Tamoxifen Functions As a Molecular Agonist Inducing Cell Cycle-Associated Genes in Breast Cancer Cells

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Abstract
Tamoxifen is a widely used breast cancer therapeutic and preventative agent. Although functioning as an estrogen antagonist at the cellular level, transcriptional profiling revealed that at the molecular level, tamoxifen functions largely as an agonist, virtually recapitulating the gene expression profile induced in breast cancer cells by estrogen. Remarkably, tamoxifen induces transcription factors and genes involved in promoting cell cycle progression including fos, myc, myb, cdc23a, cyclins E and A2, and stk15 with kinetics that paralleled that of cells cycling in response to estrogen, even though tamoxifen-treated cells are not transiting through the cell cycle. Induction of cell cycle-associated genes was specific for tamoxifen, and did not occur with raloxifene. However, cyclin D1 was a key estrogen-induced gene not expressed in response to tamoxifen or raloxifene but constitutively expressed in tamoxifen-resistant cells.

Introduction
Modulation of hormone action is an important therapeutic strategy for treating hormone-responsive breast cancer. The nonsteroidal triphenylethyleno tamoxifen is currently prescribed as a chemotherapeutic modulator of estrogen (E2) action in estrogen receptor (ER)-positive tumors. Tamoxifen treatment increases overall survival rate and significantly reduces the incidence of contralateral disease in early breast cancer and the risk of recurrence (1). Tamoxifen also reduces the incidence of breast cancer in high-risk women and is the first chemopreventive agent for breast cancer in pre- and postmenopausal women (2). In addition to the well-characterized antagonist properties of tamoxifen in the breast, tamoxifen also functions as an antagonist in uterine myometrial cells and reduces tumor incidence and volume in a rat model of spontaneous leiomyoma (3, 4). Paradoxically, tamoxifen functions as an agonist in the uterine endometrium (5, 6), bone (7), and cardiovascular system (8). Because of this mixed agonist and antagonist activity in specific cell types, tamoxifen has been termed a selective estrogen receptor modulator (SERM). It is a goal of current endocrine research to develop new SERMs that, like E2, are protective of the skeletal, cardiovascular, and nervous systems, but lack the proliferative activity of E2 that could potentially lead to tumor development in the breast and uterus.

Antagonism has traditionally been attributed to tamoxifen’s antiestrogenic activity, in which the active metabolite of tamoxifen, 4-hydroxytamoxifen (OHT), directly competes with E2 for binding to the ER. In contrast to E2, this ligand-receptor complex recruits corepressors, rather than coactivators, to the promoter regions of estrogen-responsive genes, blocking their transcription (9–11). The mechanisms responsible for the agonist properties of tamoxifen are less clear but appear to involve the transactivation of responsive genes in a promoter- and cell-specific context through the AF-1 transactivation function of the ER (12, 13). Interestingly, even in cells in which tamoxifen functions as an antagonist, such as the breast, tamoxifen can induce the expression of some estrogen-responsive genes such as fos (14), transforming growth factor β (15), progesterone receptor (16), and retinoic acid receptor α (17). Thus, tamoxifen can exhibit agonism on the molecular level even in cell types in which it functions as an antagonist. Furthermore, breast tumors often become resistant to tamoxifen treatment, displaying inappropriate agonist responses such as altered gene expression and growth factor signaling (18). Thus, it is of clinical importance to identify genes that determine the agonist and/or antagonist activities of SERMs such as tamoxifen in the breast and other target tissues. Here we employed a functional genomics approach to identify gene expression profiles induced by a prototypical agonist (E2) and antagonist (OHT) in MCF-7 breast cancer cells, a cell type for which the antagonist properties of OHT have been well characterized.

