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Identification of gene regulation patterns underlying both E₂- and tamoxifen-stimulated cell growth through global gene expression profiling in breast cancer cells

Ping Fan¹, Heather E. Cunliffe^{2,3}, Obi L. Griffith⁴, Fadeke A. Agboke¹, Pilar Ramos², Joe W. Gray⁵, and V. Craig Jordan^{1,*}

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC 20057

²Computational Biology Division, The Translational Genomics Research Institute, Phoenix, AZ 85004

⁴Department of Medicine, Division of Oncology, The Genome Institute, Washington University, St. Louis, MO 63108

⁵Biomedical Engineering Department, Oregon Health and Science University, Portland, OR 97239

Abstract

Purpose—A c-Src inhibitor blocks estrogen (E₂)-induced stress and converts E₂ responses from inducing apoptosis to growth stimulation in E₂-deprived breast cancer cells. A reprogrammed cell line, MCF-7:PF, results in a functional estrogen receptor (ER). We addressed the question of whether the selective ER modulator 4-hydroxytamoxifen (4-OHT) could target ER to prevent E₂-stimulated growth in MCF-7:PF cells.

Methods—Expression of mRNA was measured through real-time RT-PCR. Global gene expression profile was analyzed through microarray. Transcriptome profiles were screened by RNA-sequencing.

Results—Unexpectedly, both 4-OHT and E₂ stimulated cell growth in a concentration-dependent manner. Expression profiling showed a remarkable overlap in genes regulated in the same direction by E₂ and 4-OHT. Pathway enrichment analysis of the 280 genes commonly deregulated in MCF-7:PF cells by 4-OHT and E₂ revealed functions mainly related to membrane, cytoplasm, and metabolic processes. Further analysis of 98 genes up-regulated by both 4-OHT and E₂ uncovered a significant enrichment in genes associated with membrane remodeling, cytoskeleton

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*Address correspondence to this author: V. Craig Jordan OBE, PhD, DSc, FMedSci, Lombardi Comprehensive Cancer Center, Georgetown University, E507A Research Bldg, 3970 Reservoir RD NW, Washington D.C. 20057, Tel: (202) 687 2897; ycj2@georgetown.edu.

³Current address: Department of Pathology, Dunedin School of Medicine, University of Otago, Dunedin 9054, New Zealand

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reorganization, cytoplasmic adapter proteins, cytoplasm organelles proteins, and related processes. 4-OHT was more potent than E₂ in up-regulating some membrane remodeling molecules, such as *EHD2*, *FHL2*, *HOMER3* and *RHOF*. In contrast, 4-OHT acted as an antagonist to inhibit expression of the majority of enriched membrane-associated genes in wild-type MCF-7 cells.

Conclusions—Long-term selection pressure has changed the cell population responses to 4-OHT. Membrane-associated signaling is critical for 4-OHT-stimulated cell growth in MCF-7:PF cells. This study provides a rationale for the further investigation of target therapy for tamoxifen resistant patients.

Keywords

tamoxifen; estrogen; membrane-associated molecules; gene expression profiling

1. Introduction

Estrogen (E₂) plays a pivotal role in the development and progression of breast cancers. As a result, blockade of the E₂ signal through either aromatase inhibitors (AIs) or tamoxifen are important therapeutic strategies to treat or prevent estrogen receptor (ER) positive breast cancers (1). However, the acquired resistance to anti-estrogen therapies is still a challenge in the clinic. Laboratory findings that re-transplantation of tamoxifen-stimulated MCF-7 tumors into successive generations of athymic mice over 5 years results in the selection of a resistant tumor cell population that is killed by physiological levels of E₂ (2, 3), has resulted in the new biology of E₂-induced apoptosis (4-6). Indeed, E₂-induced apoptosis has been used to treat breast cancer after failure of AI therapy (7) and used to explain the action of E₂ replacement therapy for postmenopausal women in their 60s having a lower incidence of breast cancer and mortality (8). All of these clinical relevant findings encouraged us to investigate the mechanisms underlying E₂-induced apoptosis in long-term E₂-deprived breast cancer.

