Adaptation to AI Therapy in Breast Cancer Can Induce Dynamic Alterations in ER Activity Resulting in Estrogen-Independent Metastatic Tumors

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Abstract

Purpose: Acquired resistance to aromatase inhibitor (AI) therapy is a major clinical problem in the treatment of breast cancer. The detailed mechanisms of how tumor cells develop this resistance remain unclear. Here, the adapted function of estrogen receptor (ER) to an estrogen-depleted environment following AI treatment is reported.

Experimental Design: Global ER chromatin immuno-precipitation (ChIP)-seq analysis of AI-resistant cells identified steroid-independent ER target genes. Matched patient tumor samples, collected before and after AI treatment, were used to assess ER activity.

Results: Maintained ER activity was observed in patient tumors following neoadjuvant AI therapy. Genome-wide ER-DNA-binding analysis in AI-resistant cell lines identified a subset of classic ligand-dependent ER target genes that develop steroid independence. The Kaplan–Meier analysis revealed a significant association between tumors, which fail to decrease this steroid-independent ER target gene set in response to neoadjuvant AI therapy, and poor disease-free survival and overall survival (n = 72 matched patient tumor samples, P = 0.00339 and 0.00155, respectively). The adaptive ER response to AI treatment was highlighted by the ER/AIB1 target gene, early growth response 3 (EGR3). Elevated levels of EGR3 were detected in endocrine-resistant local disease recurrent patient tumors in comparison with matched primary tissue. However, evidence from distant metastatic tumors demonstrates that the ER signaling network may undergo further adaptations with disease progression as estrogen-independent ER target gene expression is routinely lost in established metastatic tumors.

Conclusions: Overall, these data provide evidence of a dynamic ER response to endocrine treatment that may provide vital clues for overcoming the clinical issue of therapy resistance.

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Introduction

Aromatase inhibitor (AI) therapy is now the first-line treatment for estrogen receptor (ER)–positive postmenopausal breast cancer patients. With prolonged exposure, a significant number of patients develop AI resistance (1). Several molecular mechanisms of acquired resistance have been described, and these include enhanced signaling through growth factor pathways as well as ligand-independent ER function (2, 3).

ER/growth factor cross-talk and in particular amplification of the growth factor receptor HER2 has been associated with resistance to endocrine therapy in both preclinical and clinical studies (4–6). Recently, clinical and proteomic work has implicated PI3K as a central node in AI-resistant second messenger signaling networks (2, 7–9). Activation of PI3K has been shown to induce ER phosphorylation and promote estrogen-independent ER transcriptional activity (10, 11). However, molecular studies in models of AI resistance suggest that PI3K may not be directly responsible for ligand-independent ER signaling following prolonged steroid deprivation (12).

Clinical trial data, which describe the efficacy of the ER disrupter fulvestrant as a second-line therapy in patients who progressed on an AI treatment, suggest that a functional ER remains after development of AI resistance (13, 14). Though molecular studies have suggested that mutations in the ligand-binding domain may be important in determining response to endocrine therapy (15, 16), ER mutations in primary patient breast cancers were found to be uncommon (17). More recently however, reports are emerging of ligand-binding domain ER mutations in endocrine-related metastasis which can confer estrogen-independent activity of the receptor and contribute to...
Translational Relevance

Aromatase inhibitors (AI) are the treatment of choice for postmenopausal women with ER-positive breast tumors. Although effective, resistance to AIs, through various mechanisms, is an ongoing clinical problem. The current study is the first to profile ER activity in the estrogen-depleted AI-resistant setting. Rather than being silenced in response to AI therapy, the ER can undergo dynamic adaptations to regulate transcription in an estrogen-independent manner. Furthermore, by the time metastatic disease has developed, a small but significant subset of tumors has adapted to survive without ER activity.

From the clinical perspective, this study highlights two important issues: First, the need for more efficient drugs to completely block ER signaling and, second, the need to re-assess where possible the ER status during disease progression, particularly at the metastatic stage, in order to select the most appropriate treatment.

the development of resistance (18,19). Furthermore, global analysis of ER-binding events has revealed altered DNA-binding dynamics and a gain of new target genes in tumors from patients with a poor response to treatment (20).

