Decreased Chicken Ovalbumin Upstream Promoter Transcription Factor II Expression in Tamoxifen-Resistant Breast Cancer Cells

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Abstract

Tamoxifen (TAM) is successfully used for the treatment and prevention of breast cancer. However, many patients that are initially TAM responsive develop tumors that are antiestrogen/TAM resistant (TAM-R). The mechanism behind TAM resistance in estrogen receptor α (ERα)–positive tumors is not understood. The orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor (COUP-TF)-I interacts directly with 4-hydroxytamoxifen (4-OHT)- and estrogen (E2)-occupied ERα, corepressors NCoR and SMRT, and inhibits E2-induced gene transcription in breast cancer cells. Here we tested the hypothesis that reduced COUP-TFI and COUP-TFII correlate with TAM resistance. We report for the first time that COUP-TFII, but not COUP-TFI, is reduced in three antiestrogen/TAM-R cell lines derived from TAM-sensitive (TAM-S) MCF-7 human breast cancer cells and in MDA-MB-231 cells compared with MCF-7. ERα and ERβ protein expression was not different between TAM-S and TAM-R cells, but progesterone receptor (PR) was decreased in TAM-R cells. Further, E2 increased COUP-TFII transcription in MCF-7, but not TAM-R, cells. Importantly, reexpression of COUP-TFII in TAM-S cells to levels comparable to those in MCF-7 was shown to increase 4-OHT-mediated growth inhibition and increased apoptosis. Conversely, knockdown of COUP-TFII in TAM-S MCF-7 cells blocked growth inhibitory activity and increased 4-OHT agonist activity. 4-OHT increased COUP-TFII-ERα interaction ~2-fold in MCF-7 cells. COUP-TFII expression in TAM-R cells also inhibited 4-OHT-induced endogenous PR and p52 mRNA expression. These data indicate that reduced COUP-TFII expression correlates with acquired TAM resistance in human breast cancer cell lines and that COUP-TFII plays a role in regulating the growth inhibitory activity of TAM in breast cancer cells. (Cancer Res 2006; 66(20): 10188-98)

Introduction

Currently, breast cancer is the second leading cause of cancer deaths in women in the United States, surpassed only by lung cancer (1). The selective estrogen receptor modulator tamoxifen (TAM) is a widely used endocrine therapy for the prevention and treatment of estrogen receptor (ER)–positive breast cancer (2). TAM and its active metabolite, 4-hydroxytamoxifen (4-OHT), are antiestrogens that inhibit tumor cell proliferation by competing with estrogens for ER and thereby inhibiting transcription of estrogen target genes (2). In addition to being effective against existing tumors, TAM is used as a breast cancer preventative treatment in high-risk patients (2, 3). Expression of ERs is used as a clinical marker to predict patient response to treatment and survival (4). However, 35% to 50% of ERα-positive breast tumors that initially respond to TAM develop acquired TAM resistance, no longer responding to the growth inhibitory effect of TAM (2). TAM resistance is a significant clinical problem resulting in the requirement for alternative therapies. In some cases, antiestrogen resistance results from a loss of ERα expression (5), but in many TAM-resistant (TAM-R) tumors, ERα is still expressed (2).

Although many cellular changes have been implicated in the development of acquired TAM resistance [ref. 2; e.g., overexpression or activation of growth signaling pathways including protein kinase C, epidermal growth factor receptor, HER-2/neu/ErbB2 (6, 7), and Cas (8)], the precise mechanism behind acquired TAM resistance is not known. Thus, the elucidation of biomarkers of antiestrogen resistance could impact treatment and disease outcome. Alternative mechanisms proposed to contribute to acquired TAM resistance include decreased ERβ, increased expression of the ERβ variant, ERβcx (9), and increased expression of coactivators or decreased expression of corepressors (9–12). For example, increased expression of the coactivator AIB1 and loss of corepressor NCoR have been implicated in the development of hormone-independent and antiestrogen-resistant breast cancer (13, 14). Clearly, it is likely that acquired TAM resistance is not the result of one cellular change but rather represents a multifactorial phenotype evolving from alterations in many signaling pathways that interact in a complex manner.

Chicken ovalbumin upstream promoter transcription factors (COUP-TF) are orphan members of the nuclear receptor superfamily that activate or repress gene transcription by directly binding DNA sequences containing imperfect 5'-AGGTCA-3' direct repeats (15). There are three members of the human COUP-TF family: COUP-TFI (NR2F1, NM_005654), COUP-TFII (NR2F2, NM_021005), and ErbA-related protein 2 (NR2F6, NM_005234; ref. 16). COUP-TFI and COUP-TFII proteins are 95% homologous and evolutionarily conserved in the DNA binding domain as well as the ligand-binding domain, strongly suggesting that they are primordial members of the nuclear receptor family that have important biological functions (15). It is noteworthy that highly homologous COUP-TFI and COUP-TFII are located on separate chromosomes, chromosomes 3 and 15, respectively, suggesting...
distinct and independent functions. Consistent with this suggestion, mice null for COUP-TFI or COUP-TFII exhibit perinatal and embryonic lethality, respectively (16). ErbA-related protein 2 is less conserved and little is known about its expression and function (17).

COUP-TF interacts with other nuclear receptors, including ER, the retinoid X receptor, peroxisome proliferator–activated receptors, and the vitamin D receptor (18–21), via direct interaction with receptor ligand-binding domain (22). In general, COUP-TF inhibits the transcriptional activity of other nuclear receptors by competing for their DNA binding sites or by heterodimerization with the class II nuclear receptor heterodimer partner retinoid X receptor, thereby preventing gene expression (22). In addition, like thyroid hormone receptor and retinoic acid receptor, unliganded DNA-bound COUP-TF represses gene expression by an active silencing chromatin structure that blocks transcriptional activation of target genes (22).

