Research Article

ERRγ Mediates Tamoxifen Resistance in Novel Models of Invasive Lobular Breast Cancer

Rebecca B. Riggins,[1] Jennifer P-J. Lan,[1] Uwe Klimach,[1] Alan Zwart,[1] Luciane R. Cavalli,[1] Bassem R. Haddad,[1] Li Chen,[2] Ting Gong,[2] Jianhua Xuan,[2] Stephen P. Ethier,[3] and Robert Clarke[1]

[1]Lombardi Comprehensive Cancer Center and Department of Oncology, Georgetown University School of Medicine, Washington, District of Columbia; [2]Department of Electrical and Computer Engineering, Virginia Polytechnic Institute and State University, Arlington, Virginia; and [3]Barbara Ann Karmanos Cancer Institute and Department of Pathology, Wayne State University, Detroit, Michigan

Abstract

One-third of all estrogen receptor (ER)–positive breast tumors treated with endocrine therapy fail to respond, and the remainder is likely to relapse in the future. Almost all data on endocrine resistance has been obtained in models of invasive ductal carcinoma (IDC). However, invasive lobular carcinomas (ILC) comprise up to 15% of newly diagnosed invasive breast cancers each year and, whereas the incidence of IDC has remained relatively constant during the last 20 years, the prevalence of ILC continues to increase among postmenopausal women. We report a new model of Tamoxifen (TAM)-resistant invasive lobular breast carcinoma cells that provides novel insights into the molecular mechanisms of endocrine resistance. SUM44 cells express ER and are sensitive to the growth inhibitory effects of antiestrogens. Selection for resistance to 4-hydroxytamoxifen led to the development of the SUM44/LCCTam cell line, which exhibits decreased expression of ERα and increased expression of the estrogen-related receptor γ (ERRγ). Knockdown of ERRγ in SUM44/LCCTam cells by siRNA restores TAM sensitivity, and overexpression of ERRγ blocks the growth-inhibitory effects of TAM in SUM44 and MDA-MB-134 VI lobular breast cancer cells. ERRγ-driven transcription is also increased in SUM44/LCCTam, and inhibition of activator protein 1 (AP1) can restore or enhance TAM sensitivity. These data support a role for ERRγ/AP1 signaling in the development of TAM resistance and suggest that expression of ERRγ may be a marker of poor TAM response. [Cancer Res 2008;68(21):8908–17]

Introduction

Breast cancer is the second-most common cause of cancer-related death in women (1). One of the challenges in treating breast cancer is addressing the biological heterogeneity evident in the existence of several histologic and molecular subtypes. Two of the major histologic breast cancer classifications are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). Currently, ILCs comprise up to 15% of invasive breast cancer diagnoses annually (2). Although the incidence of IDC has remained relatively constant during the last 20 years, a significant increase in ILC diagnosis is evident among postmenopausal women in Western Europe and the United States (reviewed in ref. 3). Although the increased use of estrogen plus progestin hormone replacement therapy for relief of perimenopausal and postmenopausal symptoms during this same time period may have contributed to the increase in ILC incidence (3), the precise mechanism(s) remains uncertain.

The clinical and pathologic features of lobular tumors are unique. ILC typically invades in a linear pattern, creating a longer, thinner mass, which is more difficult to detect by mammography, ultrasound, or breast self-exam (3). ILCs have a greater tendency to be bilateral, and women with this type of breast cancer are frequently older and have larger tumors at the time of their diagnosis (3). A higher incidence of ILC has been reported among women who initially present to the clinic with metastatic breast cancer (4). Although recent clinical studies imply that ILC is less responsive to neoadjuvant cytotoxic chemotherapy as a precursor to breast-conserving surgery (5, 6), there are conflicting reports as to whether patients diagnosed with ILC have a poorer, equivalent, or improved prognosis and overall survival when compared with IDC (reviewed in ref. 3).

Breast cancer patients whose tumors express estrogen receptor (ER) α (ERα) may be offered endocrine or antiestrogen therapy in addition to or in place of conventional chemotherapies. Currently, the most widely used antiestrogen is the triphenylethylene Tamoxifen (TAM), which functions as a partial antagonist by competing with estrogen for binding to the ER. TAM is known to induce a statistically significant improvement in the overall survival rate from breast cancer (7), and ∼70% of all ER-positive (ER+)/progesterone receptor (PR)-positive (PR+) breast cancers will respond to TAM. When compared with IDC, a significantly greater percentage of ILC tumors are ER+/PR+ (discussed in ref. 3), suggesting that women diagnosed with this tumor subtype should be ideal candidates for endocrine therapy. However, study results differ as to whether ILC patients experience a better or worse risk of mortality than IDC patients after antiestrogen treatment (8, 9).

Regardless of tumor subtype, the development of endocrine resistance is a pervasive clinical problem (10–12). One-third of ER+/PR+ breast tumors treated with TAM do not respond to initial treatment, and the remaining 70% are still at risk to relapse in the future. A number of mechanisms have been proposed to control antiestrogen resistance in ER+ breast cancer (13), but many details of these mechanisms continue to be unclear. Studying endocrine resistance specifically in ILC has not been possible because of the lack of appropriate models; the most common models of resistance (notably MCF-7 cells) are derived from ductal adenocarcinomas (14).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Rebecca B. Riggins, Room E407 Research Building, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, 3970 Reservoir Road Northwest, Washington, DC 20057. Phone: 202-687-7451; Fax: 202-687-7505; E-mail: rbr7@georgetown.edu.

©2008 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-2669

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2598947/8908.pdf by guest on 14 December 2024

Given the unique clinical and molecular features of lobular tumors, and the suggestion that ILC tumors may respond less well to endocrine therapy, we have developed an ILC-specific cell culture model of endocrine resistance. The SUM44 breast cancer cell line was isolated from an ILC metastasis (15), is ER+/PR+, and displays other common features of ILC such as the loss of E-cadherin (16). We show that SUM44 cells contain functional ER and are sensitive to growth inhibition by antiestrogens. Selection of SUM44 cells against 4-hydroxytamoxifen (4HT) led to the establishment of the SUM44/LCCTam cell line, which is stably resistant to TAM. We then identified candidate genes associated with the endocrine resistant phenotype in SUM44/LCCTam cells and found changes in the expression of ERα and the estrogen-related receptor γ (ERRγ). Our mechanistic studies show that knockdown of ERRγ in the resistant cell line, and overexpression of ERRγ in endocrine-responsive lobular breast cancer cells, modulates TAM sensitivity. Finally, we show that ERRγ-driven transcription is increased in the resistant SUM44/LCCTam cell line, and inhibition of activator protein 1 (AP1) can restore or enhance TAM sensitivity in this model system.

Materials and Methods

Cell culture and reagents. All cells were shown to be free of *Mycoplasma* spp. contamination and maintained in a humidified incubator at 37°C in an atmosphere containing 95% air/5% CO_2. Routine tissue culture reagents (culture medium and additives, PBS, trypsin, etc.) were purchased from Invitrogen.

SUM44 cells were routinely cultured in serum-free medium plus insulin and hydrocortisone (SFIH) as described previously (15). LCCTam cells were maintained in SFIH containing 500 nmol/L 4HT (Sigma). LCCTam cells were cultured in SFIH in the absence of 4HT for 1 wk before all experiments. When SUM44 and LCCTam were passaged, cells were seeded in SFIH containing 2% fetal bovine serum (FBS) for the first 24 h to neutralize trypsin and promote cell attachment. MCF-7 cells were originally obtained from Dr. Marvin Rich (Karmanos Cancer Center, Detroit, MI), and MDA-MB-134 VI breast cancer cells were purchased from American Type Culture Collection; both were maintained in improved minimal essential medium with phenol red supplemented with 5% FBS.

17β-Estradiol (estradiol, E2) was purchased from Sigma; Fulvestrant (ICI 182,780; Fulv) and the c-JUN peptide inhibitor were purchased from Tocris Bioscience. The 3xERE-tk-luc promoter-reporter plasmid was kindly provided by Dr. Malcolm G. Parker (Imperial College, London, United Kingdom; ref. 17), 3xSF1RE-luciferase was a gift from Dr. Jean-Marc Vanacker (Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Lyon, France; ref. 18), and 3xAP1-luciferase was generously provided by Dr. Richard Pestell (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). The plasmid encoding wild-type murine ERRγ bearing an NH_2-terminal hemagglutinin (HA) tag (pSG5-HA-ERR3) was a gift from Dr. Michael Stallcup (Keck School of Medicine, University of Southern California, Los Angeles, CA; ref. 19). Small inhibitory RNA (siRNA) oligonucleotide duplexes directed against ERRγ (siGENOME SMARTpool), nonsilencing control oligonucleotides, and the DharmaFECT 1 reagent were purchased from Dharmacon. The FuGene 6 transfection reagent was purchased from Roche.