The function of oncogene c-Src is closely associated with the ER and has been reported to mediate resistance to endocrine therapy in breast cancer cells (9, 10). Our previous findings show that c-Src activity is increased in two long-term E₂-deprived breast cancer cells (11), which mediates the non-genomic and genomic pathways of E₂ (12). In addition to the role of c-Src in growth, the oncogene participates in stress responses induced by E₂ (12), which are critical to induce apoptosis (12-14). Inhibition of c-Src blocks E₂-induced apoptosis (12,15). Unexpectedly, a resulting cell line, MCF-7:PF, is established when treatment is extended for 2 months to mimic clinical trials (15). Rather than undergo apoptosis a new E₂-stimulated population emerged (15). It raises the question whether the antiestrogen 4-hydroxytamoxifen (4-OHT) can block the E₂-stimulated growth in MCF-7:PF cells.

In this study, we found that 4-OHT stimulated MCF-7:PF cell growth in an ER-dependent manner and could not block E₂-stimulated growth. Global gene expression microarray revealed a remarkable overlap in genes deregulated in the same direction by E₂ and 4-OHT in MCF-7:PF cells, which could be blocked by the pure antiestrogen ICI 182,780 (ICI). Pathway enrichment analysis of the 280 genes commonly deregulated by 4-OHT and E₂ revealed the top 13 functions are mainly related to membrane, cytoplasm, and metabolic

processes. Together, these results suggest that membrane-associated signaling may be involved in the tamoxifen-stimulated growth.

2. Materials and Methods

2.1 Materials

Estradiol (E₂) and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma-Aldrich (St. Louis, MO); ICI 182,780 (ICI) was purchased from Tocris (Park Ellisville, MO). c-Src inhibitor, PP2, and IGF-1R β inhibitor, AG1024, were purchased from CalBiochem (San Diego, CA).

2.2 Cell culture conditions and cell proliferation assays

The ER-positive wild-type human breast cancer MCF-7 cells, estrogen-deprived MCF-7:5C cells, and MCF-7:PF cells were cultured as previously described (15). The DNA fingerprinting pattern of all cell lines are consistent with the report by the ATCC (15). The DNA content of the cells, a measure of proliferation, was determined as previously described (15) using a DNA fluorescence Quantitation kit (Bio-Rad Laboratories, Hercules, CA).

2.3 Quantitative real-time RT-PCR

Quantitative real-time RT-PCR assays were conducted as previously described (12) using the SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA) and a 7900HT Fast Real-time PCR System (Applied Biosystems). All primers were synthesized by Integrated DNA Technologies (San Diego, CA).

2.4 RNA sequencing (RNA-seq) analysis

In brief, wild-type MCF-7 cells were treated with vehicle control (0.1% ethanol), E₂ (10⁻⁹ mol/L), and 4-OHT (10⁻⁶ mol/L) for 24 hours. Total RNA was isolated with an RNeasy Micro kit (Qiagen, Valencia, CA). These RNA samples were first converted into a library of cDNA fragments. Sequencing adaptors were subsequently added to each cDNA fragment and a 2 $\times$ 100 bp paired-end sequence was obtained from each cDNA using high-throughput sequencing technology (Illumina GAII). An average of 88.7 million such reads was produced for each sample. Of these, 43.8 million reads mapped to known transcripts on average, resulting in an average coverage of 47.1 $\times$ across all known gene loci. Analysis was performed with the ALEXA-seq software package as previously described (12, 16). Briefly, this approach comprised (i) creation of a database of expression and alternative expression sequence 'features' (genes, transcripts, exons, junctions, boundaries, introns, and intergenic sequences) based on Ensembl (53_360) gene models, (ii) alignment of short paired-end sequence reads to these features using BWA (0.5.8a) and BLAST (2.2.18), (iii) identification of features that are expressed above background while taking into account locus-by-locus noise and (iv) assignment of FPKM-like values for each feature type, normalized to feature size and total library coverage. Gene expression data was deposited in Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) with accession number GSE59536.