COUP-TF has been reported to be either a negative (24, 25) or positive (26, 27) regulator of ERα transcriptional activity. Earlier we reported that COUP-TF copurified with ERα from calf uterus and commonunoprecipitated with ERα in MCF-7 human breast cancer cells (28). COUP-TF shows stronger interaction with TAM-azidirine–liganded ERα than estradiol (E2)-liganded ERα in vitro (24). Because COUP-TF interacts with the corepressors NCoR and SMRT (23), which also interact with 4-OHT-ERα (29), it is possible that COUP-TF plays a role in inhibiting gene transcription in response to 4-OHT or other antiestrogens. Based on these observations, we hypothesized that COUP-TF may act as a negative regulator of ER transcription in the TAM-sensitive (TAM-S) MCF-7 breast cancer cell line and that a decrease in COUP-TF expression could contribute to the development of TAM resistance and breast cancer progression.

Support for a role for COUP-TF in breast tumorigenesis comes from a report that COUP-TFI mRNA expression was reduced in 3 of 12 commonly studied breast cancer cell lines, including SK-BR-3, MDA-MB-435, and MDA-MB-468, with the highest COUP-TFI expression in estrogen-dependent TAM-S MCF-7 and T47D cells (20). However, no one has examined the expression of COUP-TF in TAM-S versus TAM-R breast cancer cells or as a biomarker for TAM sensitivity/prognosis in human breast tumors. Here, we show that the expression of COUP-TFI, but not COUP-TFI, is reduced at both the mRNA and protein levels in TAM-R compared with TAM-S human breast cancer cell lines. By using small interfering RNA (siRNA) to knock down the expression of COUP-TFI in TAM-S MCF-7 cells, we show that decreased COUP-TFI expression reduces the growth inhibitory activity of 4-OHT and ICI 182,780 in these cells without affecting ERα or ERβ expression. Concordantly, overexpression of COUP-TFI by stable transfection of TAM-R and LY2 cells results in reduced cell proliferation with 4-OHT, a response not seen in the TAM-R parental cell lines. Together these data support our hypothesis that reduced COUP-TFI expression may play a role in antiestrogen/TAM resistance in breast cancer.

Materials and Methods

Chemicals. E2 was purchased from Sigma (St. Louis, MO). 4-OHT was purchased from Research Biochemicals International (Natick, MA). ICI 182,780 was purchased from Tocris (Ellisville, MO).

Antibodies. Antibodies to COUP-TFI and COUP-TFII were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and from Research and Diagnostic Systems (Minneapolis, MN). Rabbit polyclonal antibodies raised against peptides unique to the NH2-terminal regions of human COUP-TFI were purchased from NeoMarkers (Freemont, CA), Santa Cruz Biotechnology, Genetex (San Antonio, TX), Sigma, and Research Diagnostics (Flanders, NJ), respectively.

Cell culture and treatment description. MCF-7/Karmanos were obtained from Dr. Robert Pauley and Steven J. Santner (Karmanos Cancer Institute, Detroit, MI). MCF-7/LCC1, MCF-7/LCC2, MCF-7/LCC9, and LY2 were kindly provided by Dr. Robert Clarke. MCF-10A, MCF-7/ATCC and MDA-MB-231 were purchased from American Type Tissue Collection (ATCC; Manassas, VA). Characteristics of these cell lines are listed in Table 1. Cells were maintained in vented flasks containing improved MEM (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained in a humidified 37°C incubator containing 5% CO2.

Protein isolation. Whole-cell extracts were in 500 μL of modified radioimmunoprecipitation assay buffer (10 mmol/L sodium phosphate (pH 7.2), 1% NP40, 1% Na-deoxycholate, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 http://www.ebi.ac.uk.

| Table 1. Characteristics of the breast cancer cell lines used in this study |
|-----------------------------|-----------------------------------------------|
| Cell line | Reported phenotypic characteristics: ERα, ERβ, and PR RNA expression and effects of E2 and antiestrogens on cell proliferation |
| MCF-7/ATCC | ERα++; ERβ+; E2 < ERα; PR++; estrogen dependent, stimulated by E2 tamoxifen and ICI 182,780 sensitive (TAM-S). |
| MCF-7/Karmanos | Identical to MCF-7/ATCC but more E2 sensitive (32). |
| MCF7/LCC1 | ERα++, ERβ+, and PR– (48); E2 independent but growth stimulated by E2, TAM-R; ICI 182,780 sensitive (34). |
| MCF7/LCC2 | ERα++, ERβ+, and PR– (48); E2 independent; TAM-R; ICI 182,780 sensitive (34). |
| MCF7/LCC9 | ERα++, ERβ+, and PR– (48); E2 independent (45); TAM and ICI 182,780 resistant (45). |
| MCF7/LY2 | ERα+; PR–; E2 independent; TAM, ICI 182,780, and LY117018 resistant (49). |
| MDA-MB-231 | ERα+; COOH-terminally truncated ERβ (37); PR–; E2 independent; TAM, ICI 182,780, ICI 164,682, and LY117018 resistant. |