Results
Similar Gene Expression Profiles Induced by OHT and E2
Microarray analyses were performed using total RNA isolated from MCF-7 cells growth arrested in hormone-depleted...
medium and treated with vehicle, 10 nM OHT, or 10 nM E2 over five time points ranging from 0.5 to 24 h. Cy3 or Cy5 fluorescently labeled cDNAs from vehicle versus OHT- or E2-treated cells were simultaneously hybridized to target sequences on the ToxChip, containing 1901 human genes (http://dir.niehs.nih.gov/microarray/chips.htm). The complete data set containing the Cy3/Cy5 intensity ratios for all target genes on the ToxChip may be viewed at http://dir.niehs.nih.gov/microarray/datasets/MCF-7_estrogen_tamoxifen.txt. For each hybridization, genes significantly induced or repressed with treatment were identified as those lying outside of a 95% confidence interval (CI), based on normalization of the Cy3/Cy5 intensity ratio distribution for a set of 72 control genes or, occasionally, for all genes on the chip (19). To increase sensitivity and minimize the number of false positives, four to eight replicate hybridizations were performed in which the Cy3 and Cy5 labels were reversed for vehicle- and treated-cDNAs and the average log2-transformed intensity ratios were calculated for treatment/vehicle. For higher order comparative analyses (i.e., clustering) of OHT versus E2 treatment, a data set was compiled containing 126 genes that were significantly induced or repressed in at least three of four hybridizations in at least one treatment group at any one time point.

The average log2-transformed expression data for these 126 genes were analyzed using a two-dimensional hierarchical clustering method that grouped genes with similar expression patterns in one dimension and treatment groups in the other (20) (Fig. 1A). Strikingly, many of these genes were regulated by E2 and OHT in a similar manner, as evidenced by the clusters in the dendrogram being driven by the duration of treatment as opposed to ligand (Fig. 1B). The 0.5-h E2 and OHT treatments were more similar to each other than to any of the other treatment groups, although very few changes in gene expression were detected at this early time point. Within the second major cluster, the 2-h E2 and OHT treatment groups clustered together, whereas the 4- and 12-h OHT treatment groups showed little similarity to other treatments within this cluster. However, the 24-h OHT treatment was most similar to the 4-, 12-, and 24-h E2 treatment groups. OHT recapitulation of E2-induced changes in gene expression in MCF-7 cells was reproduced in an independent microarray experiment in which OHT treatments clustered closely with E2 treatments (data not shown).

Although OHT recapitulated the E2-induced expression pattern with similar kinetics for the majority of genes in the compiled data set, the magnitude of OHT-induced gene expression was decreased relative to E2 treatment. This attenuated response is evident on visual inspection of the decreased signal intensities for genes modulated by OHT in the clustering diagram which was confirmed in an independent microarray analysis with 12- and 24-h E2 and OHT treatments (Fig. 1 and data not shown). In addition, several genes validated on the RNA and protein level using Northern and Western analyses (see below) exhibited a similar profile: response to OHT was attenuated in comparison to E2 treatment (Table 1). Although there were both quantitative and qualitative differences in expression of many genes in the compiled data set in response to E2 or OHT, it is important to consider the overall expression pattern for detecting important trends in the data that highlight functional differences between ligands. It was notable that, despite tamoxifen’s well-characterized antiestrogenic effects in breast cells, OHT treatment largely recapitulated the effect of E2 for the expression of the majority of the 126 genes affected by E2.

To confirm that the MCF-7 cells used in these experiments proliferated in response to E2 but not OHT, we performed several growth kinetic assays. Doses of E2 ranging from 1 to 100 nM significantly stimulated an increase in MCF-7 cell number on days 5, 7, and 9, whereas the same doses of OHT failed to do so (Fig. 2A). Cell cycle kinetics analyzed by fluorescence-activated cell sorting (FACS) analysis confirmed that cells grown in hormone-deficient media and treated with 10 nM OHT remained growth arrested in G0/G1 (Fig. 2B). In contrast, treatment with 10 nM E2 stimulated approximately 30% of cells to enter S-phase by 12 h, and by 24 h, 52% had exited G0/G1. The percentage of OHT-treated cells in G0/G1 remained relatively constant, approximately 78%, throughout this 48-h period, similar to the percentage of cells in G0/G1 observed with vehicle treatment (83%). Thus, OHT treatment failed to stimulate MCF-7 cells to enter the cell cycle, consistent with earlier reports (21, 22).