Clinical and sequencing data describing somatic mutations and altered ER activity do not fully explain continued metastatic disease progression. There is now evidence that a functioning ER may not be required for sustained tumor growth at established metastatic sites in all patients. Receptor switching between the primary and secondary tumors in ER-positive breast cancer has been reported, with up to 20% of tumors losing ER and/or progesterone receptor (PR) with a reciprocal gain of HER2 (21–23). These clinical data raise the possibility that once metastatic tumors have formed, a significant number of these become steroid receptor independent.

In this study, we investigate the functional and clinical consequences of altered ER action in response to prolonged estrogen deprivation. In a model of resistance to the AI letrozole, we report a global loss of ER binding and identify a subset of classic ligand-dependent ER target genes which become estrogen independent. Initial adaptation to estrogen deprivation, manifested by ligand-independent ER activation, is found to contribute to the development of local endocrine resistance in vitro. At a clinical level, failure to regulate the steroid-independent ER gene set following neoadjuvant treatment associates with poor long-term response to AI therapy in breast cancer patients. Further, ER adaptation can occur with the establishment of metastatic tumors, where loss of ER function and target gene expression can lead to the development of a fully estrogen-independent endocrine-resistant tumor.

Materials and Methods

Cell lines and treatments

Endocrine-sensitive MCF7 breast cancer cells were obtained from the American Type Culture Collection (ATCC). Endocrine-resistant LY2 cells were a kind gift from R. Clarke, Georgetown University, Washington DC (24). Cells were grown as previously described (25). AI-sensitive cells (MCF7-Aro) were developed by stable transfection of the aromatase gene (CYP19; Invitrogen). MCF7-Aro cells were cultured in MEM supplemented with 10% FCS, 1% l-Glutamine, 1% pen/strep, and 200 mg/mL Gentamicin (G418; Gibco Invitrogen). Al-resistant (LertR and AnaR) cells were generated by long-term culture of MCF7-Aro cells with an AI (letrozole, 10−6 mol/L; Novartis or anastrozole, 10−6 mol/L, AstraZeneca) and aromastenedione (25 × 10−5 mol/L; Sigma Aldrich) in MEM supplemented with 10% charcoal–dextran-stripped FCS, 1% l-Glutamine, 1% pen/strep, and 200 mg/mL Gentamicin. Cells were maintained in steroid-depleted medium (phenol red–free MEM with 10% charcoal–dextran-stripped FCS, 1% Glutamine, and 1% pen/strep) for 72 hours prior to treatment with hormones (estriadiol, 10−7 mol/L; Sigma Aldrich or aromastenedione, 10−7 mol/L; Sigma Aldrich) or letrozole (10−8 mol/L; Novartis). All cells were maintained at 37°C, 5% CO2 in a humidified incubator. All cell lines were authenticated according to the ATCC guidelines (Supplementary Table S1).

Transfections

siRNA directed against AIB1 (Ambion, AM16706 and Dharmacon, L-003759-00-0005), ERα (Ambion, 4392421 and Dharmacon, LQ-003401-00-0002), and EGR3 (Qiagen, GS1960) were used to knock down gene expression. Multiple siRNAs from the EGR3 and ER SMARTpools were compared, and the most effective was selected for further studies (EGR3 siRNA-6 and ER siRNA-4; Supplementary Figs. S1 and S2). Transfections were carried out using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions.

Cell growth and cell motility assays

The Cellomics Cell Motility Kit (Thermo Scientific; K0800011) was used to assess individual cell movement after 24 hours as per the manufacturer’s instructions using cells seeded at 1 × 104 cells/mL. Mean track areas (minimum of 90 cell tracks per condition) were analyzed with Olympus cell imaging software and compared with a Student t test.

