), and GO analysis of genes near p53 binding sites (<30 kb) revealed a considerable enrichment for apoptosis biological process categories with significant *P* values (Fig. S5A). The distribution profile of the p53 binding sites was similar to other transcription factors where a majority of sites are found at distal intergenic regions (56%) and a minority at promoters (4%) (Fig. S5B). There was also a significant enrichment for p53 binding sites within introns (34%) and a small number of sites in the 5′ and 3′ UTRs (1% each) and immediately downstream of genes (2%) (Fig. S5B).

The 179 commonly regulated genes were interrogated by using Oncomine Concepts analysis (Compendia Biosciences) against publicly available primary breast tumor datasets, and significant associations were graphically represented in an interaction network by using Cytoscape (http://www.cytoscape.org). In this network, a node represents a dataset, and each edge represents a significant association with *P < 0.01* and an odds ratio > 4. The 179 genes (shown in purple) demonstrated significant association with patient datasets exhibiting metastasis (blue circle), death (green circles), and recurrence (red circles) within 1, 3, or 5 y. The node size is proportional to the number of associated genes in each dataset. The van de Vlijter dataset is shown on the right as an example of genes up-regulated in patients with recurrence at 3 y.
Using an integrative approach, we compared the p53 and ER cistromes (Fig. 3) with the 179 differentially expressed genes to determine which are directly affected by both transcription factors. We observed that there were 155 genes with an ER binding site and 38 genes with a p53 binding site within 100 kb of the transcription start site (Fig. S6). Of these genes, 36 had both a p53 and ER binding site that may directly influence their expression. These genes were enriched for cell cycle and apoptotic GO term categories (Fig. S7) and included targets previously reported to be associated with apoptosis and cell growth control, including cyclin-dependent kinase inhibitor 1 (p21), tumor necrosis factor (TNF) receptor-associated factor 4 (TRAF4), B-cell translocation gene 2 (BTG2), and activating transcription factor 3 (ATF3).

**ER Blocks p53-Mediated Transcription.** We compared the expression of the 36 genes that were differentially expressed and possessed an ER and p53 binding site using gene expression microarray data from MCF7 cells that were treated with E2 or doxorubicin (Fig. 4A). GO term analysis of this gene set demonstrated enrichment for genes correlated with the cell cycle and apoptosis.

We examined the consequence of dual treatment with E2 and doxorubicin on the expression of genes that have been reported to participate in cell cycle arrest and apoptosis induction, i.e., genes with common sites from cluster 1. For this purpose, we examined the expression of the ATF3, BTG2, and TRAF4 genes. ATF3 is an ATF/CCAAT enhancer-binding protein (CREB) family member that can heterodimerize with Jun AP-1 family members to activate transcription (21–23). It has been reported to interact with and stabilize p53 by blocking its ubiquitination. BTG2 is a gene that was found to be suppressed in breast cancer (24, 25), and its up-regulation was shown to be correlated with better survival in patients with breast cancer (26). This gene was also found to be regulated in response to DNA-damaging stimuli in a p53-dependent manner (27, 28). TRAF4 is a TNF protein family member that was identified as a p53 target and shown to be proapoptotic when overexpressed in breast cancer cell lines (29, 30).

Although treatment with doxorubicin induced the expression of the ATF3, BTG2, and TRAF4 genes, these genes were repressed by E2 (Fig. 4B). Combined treatment with E2 and doxorubicin led to an inhibition of the expression of these genes, demonstrating that E2 plays an inhibitory role for these genes even in the presence of doxorubicin. To test the effects of ER antagonists, we treated the cells in the presence of doxorubicin with either fulvestrant or tamoxifen. Strikingly, whereas the full antagonist fulvestrant facilitated the expression of ATF3, BTG2, and TRAF4, the partial antagonist tamoxifen inhibited their expression in a manner that is similar to E2 treatment. We observed similar results when stimulating with E2 in combination with nutlin-3, demonstrating that elevated p53 levels are sufficient for the p53-mediated activation of these genes in MCF7 cells (Fig. 4C).

To address whether ER blocks p53-mediated expression through the prevention of p53 binding as has been suggested (15, 31), we examined the p53 binding sites near the ATF3, BTG2, and TRAF4 genes (Fig. 4D). Using a targeted ChIP approach, we found strong p53 binding at each of these sites upon doxorubicin treatment which was unaffected by the addition of E2. A similar effect was observed with nutlin treatment (Fig. 4E). In addition, we measured the effect of E2 on the p53 protein level by performing a Western blot using lysates from MCF7 cells treated under the ChIP conditions. We found that, whereas doxorubicin and nutlin led to an increase in p53 protein, E2 had no effect on p53 protein levels. These data suggest that although ER does not displace p53 binding or affect the p53 expression level, it independently targets and represses p53 target gene expression.