NOTE: From top to bottom, the cell lines have an increasing antiestrogens-resistant phenotype. MCF-7 cells are antiestrogen/tamoxifen sensitive; the other cell lines are antiestrogen/tamoxifen resistant.
Western blot analysis. Thirty micrograms of protein were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in a solution consisting of 5% dried milk, 0.1% Tween (polyoxyethylene 20-sorbitan monolaureate), and 1× TBS (TBS-Tween), or 3% bovine serum albumin (BSA) in 1× TBS-Tween, and subsequently incubated with primary antibodies overnight at 4°C. Membranes were incubated in 1:1000 dilution of horseradish peroxidase–conjugated secondary antibodies (Pierce, Rockford, IL). Immunoreacting bands were visualized with SuperSignal West (Pierce) chemiluminescence kit on Kodak BioMax ML film (Eastman Kodak, Rochester, NY). Membranes were first probed for ERα, ERβ, PR, COUP-TFI, or COUP-TFII, and then stripped and reprobed for β-actin or GAPDH. Resulting immunoblots were scanned into Adobe Photoshop 7.0 using a Microtek ScanMaker III scanner (Carson, CA). Un-Scan-It (Silk Scientific, Orem, UT) was used to quantitate the integrated optical densities (IOD) for each band. The IOD for ERα, ERβ, PR, COUP-TFI, and COUP-TFII were divided by concordant β-actin integrated absorbances in the same blot. For comparison between experiments, the β-actin-normalized pixel values for ERα, ERβ, COUP-TFI, and COUP-TFII from MCF-7 (ATCC) was set to 1. In experiments with hormone treatments, the ethanol value was set to 1.

Preparation of COUP-TF proteins. COUP-TFI and COUP-TFII proteins were synthesized with the PROTEINscript II T7 kit, an in vitro transcription and translation system using rabbit reticulocyte lysate (Ambion, Austin, TX). The relative amounts of the translated proteins were determined by quantitating the [35S]methionine–labeled protein in SDS-PAGE and normalizing it relative to the content of methionine in each protein.

Cell proliferation assays. Cell proliferation was determined with Cell Titer 96 AQueous One 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay from Promega (Madison, WI) according to the instructions of the manufacturer. For ligand responses, 1.5 × 10^4 cells per well were plated in 96-well plates in growth medium and allowed to adhere for 24 hours, after which the medium was replaced with phenol red–free IMEM supplemented with 3% dextran–coated, charcoal-stripped FBS for 24 hours before treatment with vehicle control (i.e., ethanol, E2, 4-OHT, or ICI 182,780, alone or in combination) for 4 days with treatments and medium replenished after 48 hours. For serum responses, 2 × 10^5 cells per well were plated as above in growth medium. After 24 hours, the growth medium was removed and cells were rinsed with sterile PBS, and 100 μL of phenol red–free IMEM medium supplemented with 0% to 10% FBS were added. MTT assays were done on day 4.

For measuring cell proliferation by bromodeoxyuridine (BrdUrd) incorporation, MCF-7 cells were transiently transfected with pCR3.1 parental vector or pCR3.1-mCOUP-TFI and plated in 96-well plates in phenol red–free IMEM supplemented with 3% dextran–coated, charcoal-stripped FBS for 24 hours. Treatments (vehicle control, i.e., ethanol, E2, 4-OHT, or ICI 182,780, alone or in combination) for 4 days with treatments and medium replenished after 48 hours. For serum responses, 2 × 10^5 cells per well were plated as above in growth medium. After 24 hours, the growth medium was removed and cells were rinsed with sterile PBS, and 100 μL of phenol red–free IMEM medium supplemented with 6% to 10% FBS were added. MTT assays were done on day 4.

To measure cell proliferation by bromodeoxyuridine (BrdUrd) incorporation, MCF-7 cells were transiently transfected with pCR3.1 parental vector or pCR3.1-mCOUP-TFI and plated in 96-well plates in phenol red–free IMEM supplemented with 3% dextran–coated, charcoal-stripped FBS for 24 hours. Treatments (vehicle control, i.e., ethanol, E2, 4-OHT, or ICI 182,780, alone or in combination) for 4 days with treatments and medium replenished after 48 hours. For serum responses, 2 × 10^5 cells per well were plated as above in growth medium. After 24 hours, the growth medium was removed and cells were rinsed with sterile PBS, and 100 μL of phenol red–free IMEM medium supplemented with 0% to 10% FBS were added. MTT assays were done on day 4.

RNA isolation, reverse transcription-PCR, and quantitative real-time reverse transcription-PCR. RNA was extracted with Trizol reagent (Invitrogen) according to the protocol of the manufacturer, followed by purification on RNeasy columns (Qiagen, Valencia, CA). Total RNA was reverse transcribed with random hexamers and the High-Capacity cDNA Expression Kit (PE Applied Biosystems, Foster City, CA). QIAquick PCR purification kit (Qiagen) was used to purify cDNA.

TagMan probes and primers for ERα, COUP-TFI, COUP-TFII, and control gene 18S rRNA were purchased as Assays-on-Demand Gene Expression Products (PE Applied Biosystems). Additional COUP-TFI and GAPDH primers were identical to those used in ref. 30. Expression of each target gene was determined in triplicate with purified sample cDNA and normalized to 18S. A standard curve for input cDNA was generated using serial dilutions of MCF-7 untreated RNA. Semiquantitative reverse transcription-PCR (RT-PCR) was done with 35 PCR cycles of 1 minute at 95°C, an annealing step of 1 minute at 60°C, and an extension step of 90 seconds at 72°C. PCR products were separated on a 2% agarose gel, then scanned and quantitated using Un-Scan-IT software as described above. Quantitative RT-PCR was done with the ABI PRISM 7900 SDS 2.1 (PE Applied Biosystems) by relative quantification with standard thermal cycles. Analysis and fold differences were determined using the comparative CT method. Fold change was calculated from the ΔΔCT values with the formula 2−ΔΔCT and data were presented as relative to expression in ethanol-treated (control) MCF-7 unless otherwise indicated.