Luciferase promoter-reporter assays. Cells were seeded in SFIH at a density of 9×10^4 cells per well in 12-well plastic tissue culture dishes for 24 to 48 h before transfection with 0.6 μg luciferase promoter-reporter construct and 0.2 μg phRL-SV40 Renilla internal control (Promega). The following day, transfected cells were refed with SFIH, or SFIH containing 10 nmol/L E2, 1,000 nmol/L 4HT, 100 nmol/L Fulv, 20 μmol/L c-JUN peptide inhibitor, or ethanol vehicle as indicated in each figure for a further 24 h before lysis and measurement of luciferase activity by using the Dual Luciferase Assay kit (Promega) as described previously (20). Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold).

Proliferation assays. Cells were seeded in SFIH at a density of 2 to 3×10^4 per well in 24-well plastic tissue culture dishes 1 d before the addition of the indicated concentrations of drug or ethanol vehicle. Cells were cultured for 6 d with two medium changes before being trypsinized, resuspended in PBS, and counted using a Z1 Single Coulter Counter (Beckman/Coulter). At least three independent assays were performed in triplicate or quadruplicate, and the data were normalized to vehicle-treated cells.

BrdUrd ELISAs. Cells were seeded in SFIH at a density of 1×10^4 cells per well in 96-well plastic tissue culture dishes 1 d before the addition of drug or ethanol vehicle as indicated. Cells were then cultured for ~54 h before the addition of BrdUrd (final concentration 10 μmol/L) for an additional 18 h (total incubation in drug, 72 h) before performing the Cell Proliferation ELISA, BrdUrd (colorimetric) assay as directed by the manufacturer (Roche). At least three independent assays were performed with five replicate wells per treatment group, and data were normalized to vehicle-treated cells.

BrdUrd immunofluorescence assays. These assays were performed as described above (drug treatment and BrdUrd addition) and by Riggins and colleagues (cell seeding and staining procedures; ref. 21) with the following modifications: ERRγ expression was detected using the HA.11 monoclonal antibody from Covance (1:500) followed by AlexaFluor594-conjugated goat anti-mouse secondary antibody (Invitrogen; 1:500), and BrdUrd incorporation was detected using the AlexaFluor488-conjugated anti-BrdUrd antibody (1:10; BD Biosciences). Cells were visualized on a Nikon E600 epifluorescence microscope at ×20 magnification.

Cell cycle analysis. Cells were seeded in SFIH at a density of 5×10^4 cells per well in 6-well plastic tissue culture dishes 1 d before the addition of 1,000 nmol/L 4HT or ethanol vehicle. Cells were then cultured for 72 h before harvesting and cell cycle analysis by the Vindelov method (22).

Derivation of SUM44/LCCTam cells. A TAM-resistant SUM44 variant was established according to previously published procedures (23). Subconfluent T-25 cm^2 tissue culture flasks of SUM44 cells were selected against increasing concentrations of 4HT, beginning with 1 nmol/L. After 3 passages of the cells at each dose, the drug concentration was increased (1→5→10→50→100→500 nmol/L), terminating at a concentration of 500 nmol/L 4HT. Cells proliferating in 500 nmol/L 4HT were designated SUM44/LCCTam (hereafter abbreviated as LCCTam). LCCTam cells were cultured in SFIH in the absence of 4HT for 1 wk before all experiments.

Comparative genomic hybridization. Normal control DNA was prepared from peripheral blood lymphocytes of a normal donor and test DNA was extracted from the cultured cell lines (SUM44 and the TAM-resistant LCCTam variant) using standard protocols, and comparative genomic hybridization (CGH) was performed as previously described (24). Gray scale images from at least 10 metaphases from each hybridization were acquired with a cooled charge-coupled device CCD camera (CH250; Photometrics) connected to a Leica DMRBE microscope equipped with fluorochrome specific optical filters TR1, TR2, TR3 (Chroma Technology). Quantitative evaluation of the hybridization was done using commercially available software (Applied Imaging). Average ratio profiles were calculated as the mean value of at least eight ratio images to identify chromosomal copy number changes in all cases (see Supplementary Fig. S1).

RNA isolation, gene expression microarray preprocessing, and data analysis. Total RNA was extracted from subconfluent T-25 cm^2 tissue culture flasks of SUM44 and LCCTam cells, then processed and arrayed as described by Gomez and colleagues (25). Microarray data quality was then assessed using several tools, including those recommended by Affymetrix and a series of additional QC measures under development in our laboratory (26). The Robust Multiple-Array Average method was used to preprocess the raw gene expression data, as implemented in the Bioconductor project.[4] We then isolated a reduced dimension data set that included genes that exhibit ≥2 fold change ($P < 0.05$) and genes with intensity ≥log2 (10) in both SUM44 and SUM44/LCCTam groups. Data visualization before and after dimensionality reduction was facilitated by multidimensional scaling as estimated using Principal Component Analysis

[4] http://bioconductor.org

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2588947/8908.pdf by guest on 14 December 2024

(PCA) and Discriminant Component Analysis (27), to ensure that the global structure of the data were not altered by dimensionality reduction procedures (see Supplementary Fig. S2). Expression data are available through the Gene Expression Omnibus database, accession GSE12708.

Real-time qPCR. Total RNA from independent cultures (not RNA from cultures used for microarray analysis) was isolated, cleaned, quantified, and reverse-transcribed as described in (25). qPCR reactions for each cDNA sample and a standard curve were performed using TaqMan Universal PCR Master Mix and the following TaqMan Gene Expression Assay primers (Applied Biosystems): ESR1, Hs00174860_m1; ESRRG, Hs00155006_m1; and the housekeeping gene RPLP0 (Hs99999902_m1) as in Gomez and colleagues (25). Expression data for each gene was estimated relative to the housekeeping control, and these data were used to calculate the ratio of expression relative to that in the parental SUM44 cell line.

Cell lysis and Western blot analysis. Subconfluent monolayers of cells were harvested, lysed, and analyzed by Western blot as in Bouker and colleagues (28). Primary antibodies for ERRγ (1:1,000), ERRα (1:500), and ERRβ (1:500) were purchased from GenWay. Antibodies for ERα (1:500) and ERβ (1:1,000) were purchased from NovoCastra and Affinity Bioreagents, respectively. Antibodies for FASN (1:500) and HMGCS2 (1:2,000) were purchased from Abcam. To confirm equal loading, membranes were reprobed using a β-actin monoclonal antibody (1:5,000) purchased from Sigma, or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) goat polyclonal antibody (1:5,000) purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated to horseradish peroxidase were purchased from GE Healthcare and Santa Cruz Biotechnology. Densitometry was performed using NIH ImageJ software[5] and images were compiled using Adobe Photoshop CS2.

ERRγ siRNA. LCCTam cells were seeded in 96-well plastic tissue culture dishes in SFIH at 1×10^4 cells per well 1 d before transfection with 100 nmol/L ERRγ (siERRγ) or nonsilencing control siRNA oligonucleotides (siC) using DharmaFECT1 (Dharmacon) according to manufacturer's specifications. The next day, cells were treated with 1,000 nmol/L 4HT or ethanol vehicle before addition of BrdUrd for an additional 18 h (total incubation in drug, 48 h). Cell Proliferation ELISA, BrdUrd (colorimetric) assays were performed as described above. In parallel, cells were seeded in 12-well dishes at a density of 9×10^4 cells per well, transfected with 100 nmol/L siC or siERRγ, and cells were lysed on the same day that BrdUrd ELISAs were performed (total transfection time, 72 h) for Western blot analysis.

Statistics. All statistical calculations were performed using SigmaStat version 3.0 (Systat). Luciferase promoter-reporter, cell proliferation, BrdUrd, real-time reverse transcription-PCR (RT-PCR), and microarray data from *in vitro* studies were compared using either Student's t test or one-way ANOVA followed by *post hoc t* test, as appropriate, and indicated in the text and figure legends. Statistical significance is defined at ≥95% confidence level, or a P value of ≤0.05.

Results

SUM44 cells have functional ER and are sensitive to growth inhibition by 4HT. The SUM44 breast cancer cell line was derived from an ILC and a high percentage of ILC tumors are ER+ (29). Although this cell line is also ER+ (15), ER functional status is unknown and SUM44 responsiveness to estrogens and antiestrogens has not previously been determined. Therefore, SUM44 cells were transfected with the 3xERE-tk-luc reporter construct and stimulated with estrogen, antiestrogen, or ethanol control (Fig. 1*A*). Estrogen (E2) modestly but significantly induces, whereas 4HT significantly decreases, ERE-luciferase activity ($P < 0.001$). We also observed that the steroidal antiestrogen Fulv decreases ERE-luciferase activity, and that both 4HT and Fulv block the E2-induced stimulation of ERE-luciferase activity ($P < 0.001$). These data suggest that the SUM44 ER responds appropriately to estrogenic and antiestrogenic stimuli.

To determine whether SUM44 cells are sensitive to growth inhibition by 4HT, cells were treated with antiestrogen as indicated for 6 d (Fig. 1*B, closed circles*). 4HT significantly inhibits the proliferation of SUM44 cells (ANOVA $P < 0.001$). The observed reduction in cell number is also reflected in an inhibition of DNA synthesis as shown by reduced BrdUrd incorporation after 72 hours of 4HT treatment (ANOVA $P < 0.001$; Fig. 1*C, closed circles*), consistent with the known cytostatic effect of 4HT (12).