Interestingly, myc and fos, both immediate-early targets of E2 and OHT action (14, 23), were strongly induced by both E2 and OHT at 0.5 h, but expression levels of these genes decreased after 2 h of OHT treatment while remaining elevated for 24 h in response to E2 treatment. The prolonged response to E2 was validated for myc at 24 h by Northern analysis (Fig. 5B). Persistent myc and fos miRNA expression in the E2-treated MCF-7 cells therefore correlated with release from growth arrest and the progressive exit from G1 under the conditions used in this study, which continued over a 24-h period. However, despite an initial up-regulation of fos and myc by OHT, these cells remained in G1 and did not enter the cell cycle.

**Genes Differentially Regulated by E2 and OHT**

Because many of the 126 genes in the compiled data set were regulated by both E2 and OHT, we hypothesized that regulation of only a few genes was responsible for the agonist activity of E2 but not OHT in MCF-7 cells. Therefore, using one-dimensional hierarchical clustering, individual clusters of genes differentially regulated by E2 and OHT were identified (Fig. 3). Among genes that were specifically up-regulated by E2 was cyclin D1, previously reported to be transcriptionally responsive to this steroid hormone (24, 25) (Fig. 3, clusters a and b). Max dimerization protein (Mad), which represses Myc-induced transcription by competing for binding to Max (26), was also up-regulated by E2 but failed to be induced by OHT. Interestingly, although both E2 and OHT up-regulated myc, only E2 induced mad; OHT had no effect on its expression. Several other E2-responsive genes previously shown to modulate ER signaling or E2 metabolism, including nuclear receptor interacting protein 1 (RIP140) (27), Fos-like antigen-1 (fra-1) (28), and cytochrome P450 1B1 (29) were also specifically induced by E2 but not OHT. In addition, four clusters were identified containing highly similar genes down-regulated by E2 but unaffected by OHT treatment (Fig. 3, clusters d–g).
One group of genes identified by hierarchical clustering exhibited what is termed “reverse pharmacology”: OHT treatment induced expression of these genes but E2 down-regulated or had no effect on their expression (Fig. 3, cluster c). Reverse pharmacology in ER signaling was first described by Montano and colleagues who reported that the expression and enzymatic activity of quinone reductase, a phase 2 detoxification enzyme, was up-regulated by OHT treatment and down-regulated by E2 treatment via ER-mediated trans-activation at an electrophile response element (30, 31). That expression of several genes not previously recognized as displaying reverse pharmacology was induced by OHT suggests that this type of regulation may affect more genes than previously thought and could contribute to the antagonism of OHT observed in the breast.

In addition to hierarchical clustering, discrimination between the response to agonist versus SERM treatment was analyzed by using a genetic algorithm/k-nearest neighbor method (GA/KNN) (32). This method employs a nonparametric pattern recognition approach, the k-nearest neighbors (KNN), and a searching tool, a genetic algorithm (GA), to generate subsets of genes that discriminate between different classes of samples. In this analysis, each treatment group and time point was treated as a distinct class, each containing the normalized log2 expression ratios from all genes in each hybridization, four for each treatment, except for the 2-h E2 treatment, for which there were eight hybridizations. Because the expression patterns of some classes (e.g., 0.5-h treatments) may be indistinguishable, a set of genes was considered discriminative when the majority of samples were correctly classified. A total of 25,000 sets of such discriminative genes was obtained, from which genes were ranked based on the frequency of selection into these 25,000 sets. The top 55 genes identified by this approach were subsequently subjected to principal component analysis to identify unifying features of the data set by reducing it into few components that retain the maximum amount of information. Examination of the first two principal components clearly demonstrates the ability of these top 55 genes to discriminate between treatment groups and/or time points (Fig. 4). Replicate hybridizations of E2-treated cells formed five distinct clusters, corresponding to treatment intervals. In contrast, data from hybridizations of OHT-treated cells clustered together regardless of the treatment time.