Cell invasion and motility assay. Invasion assays were done using the method of Albini et al. (31) with modification. Cells (2.5 × 10^4) were suspended in 500 μL of phenol red–free and serum-free IMEM containing 0.1% BSA and plated on 8-μm Matrigel–coated polyethylene terephthalate filter inserts in Boydon chambers (Bicoat Matrigel Invasion Chambers, Becton Dickinson, Bedford, MA). Noncoated membrane inserts were seeded similarly to serve as controls for determination of motility. The bottom chamber contained 0.75 mL of a mixture of equal parts normal growth medium and NIH 3T3–conditioned medium as chemotactant. The treatments were added to the upper chamber for a final concentration of 10 nmol/L E2 and 100 nmol/L 4-OHT and an equal volume of ethanol served as the vehicle control. After incubation for 24 hours in a 5% CO2 incubator at 37°C, the cells remaining on the insert were removed with a cotton swab and the cells that had penetrated Matrigel and had migrated to the lower surface of the polyethylene terephthalate membrane were fixed in methanol and stained with DiffQuik reagent (American Scientific Products, McGraw Park, IL). After drying the polyethylene terephthalate membrane, the cells were visually counted at ×100 magnification under an Olympus CKX41 microscope.

Stable transfection of breast cancer cells. LY2 cells were stably transfected with empty pCR3.1 (control) or with the pCR3.1 vector encoding mouse COUP-TFI (23) using FuGene6 (Roche) according to the instructions of the manufacturer. Twenty-four hours after transfection, selection medium containing 500 μg/mL G-418 (Invitrogen) was added. Drug-resistant clones were maintained in medium supplemented with 500 μg/mL G-418.

Apoptosis measurement. The effect of ER ligands on the induction of apoptosis was measured with a photometric enzyme immunoassay (Cell Death Detection ELISAPLUS from Roche) that quantitates cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) after induced cell death. Cells (10,000) were plated in 24-well plates in triplicate wells with normal growth medium (IMEM containing 5% FBS and penicillin/streptomycin) and allowed to attach for 24 hours. The medium was replaced with medium containing charcoal-stripped serum for 24 hours followed by treatment with ethanol, 10 nmol/L E2, 100 nmol/L 4-OHT, or 100 nmol/L ICI 182,780 for 4 days. Treatment media were replaced with fresh treatments every 48 hours. The assay was done according to the instructions of the manufacturer.

siRNA transfections. COUP-TFI siRNAs and scrambled nonspecific siRNA were purchased from Ambion. TAM-S MCF-7 cells were transiently transfected with optimal amounts of siRNA using the siPORT NeoFX siRNA transfection reagent (Ambion), according to the instructions of the manufacturer, or transfected with vector encoding siRNAs to ERα or vector control (GeneEclipse Vector-Based RNAi, Chemicon, Temecula, CA). After 48 hours, whole-cell extracts were prepared and RNA was isolated as described above. COUP-TFI expression was measured by Western blot and quantitative RT-PCR.

Statistics. Statistical analyses were done with two-tailed Student’s t test or one-way ANOVA followed by Student-Newman-Keuls or Dunnett’s post hoc tests using GraphPad Prism (San Diego, CA). Statistical significance was defined as ≥95% confidence interval or P ≤ 0.05.
Results

COUP-TFII expression is decreased in TAM-R human breast cancer cell lines. Although COUP-TFII expression was decreased in 3 of 13 commonly studied human breast cancer cell lines (20), the relationship between COUP-TF expression and TAM resistance in human breast cancer cells has not been examined. To address this question, COUP-TFI and COUP-TFII protein expression was examined in seven human breast cancer cell lines representing an established model (2) of the progression of TAM-S human breast cancer cells to an acquired TAM-R phenotype (Table 1). Because MCF-7 breast cancer cell lines are notorious for changes in phenotype between labs (32), the reported phenotypic growth responses of the cell lines were first evaluated by examining cell proliferation in response to E2, 4-OHT, and ICI 182,780, alone or in combination, using MTT assays (Supplementary Fig. S1). Examination of the effect of serum on the proliferation of the TAM-S and TAM-R cell lines revealed that antiestrogen/TAM-R cells show reduced serum dependency that correlated with their degree of antiestrogen resistance (Supplementary Fig. S2). This result agrees with studies showing that the ability of breast cancer cells to grow in the absence of added growth factors is a critical early hallmark of TAM resistance (33). Overall, our results are consistent with previous findings (34), validating these cell lines as a model system to study mechanisms of acquired TAM resistance.

Commercially available COUP-TFI and COUP-TFII antibodies (see Materials and Methods) were found to cross-react with recombinant, in vitro transcribed, translated COUP-TFI and COUP-TFII proteins, and with a number of intracellular proteins in the breast cancer cell lines in Western blots (data not shown). This lack of antibody specificity necessitated custom production of COUP-TFI and COUP-TFII polyclonal antiserum. As shown in Fig. 1A, in vitro transcribed and translated COUP-TFI and COUP-TFII were recognized only by the respective antibody, showing antibody specificity. Figure 1A shows that both COUP-TFI and COUP-TFII are expressed in MCF-7 cells. The apparent higher molecular weight of the in vitro transcribed and translated murine COUP-TF proteins may be due to different posttranslational processing in the reticulocyte lysate versus in MCF-7 cells.