Generation of a TAM-resistant SUM44 variant. Because ILCs are predominantly ER+ and TAM has been the most widely used endocrine agent for the treatment of ER+ breast cancer, we sought to develop a TAM-resistant ILC model using the SUM44 cell line. Cells were selected against increasing concentrations of 4HT, and the cell population proliferating in 500 nmol/L 4HT (within the range of clinically relevant concentrations; ref. 10) was designated SUM44/LCCTam (hereafter called LCCTam).

The basal growth rate of LCCTam is identical to that of the parental SUM44 cell line and as expected, LCCTam cells are no longer responsive to the antiproliferative effects of 4HT (Fig. 1*B, open triangles, N.S.*), and LCCTam DNA synthesis is no longer inhibited by 4HT (ANOVA $P = 0.212$; Fig. 1*C, open triangles*). To further confirm that differences in SUM44 and LCCTam cell proliferation in response to antiestrogen reflect changes in sensitivity to the cytostatic effects of 4HT, we performed cell cycle analysis. SUM44 cells treated with 1 μmol/L 4HT show a significantly greater fraction of cells arrested in the G_1 phase compared with ethanol-treated controls ($P \leq 0.001$; data not shown), whereas 4HT no longer induces an accumulation of LCCTam cells in G_1 ($P = 0.722$, data not shown). Together, these findings show that SUM44 cell growth and cell cycle progression are efficiently inhibited by 4HT, but that LCCTam cells have acquired resistance to the inhibitory effects of this antiestrogen.

Changes in the transcriptome of LCCTam cells are not associated with chromosomal aberrations. To characterize further this novel ILC cell model, we determined the pattern of, and differences in, genomic alterations and gene expression between SUM44 and LCCTam cells using CGH and Affymetrix gene expression microarray analysis, respectively. The genetic lineage of the two cell lines was confirmed to be identical by DNA fingerprinting using genetic markers at nine different loci. CGH analysis revealed changes in the DNA copy number (gains, losses, and amplifications) in both SUM44 and LCCTam (Supplementary Fig. S1). Importantly, a comparison between our CGH findings and a previously reported CGH analysis of SUM44 show a similar pattern of aberrations (30). We found no significant difference in the pattern of chromosomal alterations between the two cell lines; acquired estrogen independence also is not associated with changes in the amplification of DNA sequences (31).

In marked contrast, microarray analysis reveals a large number of changes in gene expression. We used PCA (27) to visualize the high-dimensional data set in two dimensions; SUM44 and LCCTam are linearly separable in this two-dimensional PCA projection based on the top two principal components that capture 95% of the cumulative variance in the data (Supplementary Fig. S2). Using a final cutoff of ≥2-fold change with $P \leq 0.05$ (univariate, uncorrected, T-statistic), we find that 380 genes are likely to be significantly altered: expression of 91 genes are increased and 289 genes are decreased in LCCTam versus SUM44 controls (Supplementary Table S1).

To maintain focus on the TAM-resistant phenotype observed in LCCTam cells, we first chose to investigate gene expression

[5] http://rsb.info.nih.gov/ij

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2589847/8908.pdf by guest on 14 December 2024

changes in ERs and other members of the nuclear receptor superfamily. Expression of ERα (HUGO symbol ESR1) is decreased 3.1-fold in LCCTam compared with SUM44 cells by microarray ($P = 0.0013$), which was subsequently confirmed by qPCR analysis (↓2.98-fold, $P < 0.001$; Fig. 2*A*, *white bars*). In contrast, expression of the orphan nuclear receptor ERRγ (HUGO symbol ESRRG) is 4.4-fold increased in the resistant LCCTam cells by microarray ($P = 0.01$) and 10-fold increased by qPCR ($P = 0.03$; Fig. 2*A*, *black bars*).

To confirm that differences in the mRNA expression of these receptors are maintained at the protein level, cell lysates were collected and analyzed for ERRγ and ERα expression by Western blot (Fig. 2*B*, *inset*). As observed for mRNA, ERRγ protein expression is increased (↑2.5-fold; $P = 0.03$) and ERα expression is decreased (↓2-fold; $P = 0.03$) in LCCTam cells. We also examined the protein levels of all other ERs and ERRs (ERβ, ERRα, and ERRβ) and find no differences in their expression between SUM44 and LCCTam cells (Fig. 2*C*).

ERRγ plays a functional role in TAM resistance in LCCTam cells. ERRγ is an orphan nuclear receptor with no known natural ligand that has been shown to have constitutive transcriptional activity at several DNA response elements (reviewed in refs. 32, 33). ERRγ and its family members ERRα1 and ERRβ bear some structural similarity to the ER (32, 34). Although ERRα1 has previously been shown to activate or repress estrogen response element (ERE)-mediated transcription depending on cellular context (34) and to participate in HER2-dependent signaling in BT474 breast cancer cells (35), the role of ERRγ in breast cancer therapeutic response is underexplored (36).

We hypothesized that if increased expression of ERRγ in LCCTam cells performs a functional role in the acquired TAM resistance phenotype, knockdown of receptor expression should restore TAM sensitivity. LCCTam cells were transiently transfected with siRNA oligonucleotides directed against ERRγ (siERRγ) or a nonsilencing control (siC) before treating the cells with 4HT and assessing DNA synthesis as measured by BrdUrd incorporation. A 2- to 3-fold decrease in ERRγ expression is attained by siRNA ($P < 0.001$; Fig. 3*A*). Importantly, ERRγ knockdown also partially restores sensitivity to 4HT in the LCCTam cells ($P = 0.03$ versus siERRγ ethanol and $P < 0.001$ versus siC in 1,000 nmol/L 4HT; Fig. 3*B*) with no effect on the expression of ERα (Fig. 3*A*, *inset, bottom*). These data suggest that ERRγ plays a key functional role in the LCCTam TAM resistance phenotype.

Overexpression of ERRγ induces 4HT resistance. Next, we sought to determine whether ERRγ overexpression could induce TAM resistance in endocrine-responsive breast cancer cells. SUM44 cells grown on fibronectin-coated coverslips were transiently transfected with the pSG5-HA-ERR3 plasmid, encoding the murine homologue of ERRγ, which is 100% identical to human ERRγ at the amino acid level (19), or the empty vector (pSG5). Cells were then treated with 1 μmol/L 4HT or ethanol vehicle and immunostained for BrdUrd incorporation (*green*) and ERRγ expression (HA, *red*; ref. 21). In agreement with our results in Fig. 1*C*, 4HT significantly