Not surprisingly, 13 genes identified within the agonist-specific clusters described in Fig. 3 were also identified by the GA/KNN method, including cyclin D1, fra-1, cathepsin D, parathyroid hormone, protein kinase inhibitor β, and DNAJ homolog. Hierarchical clustering was also applied to the top 55 genes identified by GA/KNN, which grouped the 4-, 12-, and 24-h E2 treatments most closely together. The 24-h OHT treatment, while clustering most closely to the later E2 treatments than the other OHT time points, exhibited either little or decreased signal intensities for these genes compared to E2 treatment, a factor most likely to have influenced the inclusion of these genes as discriminators between ligands in MCF-7 cells (see Supplemental Information).

**FIGURE 1.** Two-dimensional hierarchical clustering of genes in the compiled data set. A. (See facing page.) Expression data from treated MCF-7 cells are graphically represented as average log2 intensity ratios (treatment/vehicle). Intensities of red squares (genes induced with treatment) and green squares (genes repressed with treatment) correlate with the relative level of expression with treatment, as indicated by the calibration scale. IMAGE clone numbers are shown, along with the Human Gene Nomenclature Committee official names, when available. The dendrograms cluster treatment groups according to similarity in expression profiles (top) and genes according to similarity in expression profiles (left). Genes in the compiled data set were significantly induced or repressed (at a 95% CI) in at least three of four hybridizations in at least one treatment group. B. Enlarged view of dendrogram in Fig. 1A illustrating similarity of OHT treatment groups to that of E2 in MCF-7 cells.

Table 1. Attenuated Response of E2-Regulated Genes to OHT in MCF-7 Cells

<table>
<thead>
<tr>
<th>Genes (Method)</th>
<th>Time Point (h)</th>
<th>Vehicle ± SD</th>
<th>E2</th>
<th>OHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc (Northern)</td>
<td>2</td>
<td>0.190 ± 0.044</td>
<td>0.634* (3.3-fold)</td>
<td>0.414* (2.2-fold)</td>
</tr>
<tr>
<td>myb (Northern)</td>
<td>12</td>
<td>0.290 ± 0.048</td>
<td>0.688* (3.4-fold)</td>
<td>0.437* (2.2-fold)</td>
</tr>
<tr>
<td>stk15 (Northern)</td>
<td>24</td>
<td>0.014 ± 0.005</td>
<td>0.045* (3.2-fold)</td>
<td>0.024* (1.7-fold)</td>
</tr>
<tr>
<td>cyclin A2 (Western)</td>
<td>24</td>
<td>0.215 ± 0.014</td>
<td>2.061* (9.6-fold)</td>
<td>1.014* (4.7-fold)</td>
</tr>
<tr>
<td>cdc25a (Western)</td>
<td>48</td>
<td>0.795 ± 0.153</td>
<td>2.234* (2.8-fold)</td>
<td>1.628* (2.1-fold)</td>
</tr>
</tbody>
</table>

Note: Data presented are the net intensities of bands derived from one blot, but reproduced in two to four blots. Values were normalized to loading control, GAPDH for Northern blots, and either β-actin or nonspecific band for Western blots. Standard deviations (SDs) were calculated from multiple vehicle-treated samples (n) while n = 1 for E2- and OHT-treated samples. Asterisks denote those values that are more than 2 SD greater than the vehicle mean.