With these new antibodies, basal COUP-TFI and COUP-TFII protein expression was examined in TAM-S and antiestrogen/TAM-R breast cancer cell lines grown to identical cell densities. This is necessary for comparison of COUP-TFI between cell lines with different growth rates because COUP-TFII expression was reported to increase with cell density (30). We observed that COUP-TFII expression was similar in all the cell lines (Fig. 1B). In contrast, COUP-TFI expression was 40% to 60% lower in the four antiestrogen/TAM-R cell lines (i.e., LCC2, LCC9, LY2, and MDA-MB-231) compared with the TAM-S MCF-7 cell line (Fig. 1B). These differences were statistically significant (Fig. 1B). Our results are in agreement with an earlier report that COUP-TFI expression was reduced in MDA-MB-231 breast cancer cells (20). This is the first observation of a correlation between decreased COUP-TFI expression and TAM resistance in human breast cancer cell lines.

ERα, ERβ, and PR expression in TAM-S and TAM-R human breast cancer cell lines. ERα and PR are well-established clinical prognostic markers of antiestrogen responses. We examined protein expression of ERα, ERβ, and PR in each of the cell lines (Fig. 1C). With the exception of ERα-negative MDA-MB-231, there was no difference in ERα expression in TAM-S versus TAM-R cells. ERβ has been postulated to act as a “brake” in the “yin-yang” hypothesis where ERα has proliferative activity and ERβ has antiproliferative activity in breast cancer (35). However, a recent study of human breast tumors found no association of ERβ expression with cell proliferation, but only with aneuploidy (36). Thus, it was important to determine if ERβ expression was altered in the antiestrogen/TAM-R derivatives of MCF-7 and in MDA-MB-231 cells. Expression of full-length ERβ (61 kDa, ERβ1 isoform) was similar in all the MCF-7-derived cell lines (Fig. 1C). MDA-MB-231 expressed ~50% lower ERβ than MCF-7. A previous study using a different ERβ antibody found that MDA-MB-231 expressed only a shorter COOH-terminally truncated form of ERβ (37), but we did not detect that in this study. Differences in the ERβ antibodies are likely to be responsible for this difference. We conclude that, like ERα, ERβ expression is not altered in the TAM-R derivatives of the MCF-7 breast cancer cell line, thus obviating alterations in ER expression in the altered sensitivity to E2, TAM, and other antiestrogens in these cell lines.

We examined the effect of 24-hour treatment with E2 or 4-OHT on the protein expression of ERα, ERβ, and PR in MCF-7 versus LCC9 and LY2 as antiestrogen-resistant cell lines (Fig. 1D). E2 reduced ERα and increased ERβ protein in MCF-7 but in none of the other cell lines. 4-OHT reduced ERαs in LY2 cells. E2 and 4-OHT decreased ERβ in LCC9 and LY2 cells.

PR expression is used as a clinical marker of estrogen/antiestrogen responsiveness because E2, acting through ERα, stimulates PR expression at the transcriptional level (38). Both PR-B and PR-A expression were reduced in all TAM-R cells. In contrast, LCC1 cell lines showed higher PR-B than MCF-7. Whereas E2 increased PR expression in MCF-7 cells, neither E2 nor 4-OHT altered PR expression in LCC9 or LY2 cells (Fig. 1D). The conclusion from these data is that the antiestrogen-resistant LCC9, LY2, and MDA-MB-231 cell lines have reduced PR expression compared with the TAM-S MCF-7 and LCC1 cell lines, a result that correlates with their lack of response to E2 in MTT assays (Supplementary Fig. S1). Lastly, although MCF-7 cells from the Karmanos Cancer Center were reported to be more estrogen responsive than MCF-7 cells from ATCC (32), we observed no significant difference in ERα, ERβ, PR, or COUP-TF expression between MCF-7 cells from ATCC and MCF-7 cells from Karmanos.

E2 increases COUP-TFII expression in TAM-S MCF-7 cells. COUP-TFII mRNA expression was also reduced by 40% to 60% in the TAM-R cell lines compared with MCF-7 (Fig. 2A). These data correlate with lower COUP-TFII protein expression in these TAM-R cell lines (Fig. 1B). Treatment of MCF-7 cells with E2 increased COUP-TFII, but not COUP-TFI, mRNA (Fig. 2B, and data not shown). However, E2 did not affect COUP-TFI expression in the TAM-R LCC9 cells and reduced COUP-TFI mRNA in LY2 cells (Fig. 2B). Transient transfection of MCF-7 with siRNA to ERα decreased ERα mRNA expression by ~85% (Fig. 2C) and decreased the basal expression of PR, pS2, and COUP-TFI mRNAs. In contrast, siRNA to ERα increased ERβ mRNA by ~3.5-fold. Conversely, transfection of ERα into ERα-negative/COUP-TFI-negative MCF-10A “normal” breast cells induced COUP-TFI mRNA expression by 4-fold after 24 hours (data not shown). Whereas PR and pS2 are well-characterized estrogen-responsive genes in breast cancer, this is the first demonstration that ERα regulates COUP-TFI gene transcription.