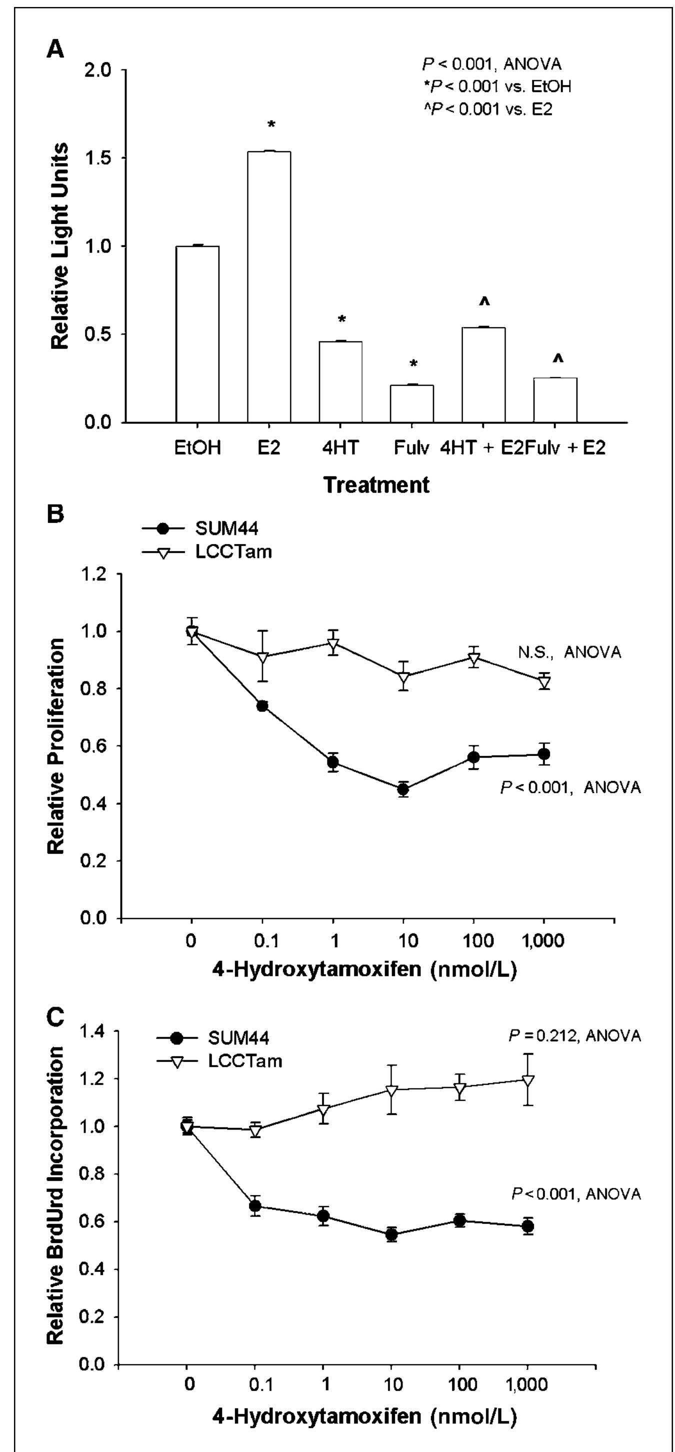


Figure 1. SUM44 cell proliferation and ER transcriptional activity are inhibited by antiestrogens, and LCCTam cells have acquired resistance to TAM. *A,* cells were seeded in 12-well tissue culture dishes, transfected with plasmids encoding 3xERE-tk-luciferase and phRL-SV40 Renilla, and treated with 10 nmol/L E2, 1,000 nmol/L 4HT, 100 nmol/L Fulv, 4HT+E2, Fulv+E2, or ethanol control for 24 h before harvest and luciferase assay. ERE-luciferase values are normalized to Renilla activity to obtain Relative Light Units, and data are presented as the mean relative to ethanol ± SE for a representative experiment performed in triplicate. ANOVA $P < 0.001$; *, $P < 0.001$ for comparisons to ethanol and ^, $P < 0.001$ for comparisons to E2 by *post hoc* Student's *t* test. *B,* cells were seeded in 24-well tissue culture dishes and treated with the indicated concentrations of 4HT for 6 d, at which time cell number was determined. *Points,* mean proliferation relative to ethanol for a representative experiment performed in quadruplicate; *bars,* SE. ANOVA $P < 0.001$ for SUM44, and not significant (*N.S.*) for LCCTam. *C,* cells were seeded in 96-well tissue culture dishes 1 d before treatment with the indicated concentrations of 4HT for a total of 72 h; BrdUrd was added for the last 18 h of culture. *Points,* mean BrdUrd incorporation relative to ethanol for a representative experiment performed in quintuplicate; *bars,* SE. ANOVA $P < 0.001$ for SUM44, and $P = 0.212$ (*N.S.*) for LCCTam.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2598947/8908.pdf by guest on 14 December 2024

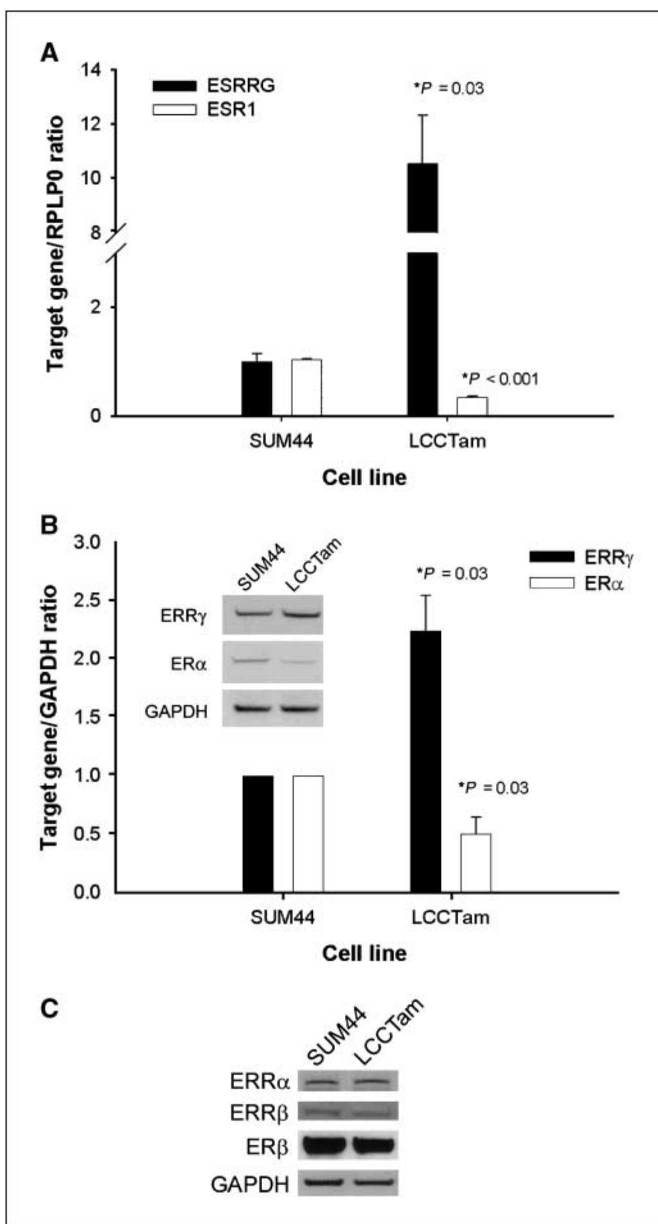


Figure 2. ERα and ERRγ mRNA and protein expression are significantly altered during the acquisition of TAM resistance. *A,* total RNA was isolated from SUM44 and LCCTam cells, reverse-transcribed, and subjected to RT-PCR to detect ERRγ (HUGO gene symbol ESRRG), ERα (*ESR1*), and the housekeeping gene RPLP0. *Columns,* mean target gene/RPLP0 ratio for three samples analyzed in triplicate; *bars,* SE. $^*P = 0.03$ for ESRRG and $^*P < 0.001$ for ESR1 in SUM44 versus LCCTam by Student's *t* test. *B,* densitometric quantification of protein expression and a representative Western blot are shown for expression of ERRγ, ERα, and the GAPDH loading control. $^*P = 0.03$ for ERRγ and ERα in SUM44 versus LCCTam by Student's *t* test. *C,* representative Western blot showing ERRα, ERRβ, and ERβ expression, and the GAPDH loading control, in SUM44 and LCCTam cells.

reduces BrdUrd incorporation in SUM44 cells transfected with the empty vector pSG5 ($P < 0.001$; Fig. 4*A*, *ii* versus *iv*, 48.9% versus 16.9% BrdUrd incorporation). However, 4HT can no longer inhibit DNA synthesis when ERRγ is overexpressed ($P < 0.001$; Fig. 4*A*, *iv* versus *viii*, 16.9% versus 53.9% BrdUrd incorporation). The effect of ERRγ overexpression is particularly striking when comparing BrdUrd incorporation in transfected versus untransfected cells in the presence of 4HT within the same field of view. In Fig. 4*A*, most ERRγ-positive (*red*) cells incorporate BrdUrd (*viii, arrowheads*), whereas ERRγ-negative cells show little-to-no BrdUrd incorporation (*viii*, *).

To confirm that ERRγ can regulate TAM resistance in breast cancer cell lines other than SUM44, we performed the same study in MDA-MB-134 VI cells, which are ER+ and TAM-sensitive (37) and are also considered to be of lobular origin (38). When transfected with the pSG5 empty vector, DNA synthesis in MDA-MB-134 VI cells is inhibited by 4HT by nearly 2-fold (49.9% versus 27.3% BrdUrd incorporation, $P < 0.001$; Fig. 4*B*). However, when ERRγ is overexpressed, these cells become significantly less responsive to the inhibitory effects of 4HT (27.3% versus 44.7% BrdUrd incorporation, $P = 0.001$; Fig. 4*B*). Together, these data show that increased expression of ERRγ can induce TAM resistance in several ER+ lobular breast cancer cell lines.