**OHT Recapitulates Estrogen Induction of Cell Cycle-Associated Genes**

Because OHT treatment failed to stimulate MCF-7 cell proliferation, we examined the expression profiles of cell cycle-related genes present on the ToxChip identified as being differentially expressed in the compiled data set (Fig. 5A). Surprisingly, similar to E2, OHT induced the expression of many cell cycle genes. Several genes that regulate transit through the cell cycle (26, 33–35), including myc, fos, myb, cyclin A2, stk15, and cdc25a, were up-regulated by both E2 and OHT treatments. We confirmed induction of these genes by both E2 and OHT at the RNA (Northern analysis) and/or protein level (Western analysis) in independent biological replicates (Fig. 5B and Table 1). Interestingly, cyclin A2, which has been reported to accumulate in G1 phase and to function at the G2/M transition, was up-regulated by OHT at 24 h, similar to its induction by E2. By this time, approximately 50% of E2-treated,
but not OHT-treated cells, have entered S-phase (Fig. 2). Thus, while cyclin A2 has been reported to be necessary for progression to G2/M, expression does not necessarily correlate with transit through the cell cycle in hormonally stimulated breast cancer cells. In addition, our microarray analysis revealed that both E2 and OHT also induced the expression of several other genes involved in replication and maintenance of genomic stability, including replication factor C, serine/threonine kinase 12 and homologues of Saccharomyces cerevisiae cdc6, and minichromosome maintenance deficient homologues 2, 3, and 7. These genes were up-regulated by OHT between 12 and 24 h, corresponding to the time of maximal numbers of cells progressing through S-phase in E2-treated cultures. Elevated expression of stk15, involved in maintaining chromosome stability during replication, in response to OHT, was observed by microarray analysis, although induction did not reach statistical significance. However, by Northern analysis, OHT was observed to reproducibly and significantly induce stk15 expression at 24 h, similar to the kinetics observed with induction by E2 (Fig. 5B and Table 1). Thus, similar to E2, treatment with the SERM OHT coordinately regulated the expression of many of the genes necessary for transit through the cell cycle in the absence of cell cycle progression (Fig. 6).

Whereas OHT appeared to prime MCF-7 cells for cell cycle progression by up-regulating the expression of many factors necessary for replication, OHT-treated cells fail to enter the cell cycle, suggesting that specific "gatekeeper"-type genes may prevent these cells from exiting G0/G1 and entering S-phase. To identify these gatekeepers, we examined the differences between E2 and OHT expression profiles during the cell cycle. Although the overall expression profiles for E2 and OHT were very similar, there were a few differences. Cell cycle-associated genes that were specifically regulated by E2 but not OHT included cyclin D1, fra-1, and uracil DNA glycosylase (UNG) (Figs. 5A and 6). That expression of cyclin D1 was increased by E2 but not OHT was confirmed at both the RNA and protein levels (Fig. 5B). E2 induction of cyclin D1 RNA expression peaked between 2 and 12 h and protein levels were induced after 4 h of treatment and peaked at 12–24 h. Thus, the inability of OHT to induce cyclin D1 identifies this key cell cycle regulator as an ideal candidate gatekeeper gene for proliferation, the expression of which may determine agonism versus antagonism in this cell type.

To confirm this, MCF-7 cells used in this study were cultured in the presence of OHT for more than 1 year to select for tamoxifen-resistant cells. As shown in Fig. 7, both parental

**FIGURE 2.** Growth kinetics of MCF-7 cells in response to treatment with E2 and OHT. A. Proliferation of MCF-7 cells in response to treatment with E2 but not OHT. Cells growth arrested in hormone-deficient media were treated in triplicate with vehicle, 10 or 100 nM E2, or OHT and cell numbers counted over multiple days. Error bars, SE; asterisks, statistical significance of P < 0.05, determined by ANOVA. B. Cell cycle distribution of MCF-7 cells in response to E2 and OHT. Cells growth arrested in hormone-deficient media were treated over a 48-h period with vehicle, 10 nM E2 or 10 nM OHT, and DNA content measured by FACS analysis. Values shown are from one independent experiment that was reproduced twice.
FIGURE 3. Agonist-specific clusters identified by one-dimensional hierarchical clustering of genes in the compiled data set. Treatment groups were organized according to treatment intervals (in hours) and genes were clustered according to similarity in expression profiles. Data are graphically represented as described in Fig. 1. Clusters a–g, lower-case letters represent clusters of genes differentially regulated by E2 and OHT, shown in greater detail to the right.
FIGURE 4. Principal component analysis for the top 55 genes selected by the GA/KNN method. Log2 expression ratios from all target sequences were analyzed using the GA/KNN method and principal component analysis was applied to the variance-covariance matrix of the data (44 hybridizations from 10 treatment groups). The principal components were obtained by projecting the original data points (log2 transformed and mean centered) onto the eigenvectors, extracted by the method of single value decomposition. A scatterplot of the first two principal components (x and y axes, respectively) is shown for each hybridization, with hybridizations from identical treatment groups shaded similarly.