For growth assays, following steroid depletion, cells were transfected with siRNA of interest, and then 24 hours later seeded out into 12-well plates at 2 × 104 cells/mL. The cells were counted manually using a hemocytometer at three different time points. Cell numbers were compared by the Student t test.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (26). Antibodies used were anti-ER (sc-543) from Santa Cruz Biotechnologies and AIB1 (sc-25742). LetR cells were treated with vehicle or aromastenedione, and MCF7 cells were treated with vehicle or estrogen for 45 minutes, cross-linked with 1% formaldehyde (F15587; Sigma Aldrich) for 10 minutes, and scraped into PBS with protease inhibitors (Complete Mini; Roche). Immunoprecipitation was performed using an antibody attached to Dynal beads (Dynabeads M-280 Sheep Anti-Rabbit IgG; Life Technologies). The proteins were then removed from the DNA by reverse crosslinking overnight, and the DNA was extracted using phenol-chloroform-isamyl alcohol (P2069; Sigma Aldrich). Real-time PCR was carried out in duplicate by SYBR Green PCR (Qiagen) using a Lightcycler (Roche), and primers are listed in Supplementary Table S2.
ChIP-seq
Cells were treated and harvested for ChIP-seq as described previously (26). Immunoprecipitation was carried out using an anti-ER antibody (sc-543) attached to Dynabeads (Dynabeads Protein A 10001D; Life Technologies). ChIP DNA was amplified as described (26), and sequenced using the Illumina Genome Analyzer-II system. Single end 36-bp ChIP-seq data were generated by the Illumina analysis pipeline version 1.6.1. ChIP-seq data from this study have been deposited in the NCBI Gene Expression Omnibus (GEO;http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE54592. LY2 and MCF7 ER ChIP-seq data have previously been reported and can be found in ArrayExpress (www.ebi.ac.uk/arrayexpress/) under accessions E-MTAB-1865 and E-MTAB-223, respectively (27, 28).

Bioinformatics
ChIP-seq reads were aligned to the hg19 genome using Bowtie (v0.12.9; ref. 29). Bowtie parameters were set to allow reads to be aligned to the genome if they mapped to one region only and if they had less than two base pair mismatches. MACS (v2.0) was applied to the ChIP-seq alignments to call peak regions (30). Peaks were called using a P-value cut-off of 1e-03. Bioinformatic analyses are described in Supplementary Methods.

Expression studies
RNA isolation and cDNA synthesis were performed as previously described (31). Real-time PCR was carried out either by SYBR Green PCR (Qiagen) using a Lightcycler (Roche) or by TaqMan probe technology (Applied Biosystems), on the ABI PRISM 7500 platform. The comparative Ct (ΔΔCt) method was applied to analyze relative gene expression levels. Primer and probe details are outlined in Supplementary Table S2.

Protein blotting
Protein from breast cancer cells was lysed, electrophoresed, and immunoblotted with the following antibodies: ER (mouse, NCL-ER-6F11; Leica Biosystems), AIB1 (rabbit, sc-25742; Santa Cruz Biotechnology), EGR3 (rabbit, sc-191; Santa Cruz Biotechnology), MREG (mouse, sc-374216; Santa Cruz Biotechnology), FOXA1 (ab23738; Abcam), and β-ACTIN (ab6276; Abcam).

Patient information and construction of tissue microarray
Patient breast tumor samples for the tissue microarray (TMA) were collected and data recorded as previously described (32). Data included pathologic characteristics (tumor stage, grade, lymph node status, ER status, recurrence) and treatment with radiotherapy, chemotherapy, tamoxifen, or AIs. Detailed follow-up data (median, 51 months) were collected on the patients to monitor recurrences. TMAs were constructed as described previously (25).

Immunohistochemistry
Immunohistochemistry (IHC) was carried out using antibodies against EGR3 (1:500, rabbit, sc-191; Santa Cruz Biotechnology), pSer118 ER (1:500, mouse, mAB2511; Cell Signaling Technology), AIB1 (3:200, rabbit, sc-25742; Santa Cruz Biotechnology), ki67 (1:200, mouse, M7240; DAKO), AR (1:50, mouse, 318-CE; Novacstra), and ER (1:50, rabbit, 790-4324; Ventana Medical Systems) with the Dako EnVision Kit (31). Antigen retrieval was done with either EDTA or sodium citrate. Primary antibody was used at the recommended dilution and incubated for 1 hour at room temperature or overnight at 4°C in the case of pSer118. Staining was assessed using a modified Allred scoring system as previously described (33). Independent observers, without knowledge of prognostic factors, scored slides. Staining was also assessed by the Aperio IHC Nuclear Image Analysis algorithm (Leica Biosystems). Paired t tests were used to compare differences in expression between matched tumor samples.