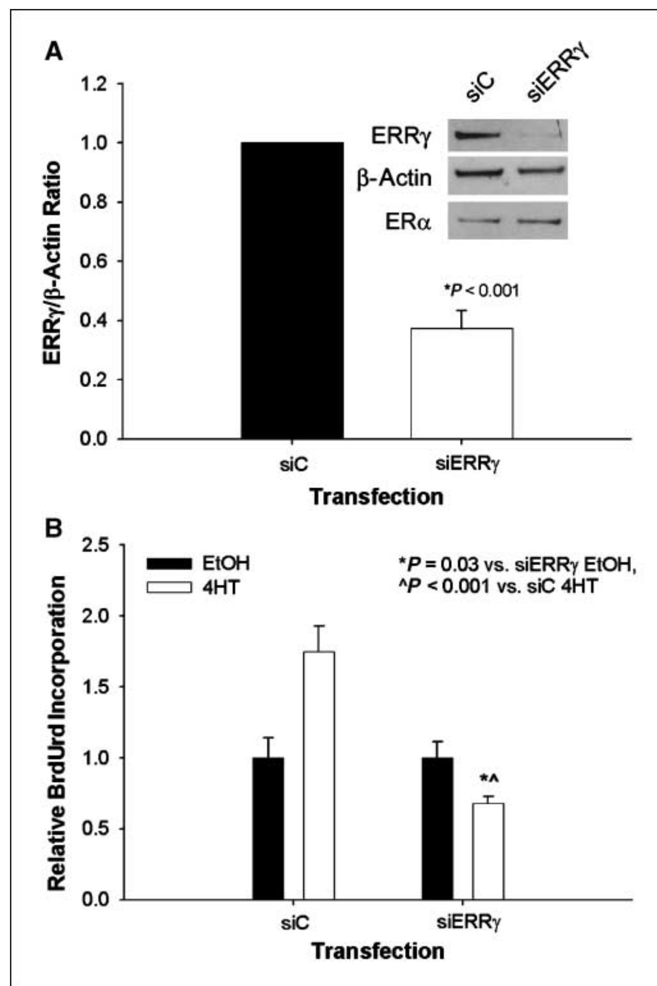


Figure 3. siRNA knockdown of ERRγ in LCCTam cells restores TAM sensitivity. *A,* cells seeded in 12-well dishes were transfected with control (*siC*) or ERRγ-specific (*siERRγ*) oligonucleotides (final concentration, 100 nmol/L) for 72 h before lysis, Western blot analysis, and densitometry. *Columns,* mean ERRγ/β-actin ratio for three independent experiments; *bars,* SE. *Inset,* a representative image. $^*P < 0.001$ for siERRγ versus siC, Student's *t* test. ERRγ knockdown has no effect on ERα expression (*inset, bottom*). *B,* cells seeded in 96-well dishes were transfected with siC or siERRγ oligonucleotides 24 h before treatment with 1,000 nmol/L 4HT or ethanol control. BrdUrd assays were performed as described above; *columns,* mean BrdUrd incorporation relative to ethanol for a representative experiment performed in quintuplicate; *bars,* SE. $^*P = 0.03$ versus siERRγ ethanol, and $^\wedge P = <0.001$ versus siC in 1,000 nmol/L 4HT by Student's *t* test.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2589847/8908.pdf by guest on 14 December 2024

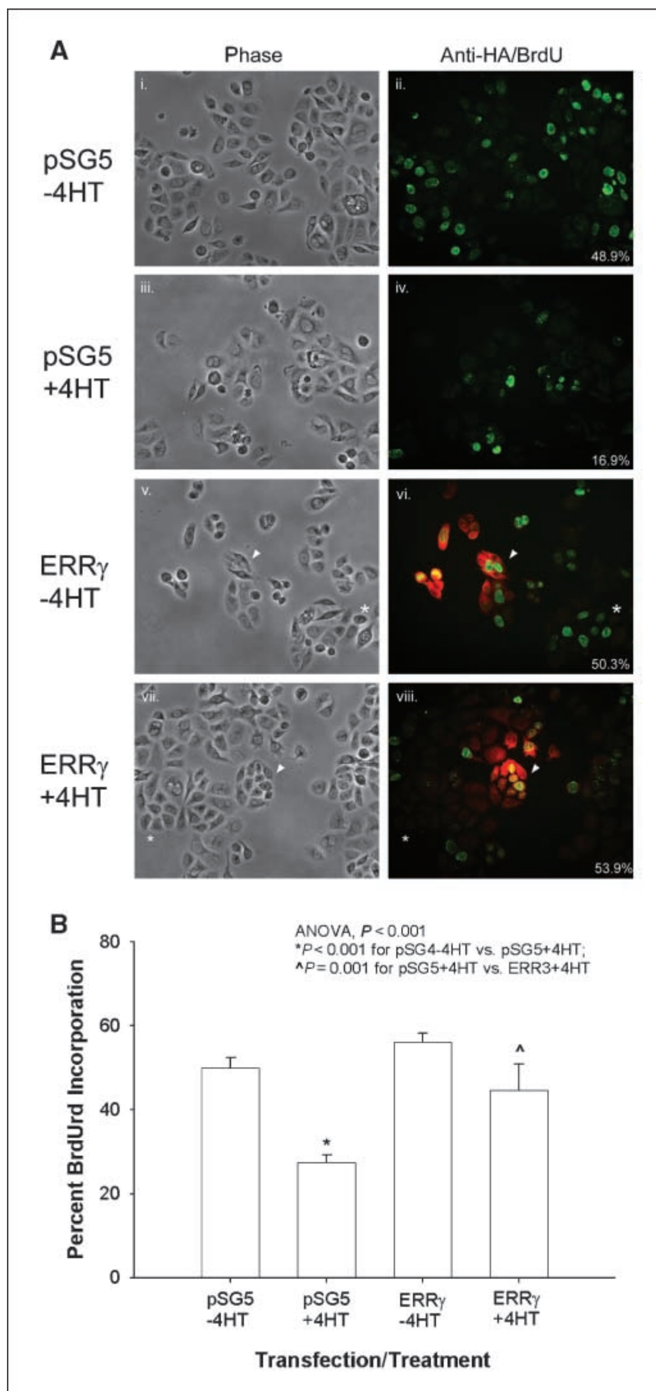


Figure 4. ERRγ overexpression in SUM44 and MDA-MB-134 VI breast cancer cells induces TAM resistance. *A,* SUM44 cells were seeded on fibronectin-coated coverslips, then transfected with pSG5-HA-ERR3 (*ERRγ*) or empty vector (*pSG5*). Cells were treated with 1,000 nmol/L 4HT or ethanol control for a total of 48 h; BrdUrd was added for the last 18 h of culture before fixation and processing for HA (*ERRγ*) and BrdUrd immunostaining. Representative phase-contrast and fluorescent images are shown (*red,* ERRγ; *green,* BrdUrd); *arrowheads,* ERRγ-positive cells; *, ERRγ-negative cells. Quantitative data are presented as the mean percent BrdUrd incorporation for a representative experiment in which 4 to 5 microscopic fields (>500 total cells) were counted per condition. ANOVA $P < 0.001$; $P < 0.001$ for pSG5-4HT versus pSG5+4HT, and $P < 0.001$ for pSG5+4HT versus ERRγ+4HT by *post hoc* Student's *t* test. *B,* MDA-MB-134 VI cells were seeded, transfected, drug treated, and stained as described in *A. Columns,* mean percent BrdUrd incorporation for a representative experiment in which 4 to 5 fields (and >500 total cells) per condition were counted; *bars,* SE. ANOVA $P < 0.001$; *, $P < 0.001$ for pSG5-4HT versus pSG5+4HT, and ^, $P = 0.001$ for pSG5+4HT versus ERRγ+4HT by *post hoc* Student's *t* test.

ERRγ-associated transcriptional activity is increased in resistant LCCTam cells. A crucial difference between ERRγ and liganded nuclear receptors like ERα is the regulation of their transcriptional activities. Whereas ERα is dependent on ligand for full activation, ERRγ and the other members of this orphan family exhibit constitutive transcriptional activity. The ERRγ DNA binding domain is ∼64% identical to that of ERα (34). Consequently ERRγ can bind to the same EREs as ERα, but it can also potently activate the steroidogenic factor-1 response element (SF1RE; ref. 32). Although none of the ERR family members are affected by E2 stimulation because their ligand binding domains cannot accommodate E2 binding (discussed in ref. 34), ERRγ transcriptional activity at EREs and SF1REs can be inhibited by 4HT (39, 40). In contrast, 4HT-bound ERRγ acquires the ability to positively regulate transcription at AP1 sites (reviewed in ref. 34).

To begin to understand the mechanism by which ERRγ up-regulation confers resistance to LCCTam cells, we examined the activity of ERE-, SF1RE-, and AP1-driven luciferase promoter-reporter constructs transiently expressed in SUM44 and LCCTam cells (Fig. 5*A*). Luciferase expression controlled by the ERE and SF1RE response elements is significantly increased by 5- and 3-fold, respectively, in LCCTam cells compared with SUM44 cells ($P < 0.005$). When LCCTam cells are cultured in 4HT ("LCCTam+4HT"), ERE-luciferase activity is somewhat reduced but still shows a nearly 2-fold increase relative to SUM44 (*black bars*; $P < 0.005$), whereas SF1RE-luciferase activity remains high (*white bars*; 3-fold above the levels in SUM44 cells; $P < 0.005$). In contrast, AP1-luciferase activity increases up to 8-fold that observed in SUM44 cells in the presence of 4HT (*hatched bars*; $P < 0.005$).

ERRγ/AP1 activity seems to drive TAM resistance in LCCTam cells. To test whether the observed robust AP1 activity plays a functional role in the TAM-resistant phenotype, we used a cell-permeable peptide fragment of c-JUN that blocks its interaction with the JUN NH_2-terminal protein kinase, resulting in strong AP1 inhibition (41). This c-JUN peptide has virtually no effect on SF1RE-luciferase activity (Fig. 5*B*, *N.S.*) but can inhibit AP1-luciferase activity by >2-fold ($P = 0.04$; Fig. 5*C*). Importantly, this level of AP1 inhibition restores 4HT-mediated growth inhibition to LCCTam cells ($P = 0.001$; Fig. 5*D*) and enhances the sensitivity of the parental SUM44 cells to the growth-inhibitory effects of 4HT ($P = 0.002$).