2.5 Gene Expression Microarrays

MCF-7:PF cells were treated for 7 days with vehicle (0.1% EtOH), E₂ (10⁻⁹ mol/L), 4-OHT (10⁻⁶ mol/L), ICI (10⁻⁶ mol/L), E₂ (10⁻⁹ mol/L) plus ICI (10⁻⁶ mol/L), or 4-OHT (10⁻⁶ mol/L) plus ICI (10⁻⁶ mol/L) in triplicate. Total RNA was isolated using the Qiagen RNeasy Micro kit (Qiagen, Valencia, CA). RNA integrity and purity were measured as previously described (13). Cy3 (sample) and Cy5 (reference) cRNA probes were prepared and co-hybridized to Agilent 4×44K Human 1A (V2) dual color Oligo Microarrays using manufacturer's protocols (Agilent Technologies, Palo Alto, CA). Common reference was derived from MCF-7:PF treated with EtOH vehicle alone. Slides were washed in an ozone-controlled environment (<0.1 ppb) and scanned using an HP Microarray Scanner (Agilent model G2505B). Data were extracted, processed and normalized using Agilent's Feature Extraction software (version FE10.5.1). Gene expression data was deposited in Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) with accession number GSE54171. To identify genes significantly deregulated in MCF-7:PF by a particular treatment condition, array feature data was filtered using their expression ratio *p* value calculated during Feature Extraction (Agilent). This software-derived gene-specific metric is an inherent objective measure of expression change significance between differentially labeled and co-hybridized probes (MCF-7:PF treated/MCF-7:PF untreated). Genes whose expression ratio was considered up- or down-regulated by this software with a ratio *p* value < 0.001 were flagged as 'statistically significant'. For genes to be significantly deregulated in a particular treatment condition relative to untreated reference, we required the expression ratio *p* values to be significant (<0.001) in all experimental replicates for that condition.

2.6 Gene Set Enrichment and Pathway Analysis

Gene set enrichment analysis was conducted using Pathway Studio version 9.0. This software identifies pre-defined pathways that are statistically implicated by Fisher's Exact Test based on our differentially expressed gene list. Significantly enriched pathways were required to pass a false discovery rate of 0.05.

2.7 Statistical Analysis

All reported values are the means ± SE. Statistical comparisons were determined with two-tailed Student's *t* tests. Results were considered statistically significant if the *p* value was <0.05. Gene expression microarrays and RNA-sequence have respective statistical analysis with special software package. Pathway Studio Version 9.0 was utilized to analyze pathway enrichment (*p* value was <0.05).

3. Results

3.1 The ER agonist activity of 4-OHT is significantly elevated in MCF-7:PF cells

Our recent publication shows the proliferative response to E₂ in the reprogrammed cell line, MCF-7:PF, occurs in an ER-dependent manner (15). Here, we addressed the question of whether 4-OHT could block E₂-stimulated growth. Unexpectedly, 4-OHT significantly stimulated cell growth in MCF-7:PF cells (Fig. 1A). The stimulation by 4-OHT could be completely blocked by ICI (Fig. 1A). Further, we examined the dose-responsive curves of 4-OHT compared with E₂ and ICI in MCF-7:PF cells (Fig. 1B). The effect of 4-OHT on cell

growth was approximately 1,000-fold less potent than E₂ (Fig. 1B). ICI exerted no effect on MCF-7:PF cells (Fig. 1B), although it blocked proliferation stimulated by E₂ and 4-OHT (15, Fig. 1A). It is well documented that 4-OHT acts as an effective inhibitor of cell growth and blocks proliferation mediated by E₂ in wild-type MCF-7 cells (Fig. S1A and S1B). In contrast, 4-OHT had no capacity to block E₂-induced cell growth in MCF-7:PF cells (Fig. 1C).

3.2 4-OHT primarily regulates ER-dependent genes to promote cell growth

To understand the molecular actions of 4-OHT and E₂ in MCF-7:PF cells, Agilent 44k dual color gene expression microarrays were performed in triplicate on MCF-7:PF cells treated with 4-OHT or E₂ with or without ICI, and co-hybridized to a common reference probe prepared from untreated MCF-7:PF cells. MCF-7:PF cells were also treated with ICI alone as a comparative control series. 1,354 genes were identified as significantly up- or down-regulated by either E₂ or 4-OHT relative to untreated MCF-7:PF cells as described in *the Materials and Methods*. Hierarchical clustering was used to visualize clustered patterns of gene expression ratio change (relative to untreated MCF-7:PF reference) for these 1,354 genes across the five treatment conditions (Fig. 2A). This analysis revealed the extent to which genes regulated by E₂ and 4-OHT in MCF-7:PF are overlapping or distinct (the two treatment groups to the left of the dendrogram), and genes whose expression pattern indicates a dependence (or lack thereof) on ER for transcriptional control by comparison of their expression behavior with ICI co-treatment (Fig. 2A). Importantly, the dendrogram showed a remarkable overlap in genes regulated in the same direction by E₂ and 4-OHT (Fig. 2A). 538 genes were identified as uniquely E₂/4-OHT regulated in an ER-dependent manner, due to ICI-mediated attenuation of their expression response to E₂ or 4-OHT, and no significant deregulation by ICI alone (Fig. 2B and S2A). 292 of these genes were significantly regulated by both E₂ and 4-OHT, 280 (96%) of which were regulated in the same direction (Fig. 2B and S2B). For example, both E₂ and 4-OHT up-regulated EH-domain containing 2 (*EHD2*), four and a half LIM domains 2 (*FHL2*), homer homolog 3 (*HOMER3*), and Ras homolog family member F (*RHOF*). EHD2 protein plays a role in membrane reorganization (17). The LIM domain protein, FHL2, interacts with filamin A to remodel the cytoskeleton and acts as a novel coactivator in regulating target genes (18). HOMER3 is a cytoplasmic scaffolding protein which mediates protein-protein interactions (19). RHOF, belonging to the Rho family GTPases, has a critical role in controlling the formation of filopodia (20).