Statistical analysis
Associations of EGR3 with clinicopathologic variables were examined using the Fisher exact test. Statistical analyses were conducted using STATA 10 data analysis software (Stata Corp. LP) and GraphPad Prism 6 (GraphPad software Inc.), and values of P < 0.05 were considered significant. Changes in gene expression on AI treatment and association with outcome were determined from the Edinburgh dataset of 72 patients treated with letrozole, performed on Affymetrix and Illumina microarrays with batch correction (34, 35). The Kaplan–Meier analysis was performed using the R Survival package. The Cluster and TreeView programs were used to generate heat maps.

Results
AI therapy can induce ligand-independent ER activity
Steroid receptor expression was maintained in breast cancer tissue from patients following neoadjuvant treatment in comparison with matched pretreatment biopsies (Supplementary Table S3; Supplementary Figs. S3 and S4). However, enhanced pSer118 ER was detected in posttreatment tissue indicating induction of ligand-independent ER activity (n = 8 breast cancer patients; P = 0.002; Fig. 1A; Supplementary Table S3 and Supplementary Fig. S3). Consistent with this observation, in cell models of resistance to the AI therapies (letrozole-resistant LetR cells and anastrozole-resistant AnaR cells), ER was found to be required for cell growth even in the absence of steroids. By contrast, knockdown of ER in a steroid-free environment in endocrine-sensitive cells (MCF7 and MCF7-Aro) had no impact on tumor cell number (Fig. 1B; Supplementary Figs. S1 and S2). The AI-resistant cell lines express higher levels of pSer118 ER than the sensitive cells (31). Taken together, these data indicate that reduced estrogen as a result of AI treatment can lead to enhanced ligand-independent ER activity.

The ER develops steroid-independent functional activity in AI-resistant cells
To examine the consequence of ER signaling following AI treatment, we investigated global ER signaling in the AI-resistant setting. ER ChIP-seq was performed in AI-resistant breast cancer cells (LetR) in the presence and absence of androstenedione. Analysis of genome-wide ER–DNA-binding events revealed that, similar to tamoxifen resistance, ER-binding events were observed to be less frequent in AI-resistant cells in comparison with those published for endocrine-sensitive MCF7 cells (27). Furthermore, steroid treatment did not result in any enhancement in ER–DNA interactions in the LetR cells (Fig. 2A), supporting a role for ligand-independent ER activation in AI-resistant cells.
Examination of the ER-binding regions identified from LetR cells in other endocrine models (MCF-7 and LY2 cells) confirms reduced ligand dependency as a feature of ER activity in endocrine resistance (Supplementary Fig. S5).

ER-binding peaks in LetR cells were significantly enriched for ERE, FOXA1, and GATA3-binding motifs (Supplementary Table S4). In addition, interactions between ER and FOXA1 were found to be unaltered in LetR cells compared with MCF7 cells indicative of a fully functional core ER–DNA complex (Supplementary Fig. S6). Previous studies from our group have observed that steroid-independent ER activity in AI resistance can occur in a promoter-specific context (31). To investigate this at a global level, the prevalence of transcription factor–binding motifs within the ER-binding peaks was compared between peaks that are unique to the vehicle-treated sample, unique to the androgen-treated sample, or common to both (Fig. 2B and C). Estrogen response elements (ERE) were found to be significantly enriched in the steroid-independent setting (vehicle only and common peaks) compared with the steroid-driven setting (androstenedione only peaks). In contrast, the steroid-driven peaks were enriched for Forkhead motifs, such as FOXA1 (Fig. 2C; Supplementary Table S5).

Steroid-independent target genes identified from ER ChIP-seq data from this study were compared with steroid-sensitive ER target genes from public microarray data sets (estrogen-treated MCF7 cells and testosterone-treated MCF7-Aro cells; refs. 32, 33). The gene set was refined based on common expression in a second independent LetR gene set (36). This analysis defined a set of ER target genes that have become...
Steroid dysregulated in AI resistance (Fig. 2D). The set of steroid-dysregulated genes are GREB1, TFF1, EGR3, MREG, TPD52L1, SIAH2, and MYB, which from hereon are referred to as the ER target gene signature (Fig. 2D and Supplementary Fig. S7). Of interest, ChIP-seq in LY2 cells confirmed that these genes are also steroid-independent ER targets in the tamoxifen-resistant setting (Supplementary Fig. S8).