Our functional data suggest that in LCCTam cells, increased ERRγ-driven AP1 transcriptional activity is most strongly associated with TAM resistance. However, endogenous ERRγ/AP1 target genes have yet to be identified; ERRγ-dependent AP1 activity has previously been reported only on heterologous promoter constructs (42). We therefore used the TRANSFAC Professional 11.1 database (43) to search the proximal promoter regions of genes up-regulated ≥2-fold in LCCTam cells for AP1 consensus sites within 5,000 bp of the start site. The MatchTM algorithm (44) was used to analyze the DNA sequences and search for potential AP1 binding sites, using Position Weight Matrices to minimize false positives. Several genes had multiple AP1 response elements in their promoter regions (Fig. 6*A*). Western blot analysis was then used to confirm the overexpression of two of these genes, HMGCS2 and FASN (Fig. 6*B*). HMGCS2 is a nuclear-encoded mitochondrial matrix gene that can regulate ketogenesis and cholesterol synthesis (45, 46), and FASN is the final enzyme of the fatty acid biosynthetic pathway (47). Components of all three processes have been

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2589447/8908.pdf by guest on 14 December 2024

implicated in the etiology or progression of breast cancer, and FASN activity can affect hormonal sensitivity in breast and endometrial cancer cells (48–50). Therefore, we suggest that HMGCS2 and FASN may be two novel ERRγ/AP1 targets in TAM-resistant breast cancer.

Discussion

In this study, we report the development of the first model of endocrine-resistant breast cancer in a cell line derived from an invasive lobular breast carcinoma, and show that the orphan nuclear receptor ERRγ and its ability to drive AP1 transcriptional activity are central to the TAM resistance phenotype.

Selection of SUM44 cells against 4HT led to the establishment of the LCCTam cell line, which is stably resistant to TAM. In the resistant LCCTam cells, we observe a significant down-regulation of ERα (although they remain ER+), accompanied by a significant increase in the expression of ERRγ. Resistance to antiestrogens has been hypothesized to take place through several diverse mechanisms (10, 12). One is loss or mutation of ERα, whereas others include alterations in the profile of

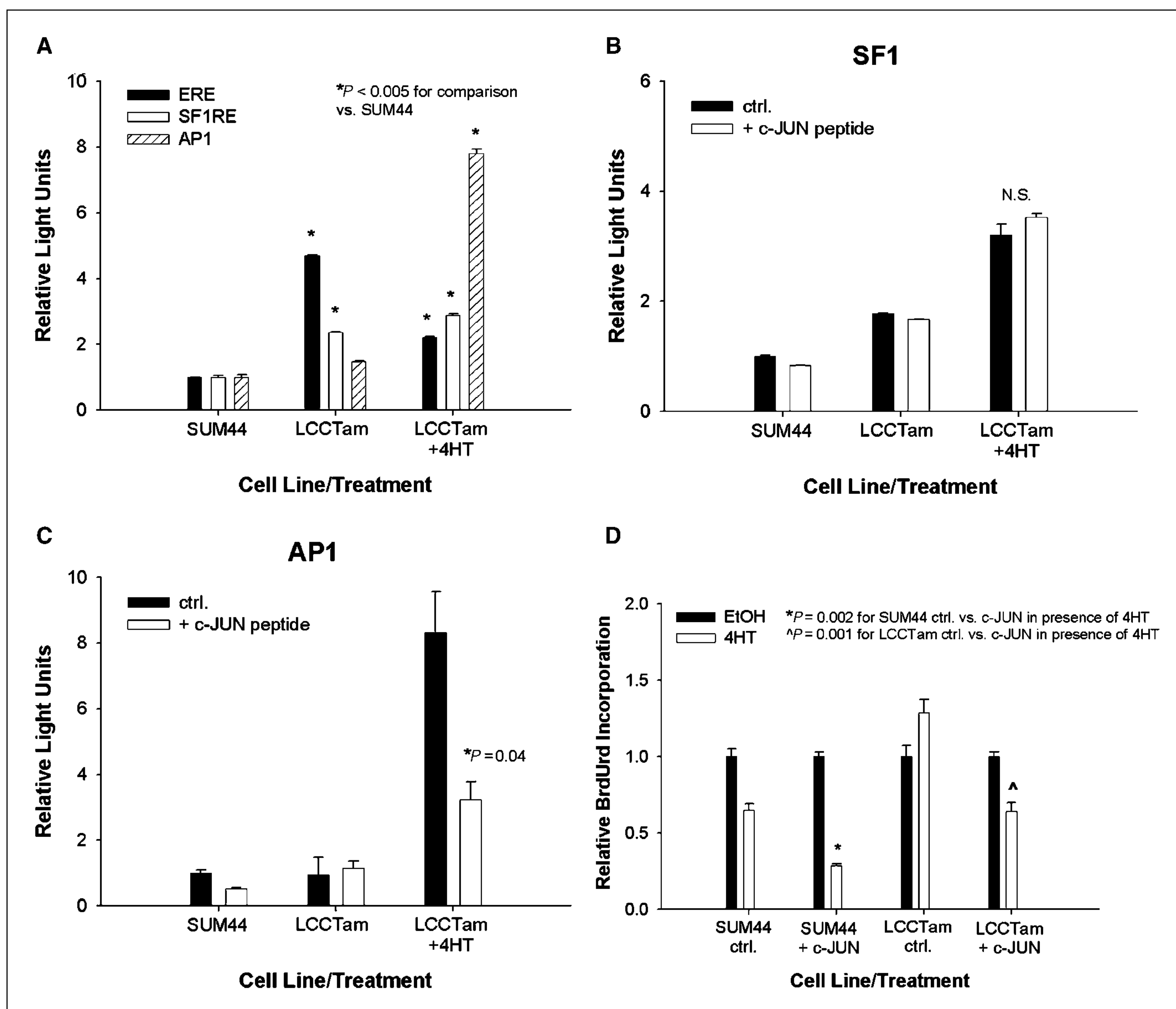
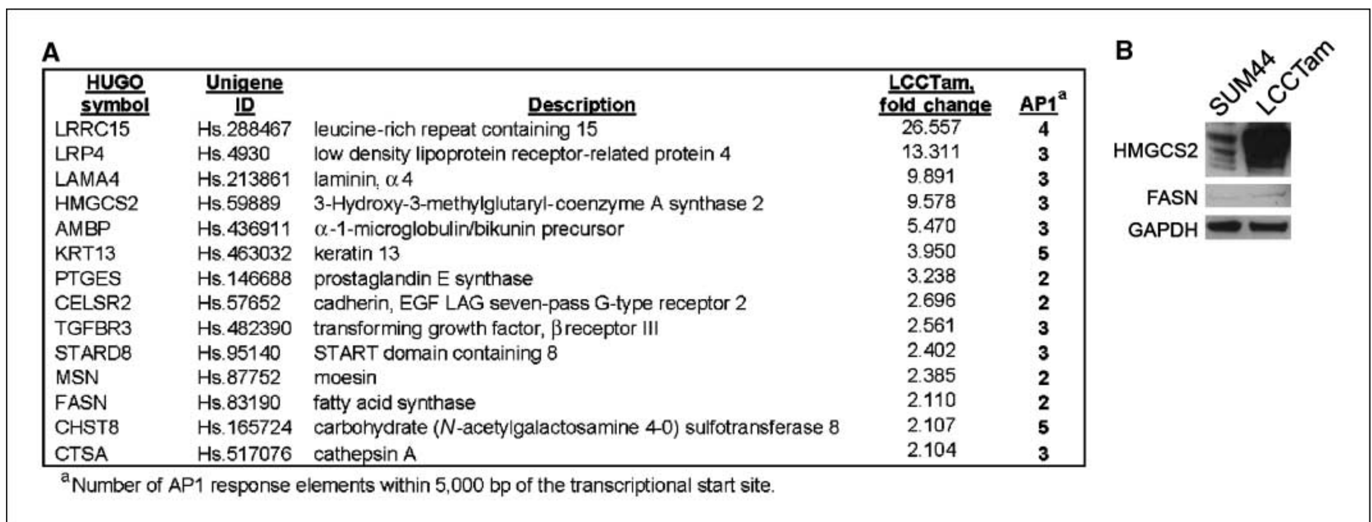