FIGURE 5. Expression data from genes involved in regulation of transit through the cell cycle in response to treatment with E2 and OHT. A. Expression data were obtained from treated MCF-7 cells in the compiled data set for genes directly involved in cell cycle, transcription factors involved in inducing entry into the cell cycle, and genes involved in replication and/or maintenance of genomic stability. Data are graphically represented as described in Fig. 1. B. Northern analysis of treated MCF-7 cells for myc, myb, stk15, and cyclin D1 (4.3 and 1.5 kb). Inset, duplicate Northern analysis highlighting the up-regulation of myc in response to E2 and OHT at early time points. Loading was assessed by expression of GAPDH RNA. C. Western analysis of treated MCF-7 cells for cyclin D1 and cyclin A2 protein expression. Loading was assessed by expression of actin (cyclin A2) or by a nonspecific band unresponsive to treatment (cyclin D1).
tamoxifen-sensitive MCF-7 cells and tamoxifen-resistant MCF-7 TAMR1 cells are estrogen responsive (Fig. 7A) and express ERs (data not shown), but in contrast to parental MCF-7 cells, MCF-7 TAMR1 cells cultured in estrogen-depleted medium proliferate in the presence of 10 nM OHT. Examination of cyclin D and cyclin A2 expression in these cells revealed that cyclin D was constitutively expressed in MCF-7 TAMR1 cells (Fig. 7B). Cyclin A2, which was maximally induced by both tamoxifen and estrogen at 24 h, was also constitutively expressed in MCF-7 TAMR1, consistent with the constitutively high levels of cyclin D expressed by these cells.

Cyclins Are Not Induced by the SERM Raloxifene

Raloxifene is a second-generation SERM with an improved agonist versus antagonist tissue profile relative to tamoxifen. Raloxifene is also an antagonist in the breast, but in contrast to tamoxifen, lacks agonism in the uterus (4, 36). To determine if, like OHT, raloxifene also induces the expression of cell cycle-associated genes, expression of cyclins D, E, and A2 was examined in MCF-7 cells exposed to estrogen, OHT, and raloxifene by Western analysis (Fig. 8). In contrast to tamoxifen, which induced cyclin E and A2 expression with the same kinetics as estrogen, raloxifene failed to induce either cyclin D or the downstream cyclins E and A2. Thus, induction of cell cycle-associated genes by SERMs is ligand specific and may even be unique to tamoxifen.

Discussion

Transcriptional profiling revealed that at the molecular level, tamoxifen functions largely as an agonist, in contrast to its action as an antagonist at the cellular level. Many more E2-responsive genes than anticipated were found to be responsive to the SERM tamoxifen in breast cancer cells. In particular, 20 of 23 cell cycle-associated genes induced by E2 were also induced by tamoxifen, including cyclins E and A2, cdc25A, and stk15. That tamoxifen recapitulated the action of E2 was unexpected because E2 is an agonist in this cell type, inducing proliferation, whereas tamoxifen is an antagonist in breast cancer cells, effectively inhibiting cell growth. This suggests that the ability of tamoxifen to illicit an agonist or antagonist response at the cellular level may be controlled by only a small subset of genes that determine whether cells proliferate in the presence of this SERM. Consequently, altered expression in tumor cells of only a very few genes may be sufficient to induce cells to proliferate in response to tamoxifen. The ability of tamoxifen to induce numerous genes required to progress through the cell cycle combined with an apparently small number of genes that discriminate between an agonist versus antagonist response could facilitate the development of tamoxifen-resistant disease.