The ER regulates target genes EGR3 and MREG independently of steroids in AI-resistant cells

The ability of AI-resistant cells to regulate ER signaling in a ligand-independent manner was investigated with in-depth studies on selected targets, EGR3 and MREG. EGR3 is a transcription factor and an early growth response gene, whereas MREG or melanoregulin is a membrane protein named for its involvement in melanocyte differentiation. Both of these genes contain an ERE within the ER-binding peak (Fig. 3A). ChIP confirmed estrogen-dependent ER recruitment in MCF7 cells and strong recruitment, independent of androgen treatment, in LetR cells, to the DNA of these gene targets (Fig. 3B). Furthermore, ER knockdown using siRNA resulted in reduced mRNA and protein expression of both EGR3 and MREG (Fig. 3C). Of interest, the binding peak located proximal to the transcriptional start site of EGR3 is located within another gene, PEBP4. Knockdown studies confirmed that ER binding regulates transcriptional activity of EGR3 but not PEBP4 (Supplementary Fig. S9). Treatment of MCF7 cells with estrogen resulted in marked upregulation of EGR3 and MREG at both
Figure 3.
ER regulates EGR3 and MREG expression independently of steroids in LetR cells. A, images from University of California Santa Cruz genome browser of LetR ER ChIP-seq vehicle sample depicting EGR3 and MREG ER-binding peaks with EREs. B, qPCR of ER ChIP in MCF7 (gray bars) and LetR (black bars) cells revealed recruitment of ER to EGR3 and MREG genes. IgG was used as an internal control. Treatments were vehicle (V), estrogen (E), and androstenedione (A). ER ChIP shows ER binding is steroid-independent in LetR cells compared with MCF7 cells. C, EGR3 and MREG expression is regulated by ER. LetR cells were transiently transfected with ER siRNA, and mRNA was analyzed by qPCR. Protein expression was also verified by Western blot. β-Actin is used as loading control. D, high expression of EGR3 and MREG becomes steroid-independent in LetR cells. MCF7 and LetR cells were treated with either E or A, and mRNA and protein were analyzed by qPCR or Western blot, respectively. Results are mean ± SEM, n = 3. *, P < 0.05; **, P < 0.01; n.s., not significant.

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mRNA and protein levels (Fig. 3D). By contrast, vehicle-treated LetR cells had high basal expression of EGR3 and MREG, and treatment with androgen did not induce significant further regulation (Fig. 3D).

AIB1 binds to the DNA at the same location as ER to regulate expression of both EGR3 and MREG

The steroid receptor coactivator, AIB1, is well known to cooperate with ER in regulating expression of target genes. The globally identified ER target genes in LetR cells were analyzed for the presence of AIB1-binding sites using publicly available data from AIB1 ChIP-seq in MCF7 cells (37). Seventy-five percent of identified ER-binding regions in LetR cells overlapped with AIB1-binding peaks. The ER-binding peaks, which had become independent of steroids, were enriched for AIB1-binding sites compared with those peaks which remained steroid regulated (Fig. 4A), suggesting a potential role for AIB1 in ligand-independent ER activity. For EGR3 and MREG, ChIP confirmed recruitment of AIB1 to the ER–DNA loci (Fig. 4B). As predicted, this recruitment was regulated by estrogen in MCF7 cells, but was steroid independent in LetR cells (Fig. 4B). Silencing of AIB1 resulted in reduced transcript and protein expression of both EGR3 and MREG, confirming the transcriptional function of AIB1 binding to the DNA (Fig. 4C; Supplementary Fig. S2). Thus, AIB1 appears to retain its steroid receptor coactivator role in AI-resistant cells and functions to coregulate expression of ER target genes in the absence of steroid.