Figure 5. ERRγ-associated transcriptional activity is increased in resistant LCCTam cells, and inhibition of AP1 restores TAM sensitivity. *A,* cells were seeded and transfected with 3xERE-, 3xSF1RE-, and 3xAP1-luciferase and phRL-SV40 Renilla, incubated, lysed, and analyzed as described for Fig. 1*A*. *LCCTam+4HT,* cells that were cultured in SFIH containing 500 nmol/L 4HT. ANOVA $P < 0.001$, and $^*P < 0.005$ for all comparisons versus SUM44 by *post hoc* Student's *t* test. *B,* cells were seeded and transfected with 3xSF1RE-luciferase and phRL-SV40 Renilla for 1 d before treatment with 20 μmol/L c-JUN peptide or PBS control (*Ctrl.*). Cells were then incubated, lysed, and analyzed as described for Fig. 1*A*; LCCTam+4HT is as described above. *C,* cells were seeded and transfected with 3xAP1-luciferase, then treated, harvested, and analyzed as described. LCCTam+4HT is as described above. ANOVA $P < 0.001$; $^*P = 0.04$ for control versus c-JUN peptide by *post hoc* Student's *t* test. *D,* cells were seeded in 96-well dishes 24 h before treatment with 1,000 nmol/L 4HT or ethanol control in the presence of 20 μmol/L c-JUN peptide or PBS control. BrdUrd assays were performed as described above; *columns,* mean BrdUrd incorporation relative to ethanol for a representative experiment performed in quintuplicate; *bars,* SE. *, $P = 0.002$ for SUM44, control versus c-JUN in the presence of 4HT, and ^, $P = 0.001$ for LCCTam, control versus c-JUN in the presence of 4HT, by Student's *t* test.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2589847/8908.pdf by guest on 14 December 2024



A

HUGO symbol	Unigene ID	Description	LCCTam, fold change	AP1[a]
LRRC15	Hs.288467	leucine-rich repeat containing 15	26.557	4
LRP4	Hs.4930	low density lipoprotein receptor-related protein 4	13.311	3
LAMA4	Hs.213861	laminin, α4	9.891	3
HMGCS2	Hs.59889	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	9.578	3
AMBP	Hs.436911	α-1-microglobulin/bikunin precursor	5.470	3
KRT13	Hs.463032	keratin 13	3.950	5
PTGES	Hs.146688	prostaglandin E synthase	3.238	2
CELSR2	Hs.57652	cadherin, EGF LAG seven-pass G-type receptor 2	2.696	2
TGFBR3	Hs.482390	transforming growth factor, β receptor III	2.561	3
STARD8	Hs.95140	START domain containing 8	2.402	3
MSN	Hs.87752	moesin	2.385	2
FASN	Hs.83190	fatty acid synthase	2.110	2
CHST8	Hs.165724	carbohydrate (*N*-acetylgalactosamine 4-0) sulfotransferase 8	2.107	5
CTSA	Hs.517076	cathepsin A	2.104	3

[a]Number of AP1 response elements within 5,000 bp of the transcriptional start site.

Figure 6. Genes overexpressed in LCCTam cells contain multiple AP1 response elements in their promoter regions. *A,* genes overexpressed by ≥2-fold in LCCTam versus SUM44 cells in the microarrays were screened for the presence of AP1 response elements in their proximal promoter regions using TRANSFAC Professional 11.1. *a,* the number of consensus sites found within 5,000 bp of the transcriptional start site. *B,* representative Western blot showing HMGCS2 and FASN expression, and the GAPDH loading control, in SUM44 and LCCTam cells.

hormone receptor coactivators and corepressors expressed by the tumor, differential metabolism of antiestrogens, and changes in the expression of additional genes that control cell proliferation and/or apoptosis (13). One or more of these mechanisms is likely contributing to the TAM-resistance phenotype of LCCTam cells. Relative to SUM44, the resistant LCCTam cells express 3-fold less ERα. However, SUM44 cells express high basal levels of ERα.[6] Consequently, the reduced level of ERα expression in LCCTam is comparable with that observed in MCF-7 breast cancer cells (~73% of basal MCF-7 ERα levels by qPCR; data not shown). Because ERα levels in MCF-7 cells are clearly sufficient to confer antiestrogen sensitivity, it is unlikely that ERα down-regulation in LCCTam is the major determinant of resistance in this model.

Our siRNA knockdown and cDNA overexpression studies are the first to show that ERRγ is an essential regulator of TAM responsiveness in lobular breast cancer cells. Until now, the role of ERRs (and specifically ERRγ) in breast cancer therapeutic response has not been well-understood. In 2002, Ariazi and colleagues (36) published a study of ERR family expression in 38 breast tumors compared with normal mammary epithelial cells (MEC). ERRγ mRNA expression is nearly 4-fold higher in breast tumors than in MECs and is positively associated with ER and PR expression. These authors conclude that the correlation of ERRγ with ER and PR is indicative of a better prognosis (36). Although this is certainly plausible, the presence of ER and PR do not always indicate hormone sensitivity in breast cancer. As discussed above, TAM therapy is ineffective in ~30% of patients with ER+/PR+ breast tumors, and the majority of initial responders who acquire resistance to TAM and other endocrine agents do so without losing detectable ER expression (10). 4HT-bound ERRγ is also known to activate transcription at AP1 sites, and elevated AP1 activity has previously been linked to TAM resistance *in vitro* (51, 52) and *in vivo* (53, 54). This is consistent with our findings that AP1 activity is robustly increased in the resistant LCCTam cells in the presence of 4HT, and that AP1 inhibition reverses the TAM-resistant phenotype of LCCTam cells while increasing the sensitivity of SUM44 cells to growth inhibition by this antiestrogen. To our knowledge, this is the first functional consequence of ERRγ-driven AP1 transcriptional activity that has been reported.

No endogenous ERRγ/AP1 target genes have yet been identified. The genes in Fig. 6*A* are strong candidates as ERRγ/AP1 targets in breast cancer. We confirmed the differential regulation of the endogenous HMGCS2 and FASN, and we propose that HMGCS2 and FASN are putative downstream targets of ERRγ in the resistant LCCTam cell line. Further assessment of their direct regulation by ERRγ/AP1 is in progress. HMGCS2 is a nuclear-encoded mitochondrial matrix gene that can regulate ketogenesis and cholesterol synthesis (45, 46) and FASN is the final enzyme of the fatty acid biosynthetic pathway (47). Components of all three processes have been implicated in the etiology or progression of breast cancer, and FASN activity can affect hormonal sensitivity in breast and endometrial cancer cells (48–50). Moreover, ERRγ has been shown to control the switch from fetal use of carbohydrates to lipid-dependent oxidative metabolism in the adult mouse heart by regulating a series of genes that drive fatty acid oxidation, oxidative phosphorylation, and mitochondrial electron transport (55). That ERRγ might also affect these metabolic processes in the context of breast cancer and TAM resistance is intriguing and will be the focus of future studies. Notably, this possibility is supported by a very recent publication by Montero and colleagues (56), which reports that increased mitochondrial cholesterol content promotes resistance to doxorubicin in hepatocellular carcinoma. Other genes in Fig. 6*A* also are of interest. High LRRC15 expression has been previously linked to invasive and aggressive behavior in breast and prostate cancer (57, 58), and MSN is a marker of basal-like breast cancers (59); our future studies will also pursue the role(s) of these genes in endocrine-resistant breast cancer and their regulation by ERRγ/AP1.

[6] D.A. Zajchowski and S.P. Ethier, unpublished data.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2598947/8908.pdf by guest on 14 December 2024

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/11/2008; accepted 8/20/2008.