Overexpression of COUP-TFI in TAM-R human breast cancer cell lines increases the antiproliferative effects of TAM and ICI 182,780. We observed a correlation between
decreased COUP-TFII expression and antiestrogen/TAM resistance in breast cancer cell lines, suggesting a possible role for COUP-TFII in the sensitivity of breast cancer cells to growth inhibition by TAM. To examine whether antiestrogen sensitivity could be restored by overexpression of COUP-TFII, antiestrogen/TAM-R LY2, LCC2, and LCC9 cells were stably transfected with a plasmid vector expressing COUP-TFII. By pooling stable clones, we “averaged” the genetic background of the stable cells (based on
random integration into the genome), thus minimizing the risk that a particular clone has undergone an unknown mutation that could affect our findings. COUP-TFII expression was increased by ~2-, 4-, and 1.7-fold in the stably transfected LY2, LCC2, and LCC9 cell lines (Fig. 3A); thus, COUP-TFII was similar to endogenous COUP-TFII expression in MCF-7 cells in the LY2-CII and LCC9-CII cell lines. ERF, ERβ, and COUP-TFII expression was not altered by COUP-TFII overexpression (data not shown).

The effect of COUP-TFII overexpression on cell proliferation in response to E2, 4-OHT, or ICI 182,780, alone or in combination, was examined. As shown in Fig. 3B to E, COUP-TFII overexpression had no effect on basal proliferation but enhanced the antiproliferative effect of 4-OHT and ICI 182,780 in LY2 and LCC9 cells. COUP-TFII overexpression had no effect on the antiproliferative activity of ICI 182,780 in the ICI 182,780–sensitive LCC2 cell line. Importantly, 4-OHT and ICI 182,780 did not affect the proliferation of vector-transfected LY2, LCC2, or LCC9 cells, indicating that increased COUP-TFII expression enhanced the antiproliferative activity of 4-OHT in all three TAM-R cell lines and enhanced ICI 182,780 sensitivity in the ICI 182,780–resistant LY2 and LCC9 cell lines. In LY2 cells, COUP-TFII overexpression resulted in a ~25% inhibition of cell proliferation by E2. In contrast, ~1.8-fold overexpression of COUP-TFII had no effect on E2-induced or 4-OHT-mediated inhibition of MCF-7 cell proliferation (Fig. 3C). Together, these results support a role for COUP-TFII in maintaining antiestrogen sensitivity in LY2, LCC2, and LCC9 cells and indicate that overexpression of COUP-TFII may play a role in reversed E2 pharmacology in the stably transfected LY2 cell line.

Expression of COUP-TFII in human lung carcinoma cell lines was reported to increase invasive and migratory abilities (39). To examine whether COUP-TFII expression correlates with the invasiveness of human breast cancer cells, we examined in vitro cell invasiveness and migration in vector-transfected versus the LY2-COUPTFII cell line. As shown in Fig. 4A, LY2-COUP-TFII cells showed a 50% decrease in motility compared with vector-transfected LY2 cells. Treatment with either E2 or 4-OHT decreased LY2 cell motility. In contrast, overexpression of COUP-TFII or treatment with E2 or 4-OHT had no effect on cell invasion. These data indicate that COUP-TFII seems to have different effects on cell motility in breast cancer cells versus lung cancer cells, with COUP-TFII inhibiting cell motility in the LY2-COUPTFII cell line.

TAM reduces MCF-7 cell viability by inducing apoptosis (2). To examine the mechanism by which overexpression of COUP-TFII in LY2 restores the antiproliferative activity of 4-OHT (Fig. 3B), we examined apoptosis in LY2 cells stably transfected with vector (negative control) or COUP-TFII. E2 or 4-OHT increased apoptosis in LY2 cells stably expressing COUP-TFII (Fig. 4B). These data are consistent with the data in the MTT assays in Fig. 3B and indicate that COUP-TFII may play a role in the apoptotic activity of 4-OHT.

**COUP-TFII knockdown in TAM-S MCF-7 cells increases cell proliferation.** We have shown that overexpression of COUP-TFII in TAM-R LY2 cells can partially restore the antiproliferative effects of 4-OHT and ICI 182,780. As a complementary approach to further investigate the role of COUP-TFII in mediating the antagonist activity of TAM, we examined whether knockdown of COUP-TFII expression in TAM-S MCF-7 cells using siRNA would decrease the antiproliferative activity of TAM. COUP-TFII mRNA was decreased by 50% in MCF-7 cells transfected with COUP-TFII siRNA compared with mock-transfected cells (Fig. 5A). No decrease in COUP-TFII expression was detected, thus showing the specificity of the COUP-TFII siRNA (Fig. 5A). An identical reduction in COUP-TFII protein was observed (Fig. 5B). No change in COUP-TFII protein was seen (data not shown). Likewise, COUP-TFII knockdown did not alter ERF or ERβ expression (Fig. 5C).

To determine the functional consequence of COUP-TFII knockdown, cell proliferation assays were done in cells treated with E2 and 4-OHT, alone or in combination. As shown in Fig. 5D, siRNA knockdown of COUP-TFII did not affect basal cell growth. However, E2-induced proliferation was blocked by both scrambled and COUP-TFII siRNA, indicating a nonspecific inhibition of cell proliferation, perhaps by an IFN response (40). IFN inhibits breast cancer cell proliferation (41). Notably, reduced COUP-TFII expression specifically increased MCF-7 proliferation with 4-OHT, alone or in combination with E2, by ~40%. These data indicate that ablating COUP-TFII expression alters the pharmacology of 4-OHT from antagonist to agonist in MCF-7 cells and supports our hypothesis that COUP-TFII plays a role in mediating TAM-antagonist activity in MCF-7 human breast cancer cells.
COUP-TFII overexpression affects endogenous PR and pS2 gene transcription in a cell-specific manner. As an additional measure of the functional consequences of stable COUP-TFII expression in TAM-R cells, we measured endogenous PR and pS2 mRNA levels by quantitative RT-PCR. E2 increased PR mRNA expression ~5-fold in MCF-7 and LCC2 cells (Fig. 6A). 4-OHT did not inhibit E2-induced PR mRNA in LCC2 cells as it did in TAM-S MCF-7 cells (Fig. 6A), reflecting the loss of 4-OHT antagonist activity in this cell line. Similar results were observed for pS2. Although lacking agonist activity alone, 4-OHT acted in a synergistic manner with E2 and increased PR expression 8-fold and pS2 transcription ~18-fold in LCC2 cells. Stable expression of COUP-TFII in LCC2 cells inhibited E2 and E2/4-OHT agonist activity for both PR and pS2 (Fig. 6A). In contrast, mRNA for PR or pS2 was not detected in the TAM-R LCC9 or LY2 cell lines with the same quantitative RT-PCR conditions used for MCF-7 and LCC cells. Stable transfection of COUP-TFII, a perturbation that increased the antiproliferative activity of E2, 4-OHT, and ICI 182,780 (Fig. 3B), had no effect on PR or pS2 expression, whether under basal conditions or in cells treated with E2 or 4-OHT (data not shown).