**Differentially Regulated Genes Are Correlated with Patient Survival.**

We next wanted to determine whether these genes, which are directly regulated by both p53 and ER, but in opposite directions, are associated with patient outcome. Using a Kaplan-Meier analysis (Fig. 5) of eight ER+ breast cancer patient datasets derived from Gyorffy et al. (32), we found that patients whose tumors had a high level of expression of genes that are up-regulated by doxorubicin and down-regulated by E2 had a better overall survival rate than those whose tumors expressed low levels of these genes. In contrast, patients whose tumors exhibited a high level of expression of the E2 up-regulated and doxorubicin...
down-regulated genes had a worse survival rate (Fig. 5). These data suggest that the genes oppositely regulated directly by both p53 and ER in MCF7 cells are relevant to the clinical behavior of ER+ breast cancers in general.

Discussion

In ER+ breast cancer, inhibiting ER action through the reduction in estrogen levels with aromatase inhibitors or the use of antagonists such as tamoxifen and fulvestrant leads to significant clinical benefit for both early stage patients and those with advanced disease. Despite frequently harboring wild-type p53, ER+ breast cancers are relatively insensitive to chemotherapy-induced apoptosis. We explored the mechanism by which ER protects ER+ breast cancers from DNA-damage-induced cell death by determining the genome-wide set of genes directly regulated by p53 and ER. We identified a small set of pro-apoptotic p53 target genes that are also directly targeted by ER. These genes are induced upon p53 binding, and this induction is blocked by ER binding. Interestingly, this is the case for full agonists such as E2 and the partial agonist tamoxifen. Clinical treatment of patients with breast cancer typically involves chemotherapies that lead to an increase in p53 expression followed by treatment with adjuvant therapy, which includes tamoxifen. Our data suggest that there are inherent deficiencies in this type of treatment. We demonstrate that treatment with E2 or tamoxifen suppresses the apoptotic response in MCF7 cells; thus, p53 activation by means of chemotherapeutic drugs before antiestrogen treatment in patients may be less effective. Moreover, our results demonstrate that tamoxifen may be insufficient for the treatment of ER+ positive breast cancers that express wild-type p53. Although tamoxifen inhibits the ER transcriptional program, it is also known to act in a similar manner to E2. (C) Similar effects were observed when treating with nutlin. (D and E) MCF7 cells were hormone starved and stimulated with doxorubicin alone or in combination with E2, fulvestrant, and tamoxifen to evaluate the expression of putative apoptosis target genes. Cotreatment with fulvestrant led to greater expression of these apoptotic genes, whereas treatment with tamoxifen inhibited in a manner similar to E2. (F) Protein lysate from MCF7 cells treated under ChIP assay conditions were assayed by immunoblot for the level of p53 protein expression, and β-actin was blotted as a control.
by ER to explain its ability to repress p53-mediated activation (11). This inhibition may involve AP-1 factors because AP-1 motifs are enriched in the ER binding sites found near the common set of ER and p53 regulated genes. AP-1 was previously shown to be capable of tethering the ER to sites in the genome, and these sites tend to be enriched for down-regulated genes (17).

In conclusion, we describe a transcriptional program that is directly regulated by both ER and p53 that explains the resistance of ER+/p53 wild-type breast cancers to apoptosis. Because the ER suppresses genes that are responsible for executing the p53-mediated apoptotic program, there is no selection for cells harboring mutant p53; rather, the selection that occurs in these cancers may actually be for increased ER signaling (44). Thus, effective treatment for these tumors may require the concomitant complete inhibition of ER signaling with drugs such as fulvestrant together with treatments that promote p53-mediated apoptosis (33–35).

Materials and Methods

Cell Culture and Reagents. MCF7 cells were grown in high-glucose DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 10% (vol/vol) heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in a humidified incubator at 37 °C and 5% CO2. Doxorubicin was purchased from Sigma and dissolved in water.

Cell Viability and Apoptotic Assays. Approximately 5 × 10^4 MCF7 cells were seeded in a 96-well plate in hormone-depleted medium and treated with vehicle alone, 10 nM E2, 100 nM fulvestrant, or 10 nM 4-OH tamoxifen. After a 2-d incubation, the medium was removed, and fresh ligand or vehicle containing medium was added with or without 10 μM doxorubicin or 5 μM nutlin. The cells were incubated for an additional 3 d, and a cell viability assay was performed by using the CellTitre 96 Non-Radioactive Cell Proliferation Assay (MTT) (Promega). This experiment was performed in triplicate and repeated three times.

Chip and Sequencing. ChiP was performed as described (45). Briefly, −5×10^6 MCF7 cells were seeded in hormone-depleted medium. After 3 d, the cells were treated with E2 or doxorubicin for 45 min or 4 h, respectively. The cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cells were washed in PBS and then lysed in 1% SDS lysis buffer, pH 8.0, and sonicated to an average size of 250 bp. The fragmented chromatin was immunoprecipitated overnight by using antibodies directed against p53 (FL-393; Santa Cruz) or ERα (HC-20; Santa Cruz). For directed quantitative PCR, the DNA was purified by using the QIAquick PCR Purification kit (Qiagen), and the samples were subjected to RT-PCR by using the SYBR green method. For ChiP-Seq, after repairing the DNA ends, sequencing adaptors (Illumina) were ligated to the purified chromatin, and the DNA was amplified for sequencing as described. To assay simultaneous recruitment, hormone-depleted MCF7 cells were treated with doxorubicin for 4 h before a 45-min incubation in the presence of E2. The cells were then processed for ChiP as described above.