3.3 Deregulation of selected genes is validated by real-time RT-PCR

A variety of genes were deregulated by both E₂ and 4-OHT (Fig. 2A). We verified some up-regulated genes (*EHD2*, *FHL2*, *HOMER3*, and *RHOF*) (Fig. 3) and down-regulated genes (*GPER*, *IGFBP3*, and *FOXO1*) by real-time RT-PCR (Fig. S3). Interestingly, G-protein-coupled estrogen receptor, GPR30 (*GPER*) was down-regulated by both E₂ and 4-OHT, which has been reported to be increased after tamoxifen treatment (21), and to be involved in tamoxifen resistance (22). Similar regulation patterns were observed in *IGFBP3* and *FOXO1* (Fig. S3). IGFBP3 serves in a negative-feedback loop to control cell growth by preventing activation of the IGF-1R (23). FOXO transcription factors mainly function to promote cell-cycle arrest and apoptosis via regulation of diverse arrays of transcription targets. AKT phosphorylation of FOXOs leads to inactivation of their transcription activities

(24). Down-regulation of two feedback effectors, *IGFBP3* and *FOXO1*, suggests that IGF-1R/PI3K/AkT axis may be activated by E₂ and 4-OHT in MCF-7:PF cells.

3.4 4-OHT stimulates a significant enrichment of genes associated with membrane function in MCF-7:PF cells

In addition to the validated genes (Fig. 3 and S3), a variety of genes were deregulated by E₂ and 4-OHT which may be involved in the coordination of a diverse range of regulatory processes for Rho-GTPase-signaling (*GTPBP3*, *RASIP1*, *CDC42EP2*, *PTAFR*, *CHML*), cellular organelle functions (*MRPS10*, *MAOA*, *ALDH1L2*, *CHST8*, *LRP8*, *RPS2*), cell metabolism or ion homeostasis (*PDZK1*, *ASPH*, *KCNK6*, *STC2*, *FAM77C*, *FADS3*, *MPPED1*, *AMZ1*), adhesion molecules or actin cytoskeleton (*CELSR2*, *TUBB6*, *FGD6*, *SH4*, *LOXL3*), and transport proteins (*SEC14L4*, *SLC16A1*, *SLC25A25*, *SERPINA6*, *SYTL5*, *SNX24*, *SEC14L4*, *NIM1*) (Table S1). Consistently, pathway enrichment analysis of the 280 genes commonly deregulated by 4-OHT and E₂ in MCF-7:PF cells revealed top 13 functions mainly related to membrane, cytoplasm, and metabolic processes (Table S2 and S3). Further analysis of 98 genes up-regulated by both 4-OHT and E₂ uncovered a significant enrichment in genes associated with membrane remodeling, cytoskeleton reorganization, cytoplasmic adapter proteins, cytoplasm organelles proteins, and related processes (Fig. 4A). There are many similarities between MCF-7:PF cells and “original” wild-type MCF-7 cells (15). A significant change is that E₂ stimulates cell growth in the two cell lines in an ER-dependent manner (15). However, they have differential response to 4-OHT (Fig. 1A and S1A). To verify whether both E₂ and 4-OHT regulate these membrane-associated genes in the same patterns or not in wild-type MCF-7 cells, we screened transcriptome profiles after E₂ and 4-OHT treatment by RNA sequence. The opposing E₂ and 4-OHT transcriptome profiles of these membrane function-associated genes were observed in wild-type MCF-7 cells, even though 4-OHT activated some genes (Fig. 4B). These data suggest that 4-OHT acts differentially on gene regulation in MCF-7:PF cells and wild-type MCF-7 cells.