Previous studies reported a significant association between AIB1 expression in breast cancer tissue and reduced disease-free survival in AI-treated patients (31). In this study, we observed a strong correlation between AIB1, ER, and EGR3 (EGR3:ER, n = 375, P < 0.0001; EGR3:AIB1, n = 212, P < 0.0001; AIB1-ER, n = 212, P < 0.0001) in the primary tumors of breast cancer patients (Fig. 4D; Supplementary Table S6).

Patient treatment with AIs induces an early response in expression of ER target gene signature

The current use of AIs as a neoadjuvant therapy allows real-time sampling and monitoring of tumor responses to endocrine therapy (Fig. 5A). Expression of our ER target gene signature was examined in a cohort of matched tumor biopsies from 50 letrozole-treated patients before, 2 weeks during and 3 months following neoadjuvant AI treatment (published dataset GSE20181; Fig. 5B; ref. 38). Treatment with AI therapy reduced expression of the dysregulated gene set, with the exception of EGR3 (Fig. 5B). For the genes TFF1, GREB1, MREG, SIAH2, and MYB, the decrease in expression was statistically significant (n = 50, P value < 0.0001).

For the gene EGR3 after an initial loss at 2-week treatment (n = 50, P = 0.0016), a significant increase in EGR3 transcript was seen following 3-month neoadjuvant therapy (P = 0.0001; Fig. 5B). This observation was confirmed at the protein level by IHC of tumor tissue from an independent cohort of AI-treated patients (n = 9, P = 0.0002; Fig. 5C; Supplementary Table S3). The response to AI therapy was modeled in vitro where enhanced EGR3 expression observed following acute 4-hour androstenedione treatment was inhibited by letrozole, whereas sustained 3-month cotreatment with the steroid and the AI inhibitor failed to reduce EGR3 expression (Fig. 5D).

At a functional level, in vitro studies in models of AI resistance confirmed a role for EGR3 in proliferation and migration of endocrine-resistant cells (Supplementary Fig. S10). In the patient setting, the clinical significance of the gene response to neoadjuvant AI therapy was examined in an extended cohort of 72 paired tumor biopsies with 10-year follow-up data (published datasets GSE20181, GSE53574, and GSE59515; ref. 34). The inability of AI therapy to inhibit the expression of the ER-dysregulated gene set was significantly associated with poor response to therapy. Breast cancer patients whose tumors displayed a combined loss of the ER target gene signature following 3-month neoadjuvant treatment had a longer disease-free survival and overall survival in comparison with patients whose tumors failed to regulate the expression levels of the gene signature in response to AI treatment (n = 72; P = 0.00339 and P = 0.00155, respectively; Fig. 5E). The target gene signature associated more strongly with survival than ER gene expression alone, although both together had improved predictive value (n = 72, disease-free survival, P = 0.00076; overall survival, P = 0.00025; Supplementary Fig. S11).

ER activity is not required to maintain growth of endocrine-resistant distant metastatic tumors

In endocrine resistance, ligand-independent ER activity is induced. This is exemplified by enhanced pSer118 ER and EGR3 observed in endocrine-resistant cell lines (LetR and AnaR; ref. 31) and in endocrine-resistant local disease recurrence patient tissue (Fig. 6A; Supplementary Table S3). With the development of metastatic disease, however, there is a growing body of clinical evidence to suggest that a significant minority of tumors lose ER activity in comparison with the primary breast cancer (Fig. 6B). In this study, in matched primary and metastatic tumors from patients who have developed a recurrence on adjuvant treatment, almost half of the patients displayed loss of ER and/or PR expression in the metastatic tissue (n = 10; Fig. 6C). Of note, loss of androgen receptor expression was also detected in some but not all patients following endocrine treatment (Supplementary Fig. S4). Consistent with the observation of loss of steroid receptors, loss of EGR3 protein expression was also found in metastatic tumors relative to the matched primary tissue following endocrine treatment (n = 10, P = 0.002; Supplementary Table S3; Fig. 6D). Furthermore, global analysis of transcript changes (RNAseq) between primary and metastatic ER-positive AI-treated patients revealed a loss of the ER target gene signature in both brain and liver metastatic tissues (Fig. 6E). Taken together, these data provide evidence that a functional ER may not be required in selected patients for maintenance of metastatic tumors secondary to endocrine treatment.