Although tamoxifen largely recapitulated the E2-induced cell cycle gene expression profile, it failed to induce expression of cyclin D1, a key regulator of the G1/S phase transition of the cell cycle. Previous reports have shown that exogenously expressed cyclin D1 is sufficient to induce proliferation as well as activate cdks and phosphorylate protein Rb in MCF-7 and T47D cells (37, 38). Neutralizing antibodies to cyclin D1 blocked E2-stimulated MCF-7 cells in G1 (39) and treatment with both the pure antiestrogen ICI 182,780 and tamoxifen down-regulated cyclin D1 expression and inhibited proliferation in mitogen-stimulated breast cancer cells (40, 41). Furthermore, there is a strong correlation between increased levels of cyclin D1 and ER expression in breast cancer (42) and it has been recently shown that specific protection again breast cancer is conferred by cyclin D1 ablation (43). Our gene expression studies further establish the importance of cyclin D1 as a mediator of E2-induced cell proliferation and suggest that cyclin D1 may act as a gatekeeper to determine agonism versus antagonism at the cellular level in breast cancer cells. Therefore, the failure of tamoxifen to induce cyclin D1 provides a plausible explanation for the inability of tamoxifen to promote cell proliferation while up-regulating numerous cell cycle-associated genes. Indeed, although tamoxifen failed to induce cyclin D1 expression in tamoxifen-sensitive MCF-7 cells, this gene was constitutively expressed in tamoxifen-resistant cells. The finding that elevated levels of cyclin D1 RNA are associated with a shorter clinical response to tamoxifen could be explained by these observations (44).

In addition to cyclin D1, fra-1 was also differentially expressed in MCF-7 cells in response to E2; fra-1 was selectively up-regulated at 4 h of E2 treatment, whereas
tamoxifen had no effect on fra-1 expression, even after 24 h of exposure. Fra-1, also known as FOSL, is a member of the fos family of leucine zipper DNA binding proteins that dimerize with jun family members to form the AP-1 transcriptional complex. fra-1 has recently been shown to be overexpressed in more aggressive breast cancer cells (45) and fra-1 protein levels have been shown to negatively correlate with ER status (46). However, fra-1 can repress E2-induced transcription from AP-1 sites when exogenously expressed in MCF-7 cells (28). Furthermore, fra-1 is constitutively expressed in some ER-negative breast cancer cells, where it is thought to play a role in the inability of exogenously expressed ER to induce transcription from AP-1 sites. In our experiments, E2 induced fra-1 expression at 4 h of treatment, and then expression rapidly declined, suggesting that fra-1 could be acting to temporally down-regulate E2-induced transcription at AP-1 sites, serving as a negative feedback mechanism for E2 action in these cells. Given the few differentially expressed genes observed in this study and the large number of genes repressed in MCF-7 cells in response to E2 but not tamoxifen, the role of fra-1 in gene repression and mediation of an agonist response in breast cancer cells should be investigated further.

In contrast to tamoxifen, the SERM raloxifene failed to induce cyclins E and A2 as well as cyclin D1. Raloxifene is a benzothiophene SERM, which like tamoxifen, is an agonist in the bone and cardiovascular system but is an antagonist in both the breast and uterus and is currently used clinically to prevent osteoporosis in postmenopausal women (47, 48). Raloxifene is now being evaluated relative to tamoxifen for postmenopausal breast cancer in the STAR trial (Study of Tamoxifen and Raloxifene) conducted by the NSABP (49, 50). Our data would predict that SERMs such as raloxifene, which do not induce cell cycle-associated genes, would have improved clinical benefit relative to tamoxifen, especially in postmenopausal breast cancer where the majority of tumors are ER-positive (51).

In conclusion, our data demonstrate that at the molecular level, tamoxifen functions largely as an agonist, in contrast to this SERM’s function as an antagonist at the cellular level. The ability of tamoxifen to induce the majority of cell cycle-associated genes induced by estrogen in breast cancer cells, in combination with acquired expression of genes such as cyclin D1 that act as gatekeepers for proliferation in these cells, could contribute to the development of tamoxifen-resistant disease. The fact that raloxifene did not induce cell cycle-associated genes suggests that other SERMs may not exhibit tamoxifen’s agonism at the molecular level and may be more beneficial in treating and preventing breast cancer.

Materials and Methods

Cell Culture and RNA Isolation

MCF-7 cells were grown in phenol red-free IMEM (BioFluids Inc., Rockville, MD) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). Tamoxifen-resistant MCF-7 (MCF-7(TAMR1)) cells were generated by maintaining MCF-7 cells in IMEM containing 5% FBS plus 1 μM OHT for >12 months. Forty-eight hours before treatment, medium was changed to IMEM containing 5% charcoal dextran stripped FBS (Hyclone Laboratories). Cells were treated with 10 nM 17β-estradiol (E2) (Sigma Chemical Co., St. Louis, MO), 10 nM OHT (Sigma), or 10 nM raloxifene (Toronto Research Chemicals, Inc., Downsview, Ontario, Canada) for microarray, Northern, Western, and FACS analyses. Doses ranging from 1 to 100 nM were used in proliferation assays. Total RNA was isolated using RNeasy kit from Qiagen (Valencia, CA).