3.5 Regulation of membrane-associated genes by 4-OHT in the presence of E₂

In agreement with our transcriptome profiles in wild-type MCF-7 (Fig. 4B), gene regulation pattern of 4-OHT is distinct from that of E₂ (25), and 4-OHT is able to block the up-regulation of many genes by E₂ in wild-type MCF-7 cells (25). To further investigate the function of 4-OHT in the regulation of genes associated with membrane functional roles in MCF-7:PF cells, we treated cells with 4-OHT in the presence of E₂. Unlike the quick regulation of ER-target genes by E₂ after a few hours (15), functional membrane-associated genes *EHD2*, *FHL2*, *HOMER3*, and *RHOF* exhibited delayed kinetics of 4-OHT and E₂ induction after 72 hours (Fig. 5). 4-OHT was unable to block deregulation of these genes by E₂ in MCF-7:PF cells (Fig. 5 and S4). Additionally, 4-OHT was more potent than E₂ to up-regulate these genes (Fig. 5). Together, these data provide evidence that 4-OHT functions as an agonist to regulate genes with membrane-associated roles in MCF-7:PF cells.

4. Discussion

Dramatic increases in patient survivorship occur following long-term adjuvant tamoxifen therapy (26, 27). However, acquired resistance to treatment develops. Strategic definition of molecular mechanisms driving the development of endocrine resistance is an important and proactive first step to improve therapy. Our knowledge of acquired TAM resistance has been primarily achieved from studies in athymic mouse models using transplanted MCF-7 cells (28, 29), in which either E₂ or a SERM stimulates the growth of acquired TAM-resistant tumors (30, 31). However, no mechanism has been established to explain this paradox. We describe the first validated model *in vitro* to decipher the gene expression profiling by both E₂ and 4-OHT in acquired SERM resistance.

A common characteristic existing between wild-type MCF-7 and MCF-7:PF cells is stimulation of cell growth by E₂ through ER (15). However, they have differential responses to 4-OHT (Fig. 1A and Fig. S1A); one is inhibited by 4-OHT (MCF-7) and another (MCF-7:PF) is stimulated by 4-OHT. In an attempt to identify gene regulation patterns by 4-OHT and E₂, global gene expression profiling was performed in MCF-7:PF cells. Supporting biological features, the ER is commonly utilized by both E₂ and 4-OHT to deregulate genes in MCF-7:PF cells (Fig. 2A). This is consistent with the findings of other group's that the majority of genes are regulated by E₂ or tamoxifen metabolites through the ER in wild-type MCF-7 cells (32-35). In contrast to the overlapping gene regulation by E₂ and 4-OHT in MCF-7:PF cells (Fig. 2A and 2B), the gene regulation pattern of 4-OHT is distinct from that of E₂ in MCF-7 cells (25), and 4-OHT is able to block the deregulation of many genes by E₂ (25). However, 4-OHT is unable to inhibit enriched genes activated by E₂ in MCF-7:PF cells (Fig. 5 and S4). Although tamoxifen can have an E₂-like agonist effect on some genes known to be regulated by E₂ in wild-type MCF-7 cells, it acts primarily to antagonize E₂ actions (34, 35). These results suggest that cellular responses to 4-OHT are dramatically altered in MCF-7:PF cells.