Grant support: Ladies Auxiliary to the VFW, the Susan G. Komen Foundation (PDF0503551), and the Department of Defense Breast Cancer Research Program (BC051851; R.B. Riggins), and Public Health Service award CA096483-01A1 from the National Cancer Institute, and BC030280 from the Department of Defense Breast Cancer Research Program (R. Clarke). Technical services were provided by the Flow Cytometry, Macromolecular Analysis, Microscopy & Imaging, and Tissue Culture Shared Resources, which are supported by Public Health Service award 1P30-CA-51008-16 (Cancer Center Support Grant to the Lombardi Comprehensive Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank past and current members of the laboratory for their critical comments and insightful discussions, as well as the staff of the Lombardi Comprehensive Cancer Center Flow Cytometry, Macromolecular Analysis, Microscopy and Imaging, and Tissue Culture Shared Resources for technical assistance.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Li CI, Anderson BO, Daling JR, Moe RE. Trends in incidence rates of invasive lobular and ductal breast carcinoma. JAMA 2003;289:1421–4.
3. Biglia N, Mariani L, Sgro L, Mininanni P, Moggio G, Sismondi P. Increased incidence of lobular breast cancer in women treated with hormone replacement therapy: implications for diagnosis, surgical and medical treatment. Endocr Relat Cancer 2007;14:549–67.
4. Jimeno A, Amador ML, Gonzalez-Cortijo L, et al. Initially metastatic breast carcinoma has a distinct disease pattern but an equivalent outcome compared with recurrent metastatic breast carcinoma. Cancer 2004;100:1833–42.
5. Tubiana-Hulin M, Stevens D, Lasry S, et al. Response to neoadjuvant chemotherapy in lobular and ductal breast carcinomas: a retrospective study on 860 patients from one institution. Ann Oncol 2006;17:1228–33.
6. Wenzel C, Bartsch R, Hussian D, et al. Invasive ductal carcinoma and invasive lobular carcinoma of breast differ in response following neoadjuvant therapy with epidoxorubicin and docetaxel + G-CSF. Breast Cancer Res Treat 2007;104:109–14.
7. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer. Cochrane Database Syst Rev 2001;CD000486.
8. Smith DB, Howell A, Wagstaff J. Infiltrating lobular carcinoma of the breast: response to endocrine therapy and survival. Eur J Cancer Clin Oncol 1987;23:979–82.
9. Rakha EA, El-Sayed ME, Powe DG, et al. Invasive lobular carcinoma of the breast: response to hormonal therapy and outcomes. Eur J Cancer 2008;44:73–83.
10. Clarke R, Skaar TC, Bouker KB, et al. Molecular and pharmacological aspects of antiestrogen resistance. J Steroid Biochem Mol Biol 2001;76:71–84.
11. Clarke R, Liu MC, Bouker KB, et al. Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene 2003;22:7316–39.
12. Riggins R, Bouton AH, Liu MC, Clarke R. Antiestrogens, aromatase inhibitors, and apoptosis in breast cancer. Vitam Horm 2005;71:201–37.
13. Riggins RB, Schrecengost RS, Guerrero MS, Bouton AH. Pathways to Tamoxifen resistance. Cancer Lett 2007;256:1–24.
14. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249–89.
15. Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL. Differential isolation of normal liminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res 1993;53:627–35.
16. van de WM, Barker N, Harkes IC, et al. Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. Cancer Res 2001;61:278–84.
17. Cowley SM, Parker MG. A comparison of transcriptional activation by ER α and ER β. J Steroid Biochem Mol Biol 1999;69:165–75.
18. Vanacker JM, Bonnelye E, Chopin-Delannoy S, Delmarre C, Cavailles V, Laudet V. Transcriptional activities of the orphan nuclear receptor ERR α (estrogen receptor-related receptor-α). Mol Endocrinol 1999;13:764–73.
19. Hong H, Yang L, Stallcup MR. Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 1999;274:22618–26.
20. Riggins R, Zwart A, Nehra N, Agarwal P, Clarke R. The NFkB inhibitor parthenolide restores ICI 182,780 (Faslodex; Fulvestrant)-induced apoptosis in antiestrogen resistant breast cancer cells. Mol Cancer Ther 2005;4:33–41.
21. Riggins RB, Thomas KS, Ta HQ, et al. Physical and functional interactions between Cas and c-Src induce tamoxifen resistance of breast cancer cells through pathways involving epidermal growth factor receptor and signal transducer and activator of transcription 5b. Cancer Res 2006;66:7007–15.
22. Vindelov LL, Christensen IJ, Nissen NI. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 1983;3:323–7.
23. Brünner N, Frandsen TL, Holst-Hansen C, et al. MCF7/LCC2: A 4-hydroxytamoxifen resistant human breast cancer variant which retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 1993;53:3229–32.
24. Figueiredo BC, Stratakis CA, Sandrini R, et al. Comparative genomic hybridization analysis of adrenocortical tumors of childhood. J Clin Endocrinol Metab 1999;84:1116–21.
25. Gomez BP, Riggins RB, Shajahan AN, et al. Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. FASEB J 2007;21:4013–27.
26. Ellis M, Davis N, Coop A, et al. Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses. Clin Cancer Res 2002;8:1155–66.
27. Liu A, Zhang Y, Gehan E, Clarke R. Block principal component analysis with application to gene microarray data classification. Stat Med 2002;21:3465–74.
28. Bouker KB, Skaar TC, Fernandez DR, et al. Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (Faslodex, Fulvestrant). Cancer Res 2004;64:4030–9.
29. Harris JR, Lippman ME, Morrow M, Osborne CK, editors. Diseases of the Breast. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2000.
30. Forozan F, Veldman R, Ammerman CA, et al. Molecular cytogenetic analysis of 11 new breast cancer cell lines. Br J Cancer 1999;81:1328–34.
31. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 1993;53:283–90.
32. Horard B, Vanacker JM. Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand. J Mol Endocrinol 2003;31:349–57.
33. Giguère V. To ERR in the estrogen pathway. Trends Endocrinol Metab 2002;13:220–5.
34. Ariazi EA, Jordan VC. Estrogen-related receptors as emerging targets in cancer and metabolic disorders. Curr Top Med Chem 2006;6:203–15.
35. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE. Estrogen-related receptor {α}1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. Mol Cancer Res 2007;5:71–85.
36. Ariazi EA, Clark GM, Mertz JE. Estrogen-related receptor α and estrogen-related receptor γ associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res 2002;62:6510–8.
37. Reiner GC, Katzenellenbogen BS. Characterization of estrogen and progesterone receptors and the dissociated regulation of growth and progesterone receptor stimulation by estrogen in MDA-MB-134 human breast cancer cells. Cancer Res 1986;46:1124–31.
38. Reis-Filho JS, Simpson PT, Turner NC, et al. FGFR1 emerges as a potential therapeutic target for lobular breast carcinomas. Clin Cancer Res 2006;12:6652–62.
39. Greschik H, Wurtz JM, Sanglier S, et al. Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. Mol Cell 2002;9:303–13.
40. Greschik H, Flaig R, Renaud JP, Moras D. Structural basis for the deactivation of the estrogen-related receptor γ by diethylstilbestrol or 4-hydroxytamoxifen and determinants of selectivity. J Biol Chem 2004;279:33639–46.
41. Holzberg D, Knight CG, Ttrich-Breiholz O, et al. Disruption of the c-JUN-JNK complex by a cell-permeable peptide containing the c-JUN δ domain induces apoptosis and affects a distinct set of interleukin-1-induced inflammatory genes. J Biol Chem 2003;278:40213–23.
42. Huppunen J, Wohlfahrt G, Aarnisalo P. Requirements for transcriptional regulation by the orphan nuclear receptor ERRγ. Mol Cell Endocrinol 2004;219:151–60.
43. Matys V, Kel-Margoulis OV, Fricke E, et al. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res 2006;34:D108–10.
44. Kel AE, Gossling E, Reuter I, Cheremushkin E, Kel-Margoulis OV, Wingender E. MATCH: a tool for searching transcription factor binding sites in DNA sequences. Nucleic Acids Res 2003;31:3576–9.
45. Mascaro C, Nadal A, Hegardt FG, Marrero PF, Haro D. Contribution of steroidogenic factor 1 to the regulation of cholesterol synthesis1. Biochem J 2000;350 Pt 3:785–90.
46. Ortiz JA, Gil-Gomez G, Casaroli-Marano RP, Vilaro S, Hegardt FG, Haro D. Transfection of the ketogenic mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase cDNA into Mev-1 cells corrects their auxotrophy for mevalonate. J Biol Chem 1994;269:28523–6.
47. Wakil SJ, Stoops JK, Joshi VC. Fatty acid synthesis and its regulation. Annu Rev Biochem 1983;52:537–79.
48. Kallinowskil F, Davel S, Vaupell P, Baessler KH, Wagner K. Glucose, lactate, and ketone body utilization by human mammary carcinomas *in vivo*. Adv Exp Med Biol 1985;191:763–73.
49. Duncan RE, El-Sohemy A, Archer MC. Dietary factors and the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase: implications for breast cancer and development. Mol Nutr Food Res 2005;49:93–100.
50. Lupu R, Menendez JA. Targeting fatty acid synthase in breast and endometrial cancer: an alternative to

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2589447/8908.pdf by guest on 14 December 2024

selective estrogen receptor modulators? Endocrinology 2006;147:4056–66.
51. Dumont JA, Bitonti AJ, Wallace CD, Baumann RJ, Cashman EA, Cross-Doersen DE. Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity. Cell Growth Differ 1996;7:351–9.
52. Zhou Y, Yau C, Gray JW, et al. Enhanced NF κ B and AP-1 transcriptional activity associated with antiestrogen resistant breast cancer. BMC Cancer 2007;7:59.
53. Johnston SR, Lu B, Scott GK, et al. Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clin Cancer Res 1999;5:251–6.
54. Schiff R, Reddy P, Ahotupa M, et al. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors *in vivo*. J Natl Cancer Inst 2000;92:1926–34.
55. Alaynick WA, Kondo RP, Xie W, et al. ERRγ directs and maintains the transition to oxidative metabolism in the postnatal heart. Cell Metab 2007;6:13–24.
56. Montero J, Morales A, Llacuna L, et al. Mitochondrial cholesterol contributes to chemotherapy resistance in hepatocellular carcinoma. Cancer Res 2008;68:5246–56.
57. Stanbrough M, Bubley GJ, Ross K, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Res 2006;66:2815–25.
58. Schuetz CS, Bonin M, Clare SE, et al. Progression-specific genes identified by expression profiling of matched ductal carcinomas *in situ* and invasive breast tumors, combining laser capture microdissection and oligonucleotide microarray analysis. Cancer Res 2006;66:5278–86.
59. Charafe-Jauffret E, Monville F, Bertucci F, et al. Moesin expression is a marker of basal breast carcinomas. Int J Cancer 2007;121:1779–85.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/21/8908/2598947/8908.pdf by guest on 14 December 2024