Immunoblotting. MCF7 cells were stimulated for 4 h with either doxorubicin or nutlin before a 45-min incubation in the presence of E2. The cells were then lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0), and the protein concentrations were determined by using the micro BCA kit (Pierce). A total of 50 μg of protein was then subjected to SDS/PAGE followed by transfer to a PVDF membrane. The membrane was immunoblotted with p53 (FL-393; Santa Cruz) and β-actin (4967; Cell Signaling Technologies) antibodies followed by secondary antibody incubation with a donkey anti-rabbit antibody (Pierce). The blots were developed by using Western Blotting Luminol Reagent (Santa Cruz).

Gene Expression Analysis. Hormone-depleted MCF7 cells were treated with PBS or doxorubicin for 12 h. The total RNA was isolated by using a combination of TRIzol (Sigma) and the RNeasy Mini Kit (Qiagen) and hybridized to Affymetrix Human Genome U133A 2.0 arrays. The expression data were normalized by using the robust multichip average (RMA) method and processed by using linear models for microarray data (LIMMA) using R. Genes with P < 0.01 were considered statistically significant. The doxorubicin-treated cells were compared with a previously generated 12-h E2-treated dataset (17). The raw data from this series were also RMA-normalized and processed by using LIMMA with the same cutoff value. For k means clustering, the log, fold change of corresponding genes from each dataset was clustered. For hierarchical clustering, Pearson correlation using pairwise complete linkage was used.

Fig. 5. Kaplan-Meier analysis of patient datasets. Kaplan-Meier analysis reveals that the common regulated genes are predictors of relapse-free survival. To determine the role of these genes in predicting relapse-free survival in patients with ER+ breast cancer, we interrogated breast cancer datasets using the clusters 1 and 2 genes containing p53 and ER binding sites. (A) Kaplan-Meier analysis reveals that the cluster 1 genes that regulated by p53 and ER demonstrate better survival when they are highly expressed in patients with ER+ breast cancer. (B) The opposite result is observed when the p53 and ER-regulated cluster 2 genes are examined.
GO and KEGG pathway analyses were performed by using the Database for Annotation, Visualization, and Integrated Discovery bioinformatics resource (46, 47). SeqPos analysis was performed by using the Cistrome and analysis line (48), and the motif distribution analysis was performed as described (17).

**RT-PCR.** For RT-PCR, total RNA was isolated from using a combination of TRIzol (Sigma) and the RNeasy Mini Kit (Qiagen). First-strand cDNA was created by using the Quantitect Reverse Transcription Kit (Qiagen) following the manufacturer’s protocol.

**Oncomine Concepts Map.** The 179 genes that were found to be commonly regulated by ERα and doxorubicin were compared with genes expressed in tumors from patients with breast cancer by using the Oncomine Concepts Map (Compendia Bioscience). Significant associations between these genes and clinical outcome were established, and Cytoscape (49) was used to generate the node connections, which represent dataset associations with P < 0.01 and odds ratio > 4. The data represented in Fig. 2C are derived from the following independent datasets: van de Vijver et al. (50), Kao et al. (51), Loi et al. (52, 53), Wang et al. (54), Hatziis et al. (55), Sotiriou et al. (56), and Desmedt et al. (57).

**ACKNOWLEDGMENTS.** This work was supported by a Terri Brodeur Breast Cancer Foundation fellowship (to S.T.B.) and National Institutes of Health Grant PO1 CA080111 (to M.B.), R01 DK074967 (to M.B.), and R01 HG004069 (to X.S.L.).
Fig. S1. Biological process GO terms of the 179 common genes.
Fig. S2. Biological process GO terms of clusters 1 and 2.
Fig. S3. Motif enrichment for the TRANSFAC p53 binding motifs.

(A) Example p53 binding sites near well-known targets. (B) KEGG pathway analysis of genes found near binding sites.
**Fig. S5.** (A) Biological process GO terms for genes found near the p53 cistrome. (B) Distribution of the p53 cistrome throughout the genome.

**Fig. S6.** Comparison of genes associated with ER and p53 binding sites. The red and blue circles represent the number of commonly regulated genes that are found within 100 kbp of an ER and p53 binding site, respectively. The purple overlap demonstrates the 36 genes that are regulated by both transcription factors.
Fig. S7. GO term analysis of the 36 overlapping genes.

Table S1. p53 cistrome

Table S2. SeqPos analysis of the p53 cistrome