Although we have extensively screened the genes deregulated by E₂ and 4-OHT in the same direction (Fig. 3, 4A, and 5A), a wide range of genes are differentially regulated by E₂ and 4-OHT in MCF-7:PF cells (Fig. 2B). Ninety-three genes are 4-OHT preferentially regulated genes; that is, they are deregulated by 4-OHT, but not by E₂ (Fig. 2B and S2A). The enrichment functions of these genes are related to adhesion molecule (*MAG*, *DOCK4*), remodeling cytoskeleton (*PMP22*, *SPAG4*), transmembrane transport of small molecules or ions (*SLC7A5*, *CAPN12*, *ATP4A*, *PITPNM2*, *SLC51B*, *PDZD2*), metabolic pathways and lipoprotein metabolism (*NPC1L1*, *DNAJA4*, *CML2*, *NAT8*, *ANGPTL4*, *GALNT5*), Rho-GTPase-activating (*OPHN1*, *P2RY5*), and transmembrane receptor protein tyrosine phosphatase activity (*PTPRH*). Most functions of 153 E₂ preferentially regulated genes are overlapped with that of 4-OHT preferentially regulated genes, which include cell-cell adhesion or re-organization of cytoskeleton (*ASAM*, *GJB2*, *EVL*, *TIMP1*), metabolism (*PTGS1*, *SAT*, *MGAT3*, *NPR3*), transport of small molecules in the cytoplasm (*RAB7B*, *TMC4*, *SYT12*, *HSPA8*, *APIS2*, *ABCC2*), and regulation of G-protein coupled receptor activity (*PTHR1*, *FZD8*, *CXCR4*, *CXCL12*, *DOCK11*). However, a dominant difference between E₂ and 4-OHT is that former has a potential to activate nuclear proteins (*RBBP8*, *TFF3*, *MYC*, *EGR3*, *AREG*, etc.) (Table S1). We validated through real-time PCR that E₂

up-regulates ER-target genes, c-Myc, Cyclin D1, and IGF-1R in MCF-7:PF cells, but 4-OHT does not (Fig. S5). Since members of the steroid hormone family classically act through transcriptional regulation at the nuclear level (36), it is necessary to investigate the functions of ER-target genes in the cell proliferation promoted by 4-OHT in this new resistant model.

Our pathway enrichment analysis revealed a significant enrichment in a variety of genes associated with actin cytoskeleton remodeling, adaptor proteins, and other membrane-related functions activated by 4-OHT and E₂ in MCF-7:PF cells (Fig. 4A). How these genes integrally coordinate to promote cell growth remains unclear. A plasma-membrane-associated protein, EHD2, is found to regulate trafficking from the plasma membrane by controlling Rho GTPases activity (37). EHD proteins are also able to bind to phospholipids in plasma membrane (38, 39) and form complexes with IGF-1R to regulate downstream Akt pathway (40). We have found that IGF-1R and c-Src are tightly linked to the PI3K/Akt pathway in MCF-7:PF cells (15). Further examination demonstrated that inhibition of IGF-1R or c-Src tyrosine kinase activities could attenuate the up-regulation of *EHD2* or *FHL2* by 4-OHT (Fig. S6). These results imply that functional interactions exist among adapter proteins and growth pathways.

In summary, a wide range of membrane-associated genes are deregulated by E₂ and 4-OHT in MCF-7:PF cells which associate with membrane remodeling, cytoskeleton reorganization, cytoplasmic adapter proteins, and cytoplasm organelles proteins. The function of these genes may link to other plasma membrane signaling, such as PI3K/Akt and IGF-1R to promote cell growth (39, 40). Certain gene, such as *FHL2* even acts as a positive regulator of transcriptional factor NF- κ B activity to promote cell regeneration (41). Here, we only showed the deregulation of gene expression levels by either E₂ or 4-OHT, whereas alteration of subcellular localization of some adaptor protein may have differential biological functions (39, 42). Furthermore, some important signal transduction molecules are activated, as seen by expressing higher serine/threonine (Akt) or tyrosine (c-Src) kinase activity, but without alteration of the amount (11, 43). As a result, it is necessary to further investigate how 4-OHT biologically regulates its signal networks to promote cell growth and offer new insights into the target therapies for resistant patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- MCF-7:PF cells are derived from MCF-7:5C cells after long-term treated with the c-Src inhibitor and E₂.
- Both 4-OHT and E₂ stimulate MCF-7:PF cell growth in a concentration-dependent manner.
- The global gene expression microarray shows a remarkable overlap in genes regulated in the same direction by E₂ and 4-OHT in MCF-7:PF cells.
- This manuscript revealed a significant enrichment in genes associated with membrane remodeling, cytoskeleton reorganization, cytoplasmic adapter proteins, cytoplasm organelles proteins, and related processes.
- In contrast, E₂ and 4-OHT have distinct transcriptome profiles observed in wild-type MCF-7 cells. This indicates that long-term selection pressure alters cell response to tamoxifen.