Thus, COUP-TFII overexpression in LCC2 cells inhibits E2 and 4-OHT agonist activity on these two endogenous ER marker genes. In contrast, COUP-TFII overexpression in the more advanced TAM-R LCC9 and LY2 cells fails to restore E2-induced pS2 or PR expression, despite expression of ERα and ERβ at levels comparable to those of MCF-7 in these cell lines (Fig. 1C, and data not shown). Taken together, these data fit with a model suggesting that COUP-TFII plays a role in the repression of ER marker gene expression in a manner that correlates with endogenous ER responses and, thus, must require additional cell-specific factors. Further, COUP-TFII does not stimulate ER agonist activity, at least at the PR and pS2 promoters. In agreement with this hypothesis, transfection of MCF-7 with siRNA to COUP-TFII did not affect basal or E2-induced PR or pS2 expression (Fig. 6B).

Increased COUP-TFII-ERα interaction in 4-OHT-treated cells. We reported that 4-OHT increased ERα interaction with GST-COUP-TFII in vitro (24). To our knowledge, no one has examined ERα-COUP-TFII interaction. To determine whether ERα interaction with COUP-TFII is influenced by 4-OHT in breast cancer cells, COUP-TFII-ERα interactions were examined by...
communoprecipitation with whole-cell extracts from TAM-S MCF-7 and TAM-R LY2 cells treated with ethanol, E2, or 4-OHT (Fig. 6D). 4-OHT, but not E2, significantly increased the amount of ERα bound to COUP-TFII in MCF-7 cells, without any alteration in total COUP-TFII (Fig. 6D, and data not shown). The reverse immunoprecipitation showed similar results (Fig. 6D). In LY2 cells, both E2 and 4-OHT increased ERα-COUPTFII interaction by ~50%, but the total percent of ERα-COUPTFII interaction was ~50% lower in the LY2 cells. In the reverse immunoprecipitation reaction, E2 had no effect whereas 4-OHT increased ERα-COUPTFII interaction by 35%. The actual amount of COUP-TFII expression in LY2 in the 10% input lane was 26% of that in MCF-7. These data are in agreement with the lower expression of COUP-TFII in LY2 versus MCF-7 (Figs. 1B and 2A). Thus, in accord with the hypothesis that COUP-TFII-ERα interaction correlates with antiestrogenic responses, LY2 cells showed reduced COUP-TFII-4-OHT-ERα interaction compared with MCF-7 cells.

Discussion

The precise mechanisms accounting for the development of acquired TAM resistance in breast cancer patients are unknown, although a variety of molecular changes have been implicated in this process and to subsequent tumor progression and metastases (2). Here, we report for the first time that the expression of the orphan nuclear COUP-TFII, but not COUP-TFI, is reduced in three antiestrogen/TAM-R breast cancer cell lines that are a model of acquired antiestrogen resistance compared with the parental MCF-7 TAM-S cell line. Further, COUP-TFII mRNA and protein levels correlate with the degree of antiestrogen/TAM sensitivity of these cells. Importantly, COUP-TFII overexpression by stable transfection restored the ability of 4-OHT to inhibit the proliferation of TAM-R LCC2, LCC9, and LY2 cells and to induce apoptosis in LY2 cells. Notably, in our study, COUP-TFII expression was increased by ~2-, 4-, and 1.7-fold in the stably transfected LY2, LCC2, and LCC9 cell lines (Fig. 5A); thus, COUP-TFII was similar to endogenous COUP-TFII expression in MCF-7 cells, indicating that 2- to 4-fold overexpression is enough to restore TAM antagonist activity as measured by cell proliferation assays. Conversely, siRNA knockdown of COUP-TFII expression in TAM-S MCF-7 cells resulted in the conversion of 4-OHT from an antagonist to an agonist as measured by cell proliferation. This reversal of 4-OHT pharmacology was not caused by altered expression of ERα or ERβ. Although COUP-TFII overexpression and knockdown by siRNA are likely to cause a number of intracellular changes that complicate interpretation, our data clearly show a functional correlation between TAM-R and decreased COUP-TFII expression.

E2 increased COUP-TFII expression in MCF-7 but not in the antiestrogen/TAM-R LCC9 and LY2 cell lines. We speculate that the lack of E2-induced COUP-TFII expression in the TAM-R cells may be related to a functional defect in the ERα transcriptional response because the reduction in endogenous COUP-TFII parallels the decrease in basal and E2-induced PR expression in these cell lines and ERα siRNA decreased basal COUP-TFII expression in MCF-7 cells. Similarly, the E2-mediated repression of COUP-TFII in LY2 cells indicates altered ER signaling compared with the TAM-S MCF-7 cell line. Future studies will be aimed at examining the mechanism by which ERα regulates COUP-TFII transcription.