Discussion

Adaptation of ER-positive breast cancer tumors to a depleted estrogen environment has been studied extensively. Work presented here and studies from other groups have demonstrated continued ER functioning in the presence of AI treatment. Altered phosphorylation status, DNA mutations, and functional alterations in the ER, including ligand-independent activation of the receptor, have been observed (18–20). However, global analyses of ER–DNA-binding events in AI-resistant breast cancer have not been reported to date. Here, we
Figure 4.
AIB1 coactivates ER to regulate expression of EGR3 and MREG in AI resistance. A, ER-binding peaks from LetR cells were compared with AIB1-binding peaks from MCF7 cells (45). Pie charts illustrate the overlap between ER and AIB1 peaks in treated and untreated LetR cells. B, qPCR of AIB1 ChIP in MCF7 (gray) and LetR (black) cells revealed recruitment of AIB1 to EGR3 and MREG promoters. IgG was used as an internal control. Treatments were vehicle (V), estrogen (E), and androstenedione (A). AIB1 ChIP shows AIB1 binding is steroid-independent in LetR cells compared with MCF7 cells. C, EGR3 and MREG expression is regulated by AIB1. LetR cells were transiently transfected with AIB1 siRNA, and mRNA was analyzed by qPCR. Protein expression was verified by Western blot. β-Actin is used as loading control. Results are mean ± SEM, n = 3. *, P < 0.05; n.s., not significant. D, patient TMA was stained for ER, AIB1, and EGR3. Images show representative positive and negative stainings. Significant associations were detected between all three proteins using the Fisher exact test.
Figure 5. Maintained expression of steroid-dysregulated genes following AI treatment associates with poor outcomes. A, tumors were sampled before, during, and after neoadjuvant AI therapy (34, 38) as illustrated. B, box plots display expression changes of steroid-dysregulated genes in neoadjuvant AI-treated patients (n = 50 patients). All genes display a decrease in expression with the exception of EGR3, which increases at 3 months. Paired t-tests pretreated versus 3 months. C, protein expression confirms a significant increase in EGR3 expression following 3-months AI treatment. Representative IHC images are shown. All images were Allred-scored, and results are displayed on the graph (n = 9 patients, paired t-test, P = 0.0002). D, MCF7-Aro cells treated with androstenedione (A) and anastrozole (AI) were analyzed by qPCR for EGR3 expression. Consistent with patient data, EGR3 expression initially decreased and subsequently increased significantly in response to AI treatment, n = 3, mean ± SEM (*, P < 0.05; **, P < 0.01). E, ranked sum of steroid-dysregulated gene expression changes is associated with poor prognosis. Red = increased and green = decreased at 3 months relative to pretreatment for 72 AI-treated patients (34). White–gray–black bars indicate significance of all possible cut points from P = 1 to 0.001. Kaplan–Meier according to expression of the ER gene signature. Reductions in the ER gene signature following neoadjuvant AI therapy associate with increased disease-free and overall survival in AI-treated breast cancer patients (n = 72; P = 0.00339 and P = 0.00155, respectively).
undertook ER ChIP-seq analysis in breast cancer cells resistant to the AI letrozole. Loss of ER binding was observed in the AI-resistant setting in comparison with the parental-sensitive cells (27). Of interest, similar reductions in ER-binding events have been reported previously for breast cancer cells resistant to tamoxifen (27). Data from this study and previous work from our group and others suggest that low levels of global ER binding observed in the AI-resistant cells are unlikely to be due to either loss of ER phosphorylation at serine 118 (31) or reduced ER interactions with the pioneer factor FOXA1 (27). Furthermore, we observed no enhancement of ER binding with steroid treatment. Rather, adaptation to sustained estrogen deprivation resulted in a subset of classic ligand-dependent ER target genes becoming estrogen independent.

The ER-dysregulated gene signature included classic ER targets, GREB1 and TFF1, as well as genes with roles in growth response (EGR3) and differentiation (MREG).