FACS Analysis

Treated MCF-7 cells were fixed in 70% ethanol and stained with propidium iodide (40 μg/ml) (BD Biosciences, Palo Alto,
DNA content was obtained by measuring 20,000 cells (post-aggregate exclusion) with the EPICS Elite flow cytometer (Coulter, Hialeah, FL) followed by analysis with Multicycle DNA analysis software (Phoenix Flow System, Inc., San Diego, CA).

**Microarray Experiments**

Microarray data were validated in two independent experiments, each with four to eight replicate hybridizations and utilizing fluorescent dye reversal. The ToxChip (version 1.0) (52) contains 1901 genes and is described at http://dir.niehs.nih.gov/microarray/chips.htm. Methods for clone preparation, probe labeling, hybridization conditions, and image acquisition can be viewed at http://dir.niehs.nih.gov/microarray/datasets/Hodges_methods.pdf.

**Data Analysis**

Cy3/Cy5 intensity ratios were calculated for each target sequence and can be viewed at http://dir.niehs.nih.gov/microarray/datasets/MCF-7_estrogen_tamoxifen.txt. IMAGE clones were annotated according to the official gene names from the Human Gene Nomenclature Committee, when available. Genes significantly induced or repressed with treatment were identified as those excluded from a 95% CI based on normalization of the Cy3/Cy5 intensity ratio distribution for a set of 72 housekeeping genes, or occasionally the distribution of intensities for all target sequences on the chip (19). For clustering analysis, data were compiled into a data set containing genes excluded from a 95% significance interval in at least three of four hybridizations for at least one treatment group. Unsupervised hierarchical clustering was performed using Cluster and Tree-view software (20). Using this program, we performed average linkage clustering using Pearson’s correlation as a similarity metric. Genetic algorithm/k-nearest neighbor (GA/KNN) method was used for sample classification from all hybridizations, based on the expression data for all target sequences and is described in Li et al. (32) and at http://dir.niehs.nih.gov/microarray/datamining/. Principal component analysis was applied to the variance-covariance matrix of genes identified by the GA/KNN method (44 hybridizations, log2 transformed) by using the method of single value decomposition (Numerical Recipes, Cambridge, MA).

**Northern Analysis**

Total RNA (5 μg) was separated on a 1% agarose-formaldehyde gel and downwardly transferred onto positively charged nylon and hybridized with [α-32P]dUTP-labeled probes in UltraHyb (Ambion, Austin, TX). RNA probes (myc, myb, and stk15) were generated using Strip-EZ RNA probe labeling kit (Ambion). Cyclin D1 DNA probe was a gift from Susan M. Fischer. To assess loading, blots were stripped and reprobed with mouse pTRI-GAPDH RNA probe (Ambion). RNA for validation of microarray data was obtained from independent biological replicates of treatment protocols used to generate RNA for microarray analysis.

**Western Analysis**

Protein extracts of treated MCF-7 cells were prepared by standard methods, separated by SDS-PAGE, and transferred to polyvinylidene difluoride. Membranes were probed with the following antibodies: cyclin D1 (Zymed, San Francisco, CA); cyclin A2 and cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA); cdc25a (Upstate Biotechnology, Inc., Lake Placid, NY); and actin (ICN Biomedicals, Aurora, OH). Protein extracts were obtained from one to two independent biological replicates of treatments used to generate microarray data.

**Supplemental Information**

Supplemental information is available on the NIEHS website:


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References


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Leslie C. Hodges, Jennifer D. Cook, Edward K. Lobenhofer, et al.


2 IMAGE clones were annotated according to the official gene names from the Human Gene Nomenclature Committee, when available. The official gene names for cyclin D1, cyclin A2, and fra-1 are CCND1, CCNA2, and FOSL1, respectively.