Figure 1. Cell response to 4-OHT

(A) Growth response to 4-OHT. MCF-7:PF cells were treated with vehicle (0.1% EtOH; con), 4-OHT(10^{-6} mol/L), ICI(10^{-6} mol/L), and 4-OHT(10^{-6} mol/L) plus ICI(10^{-6} mol/L). Cells were harvested after 7 days treatment and cell viability was quantitated by determination of total DNA. $p < 0.001$, ** compared with control. (B) Dose-responsive curves of E₂, 4-OHT, and ICI. MCF-7:PF cells were treated with titrated concentrations of E₂, 4-OHT, and ICI as indicated. Cells were harvested after 7 days treatment and total DNA was determined as above. (C) 4-OHT could not block cell growth stimulated by E₂. MCF-7:PF cells were treated with vehicle (0.1% EtOH), E₂ (10^{-9} mol/L), E₂ (10^{-9} mol/L) plus 4-OHT (10^{-11} mol/L), E₂ (10^{-9} mol/L) plus 4-OHT (10^{-10} mol/L), E₂ (10^{-9} mol/L) plus 4-OHT (10^{-9} mol/L), E₂ (10^{-9} mol/L) plus 4-OHT (10^{-8} mol/L), E₂ (10^{-9} mol/L) plus 4-OHT (10^{-7} mol/L), E₂ (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), and 4-OHT (10^{-6} mol/L) alone for a 7-day treatment. All the data shown are representative of at least three separate experiments with similar results.

Figure 2. 4-OHT regulated ER-dependent genes

Cy3-labeled cRNA probes from MCF-7:PF cells treated in triplicate with 5 selected compound combinations were competitively co-hybridized to dual color 44k microarrays (Agilent) with a common Cy5-labeled reference cRNA probe from untreated MCF-7:PF cells as described in the *Materials and Methods*. (A) 1,354 genes whose log₂ expression ratio (Cy3/Cy5) was statistically significantly regulated by 4-OHT (all 3 replicates) or E₂ (all 3 replicates). Gene expression ratios corresponding to single or combination ICI treatment are shown co-clustered to identify patterns of ER-mediated response. Color key at the right edge of the dendrogram indicates 538 genes flagged as ER-dependent, as their response to E₂ or 4-OHT was abrogated by ICI co-treatment. Genes and data corresponding to dendrogram provided in Supplemental Table S1. (B) Venn diagram of 538 ER-dependent genes significantly and consistently regulated by E₂ and/or 4-OHT. The number of genes up- and down-regulated in each treatment condition is indicated.

Figure 3. Validation of genes up-regulated by E₂ and 4-OHT by real-time RT-PCR

MCF-7:PF cells were treated for 7 days with vehicle control (0.1% EtOH), E₂ (10⁻⁹ mol/L), 4-OHT (10⁻⁶ mol/L), ICI (10⁻⁶ mol/L), E₂ (10⁻⁹ mol/L) plus ICI (10⁻⁶ mol/L), or 4-OHT (10⁻⁶ mol/L) plus ICI (10⁻⁶ mol/L) in triplicate. Cells were harvested in TRIzol. Membrane remodeling genes *EHD2* (A), *FHL2* (B), *HOMER3* (C), and *RHOF* (D) were quantitated by real-time RT-PCR. $p < 0.05$, *compared with control, $p < 0.01$, # compared with control, $p < 0.001$, ** compared with control.

Figure 4. Pathway enrichment analysis of genes regulated by E₂ or 4-OHT in MCF-7:PF and wild-type MCF-7 cells
(A) Pathway Studio software (Version 9.0) was utilized to analyze 98 genes up-regulated by both E₂ and 4-OHT in MCF-7:PF cells. (B) RNA-sequencing was performed to detect the regulation pattern of enriched genes by E₂ or 4-OHT in wild-type MCF-7 cells.

Figure 5. E₂ did not affect the gene regulation pattern by 4-OHT

MCF-7:PF cells were treated with vehicle (0.1% EtOH), E₂ (10⁻⁹ mol/L), 4-OHT (10⁻⁶ mol/L), and E₂ (10⁻⁹ mol/L) plus 4-OHT (10⁻⁶ mol/L) for 72 hours. Genes, *EHD2* (A), *FHL2* (B), *HOMER3* (C), and *RHOF* (D) were detected by real-time RT-PCR. $p < 0.05$, * compared with control, $p < 0.01$, # compared with control or indicated group, $p < 0.001$, ** compared with control or indicated group.