Although the mechanism by which COUP-TFII contributes to 4-OHT inhibition of cell proliferation in TAM-S MCF-7 cells is unknown, based on the observed increase in direct interaction (communoprecipitation) between ERα and COUP-TFII with 4-OHT,
we speculate that COUP-TFII-ERα interaction plays a role in this response. Notably, our studies of endogenous PR and pS2 expression in LCC2 cells stably transfected, and thus overexpressing COUP-TFII by ~2-fold compared with MCF-7 and 4-fold compared with LCC2 parental cells, revealed that COUP-TFII repressed E2 agonist activity. Although COUP-TFII competes with ERα for the estrogen response elements from the PR and pS2 genes and inhibits luciferase reporter activity from these gene promoters in transfected cells (24, 42), further studies will be required to address the precise mechanism accounting for the inhibitory effect of COUP-TFII.

It is also likely that COUP-TFII has mechanisms independent of ERα interaction. Regulation of gene expression by COUP-TF is cell and/or gene specific. Interestingly, COUP-TFII has been shown to reduce the expression of telomerase, a gene implicated in carcinogenesis, through binding of the promoter of the hTert gene (43). Antiestrogen-resistant breast cancer cell lines were recently reported to have increased telomerase activity (44). Thus, we speculate that the reduced expression of COUP-TFII in antiestrogen-resistant cells could relieve repression of hTert gene expression, increasing telomerase and contributing to cell immortality.

Interestingly, in TAM-R LY2 cells in which COUP-TFII was expressed at a level comparable to MCF-7 cells by stable transfection, E2 did not stimulate cell proliferation but inhibited proliferation by ~25% in a concentration-dependent manner. This reversal of E2 pharmacology in LY2 is similar to the third phase of antiestrogen resistance in a model for antiestrogen resistance in breast cancer described by Dr. V. Craig Jordan (33). According to this model, in the first phase of antiestrogen resistance, E2 stimulates while 4-OHT is tumorstatic whereas both E2 and 4-OHT stimulate proliferation in the second phase (33). The TAM-R cell lines used here (i.e., LCC2, LCC9, and LY2) have lost the ability to respond proliferatively to E2 and to be growth inhibited by TAM and other antiestrogens (2, 34, 45), corresponding to phase 2. In the third phase of antiestrogen resistance, TAM stimulates cell proliferation while E2 becomes inhibitory (33). This reversed E2 pharmacology is similar to that observed when COUP-TFII expression was knocked down by siRNA in the TAM-S MCF-7 cell line. These results are consistent with our hypothesis that a decrease in COUP-TFII expression during breast cancer progression contributes to TAM resistance. Our observation that COUP-TFII is an estrogen-responsive gene in MCF-7 cells, like PR, but not in the TAM-R LCC9 and LY2 cells supports the idea that monitoring COUP-TFII expression during antiestrogen therapy could potentially identify patients with tumors developing TAM resistance, perhaps even serving as a marker for a particular phase of acquired TAM resistance.

Decreased COUP-TFII expression has been detected in other types of human cancer. For example, microarray analysis showed that COUP-TFII was the only down-regulated gene in ovarian cancers (46). Reports on COUP-TFII expression in lung cancer have provided conflicting data. In one study, COUP-TFII expression correlated with invasiveness in human lung cancer cell lines, suggesting that COUP-TFII contributes to lung tumorigenesis (39).

Figure 5. Effect of COUP-TFII knockdown on cell proliferation. A, MCF-7 cells were mock transfected or transfected with scrambled siRNA or siRNA-COUP-TFII as described in Materials and Methods. mRNA expression of COUP-TFII and COUP-TFII was measured by quantitative RT-PCR as described in Materials and Methods. a, P < 0.001, significantly different from mock-transfected cells. B, COUP-TFII and β-actin protein expression was measured by Western blot as previously described in Materials and Methods. COUP-TFII expression was normalized by β-actin and the value set to 1 for mock-transfected cells. a, P < 0.001, significantly different from mock-transfected cells. C, ERα, ERβ, and PR mRNA expression was measured by quantitative RT-PCR as described in Materials and Methods. Columns, average of quadruplicate determinations in one representative transfection; bars, SE. The transfections have been repeated twice with similar results. a, significantly different from the same cell line treated with ethanol, vehicle control; b, significantly different from E2 alone (P < 0.05).
However, COUP-TF expression is required for cellular responses, including growth inhibition and apoptosis, induced by retinoic acid in breast cancer cells (47).

In conclusion, we report for the first time that decreased COUP-TFII expression correlates with antiestrogen resistance in breast cancer cell lines that are a model of acquired TAM resistance. We showed that knockdown of COUP-TFII by siRNA caused TAM-S MCF-7 to lose their antiproliferative response to 4-OHT. Conversely, overexpression of COUP-TFII in TAM-R cells caused inhibition of cell proliferation in response to 4-OHT and, in the case of the LY2 cell line, also by E2. These data suggest that COUP-TFII may play a role in regulating estrogenic/antiestrogenic responses, and reduced COUP-TFII expression may serve as a biomarker for the identification of tumors at risk for developing acquired TAM resistance.

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Decreased Chicken Ovalbumin Upstream Promoter Transcription Factor II Expression in Tamoxifen-Resistant Breast Cancer Cells