ER may regulate specific gene sets in a promoter-specific manner. Here, analysis of transcription factor–binding motifs revealed EREs to be enriched in the steroid-independent setting, giving rise to ER-dysregulated genes, whereas ER utilized alternative motifs, including forkhead and zinc finger–binding motifs, for steroid-dependent regulation. We have previously reported promoter-specific binding for ER and its coactivator AIB1. Meta-analysis of several gene sets (39–41) demonstrated that ER/AIB1 regulated genes which contain either full or partial EREs contribute to pathways promoting tumor progression, relative to genes that do not contain an ER response element (29). Ligand-independent
ER signaling may therefore preferentially utilize EREs over other ER-binding motifs to promote survival genes in the absence of steroid.

Associations between expression of the ER coactivator AIB1 and reduced disease-free survival in AI-treated patients have previously been reported (31). In this study, analysis of the globally identified ER and AIB1 interactome revealed steroid-independent ER genes to be enriched for AIB1 in comparison with steroid-regulated ER targets. In vitro molecular studies in models of AI resistance confirmed a role for AIB1 in ligand-independent regulation of ER targets EGR3 and MREG, and ex vivo clinical studies demonstrated a strong association between AIB1, EGR3, and ER in breast cancer patients. These data implicate AIB1 in selective ligand-independent ER transcriptional regulation.

This study focused on the adaptive role of ER in response to AI therapy, but it should be noted that several other receptors are likely to contribute to the adaptive response. The androgen receptor for example is expressed both before and after neoadjuvant AI therapy and may respond to the increased androgenic environment (42–44). Furthermore, growth factor receptor cross-talk has also been well documented in endocrine-resistant breast cancer.

Loss of classic ER target gene expression following neoadjuvant AI treatment has been well described (38, 42, 43). We observed loss of the dysregulated ER gene signature at both 2 weeks and 3 months following neoadjuvant AI treatment in the majority of breast cancer patients examined, whereas a minority did not regulate gene expression in response to estrogen ablation. The inability of AI therapy to regulate tumor levels of the dysregulated ER gene set significantly associated with subsequent response to adjuvant treatment in ER-positive breast cancer patients. These observations are consistent with recent reports of a dynamic four-gene signature following short-term AI treatment (2 weeks) as a predictive model for extended AI therapy (34). These observations support the concept that early dynamic ER gene expression alterations in response to treatment are important in determining long-term responses to endocrine therapy. Indeed, the clinical relevance, if any, of ligand-independent ER activity following neoadjuvant AI will become evident as data from current trials, combining AI therapy with ER inhibitors (fulvestrant) for the neoadjuvant treatment of invasive breast cancer, become available.

Enhanced ligand-independent ER activity, as manifest by pSer118 and EGR3 expression, was found in endocrine-resistant breast cancer. This was associated with subsequent response to adjuvant treatment in ER-positive breast cancer patients. These observations are consistent with those previously reported in our cell line models of AI resistance (31). On development of metastatic cancer in patients who failed on adjuvant endocrine treatment, further adaptations in ER signaling can be observed. Consistent with clinical studies from our group and others (21–23), we observed a loss of ER activity in a significant minority of metastatic tumors in comparison with matched primary tissue. Work from Hoefnagel and colleagues investigating receptor conversion at distant metastatic sites found that conversion of the ER target PR was more frequent than alterations in ER expression itself and that overall steroid receptor conversion was more common in the liver and brain, in comparison with other metastatic sites (44, 45). In this study, reductions in protein expression of the ER target EGR3 were found at all metastatic sites examined. Furthermore, loss of the ER gene signature at transcript level was observed in metastatic liver and brain relative to the primary tumor. These data are consistent with the concept that selected metastatic tumors can adapt to become fully independent of ER activity.

In summary, work from this study suggests that estrogen ablation leads to an adaptive ER response. An initial enhanced ligand-independent activity seen following neoadjuvant treatment and local endocrine-resistant disease can give way to loss of ER signaling in the distant metastatic setting. ER adaptability to endocrine therapy has several clinical consequences; first, the need for more efficient drugs to completely block ER signaling during first-line treatment; and second, the need to re-assess where possible the ER status during disease progression, particularly at the metastatic stage, in order to select the most appropriate treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D. Varelija, J. McBryan, L.S. Young
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