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Persistent and non-persistent changes in gene expression result from long-term estrogen exposure of MCF-7 breast cancer cells

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ABSTRACT

Life-long estrogen exposure is recognized as a major risk factor for the development of breast cancer. While the initial events in the regulation of gene expression by estrogen have been described in detail, far less is known of the role of estrogen in the long-term regulation of gene expression. In this study, we investigated the effects of long-term exposure of MCF-7 breast cancer cells to 1 nM 17β-estradiol on gene expression with the goal of distinguishing between gene expression that is continually reliant on estrogen receptor (ER) function as opposed to secondary and persistent effects that are downstream of ER. To assess the direct involvement of ER in the differential gene expression of long-term estrogen exposed (LTEE) cells in comparison with that of control cells, we exposed cultures to the selective estrogen receptor modulator raloxifene (RAL). cDNA microarray analysis showed that exposure to RAL inhibited expression of numerous characterized estrogen-regulated genes, including *PGR*, *GREB1*, and *PDZK1*. Genes that were increased in expression in LTEE cells yet were unaffected by RAL exposure included the aryl hydrocarbon receptor (*AHR*) and numerous other genes that were not previously reported to be regulated by estrogen. Epigenetic regulation was evident for the *AHR* gene; AhR transcript levels remained elevated for several cell passages after the removal of estrogen. Signal transducer and activator of transcription 1 (STAT1); STAT1-regulated genes including *ISG15*, *IFI27*, and *IFIT1*; and MHC class I genes were also up-regulated in LTEE cells and were unaffected by RAL exposure. STAT1 is commonly overexpressed in breast and other cancers, and is associated with increased resistance to radiation and chemotherapy. This is the first study to relate estrogen exposure to increased STAT1 expression in breast cancer cells, an effect that may represent an additional role of estrogen in the pathogenesis of breast cancer.

1. Introduction

A woman's lifelong exposure to endogenous and exogenous estrogens is recognized as a major risk factor for the development of breast cancer [1,2]. The use of hormone replacement therapy (HRT) is associated with increased risk of breast cancer [3], increased incidence of node-positive cancers, and increased risk of breast cancer mortality [4]. Recent declines in breast cancer incidence have been attributed to decreased use of HRT [5]. Most breast cancers, at least at their onset, are estrogen receptor (ER) positive. The ER is an established pharmacologic target in breast cancer, and to this end selective estrogen receptor modulators (SERMs) have been developed [6,7]. The clinically used SERMs, tamoxifen (TAM) and raloxifene (RAL), have shown promise not only in breast cancer treatment, but also in its prevention [8–10]. Recent clinical trials indicate that TAM and RAL have approximately equivalent efficacy in preventing invasive breast cancer in postmenopausal women with heightened risk [11]. While both TAM and RAL have been approved for breast cancer prevention in postmenopausal women [12], RAL has an additional therapeutic use in the treatment and prevention of osteoporosis in postmenopausal women, the role for which it was originally developed.

Primarily through the activation of ERα, estrogens modulate gene expression and cellular signaling pathways in breast cancer cells [13,14]. Estrogens are believed to play multiple roles in the initiation, promotion, and progression of breast cancer. Effects of estrogens in estrogen-responsive breast cancer cells include enhanced Myc-dependent signaling, up-regulation of the expression and activity of cyclin D1, and the induction of more rapid progression of cells through the cell cycle [15–17]. Many studies have focused on the initial molecular events in the regulation of gene expression by estrogen [18–23]. However, the effects of estrogens in breast cancer are not limited to the early-onset genetic and molecular changes; estrogens are believed to affect the

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development of breast cancer over a period of years. The long-term changes in gene expression elicited by estrogens are not as well understood as those involved in the initial gene-regulatory and signaling events.

Given the importance of the cellular and molecular changes that occur as a result of prolonged estrogen exposure to the development and progression of breast cancer, studies in our laboratory have focused on the long-term effects of exposure of ERα-positive breast cancer cells to physiologic levels of 17β-estradiol (E_2). We chose the MCF-7 cell line for this in-depth study because it is the most widely accepted and utilized model system for luminal A ERα-positive breast carcinoma. Due to its extensive use, there is an abundance of data on the short-term effects of estrogen on gene expression in these cells for comparison with the long-term effects that are under investigation.

Long-term estrogen exposed (LTEE) MCF-7 breast cancer cells, which were obtained by continuous exposure to medium containing 1 nM E_2 for at least 3 months, were found to differ markedly from control cells that were propagated in low-E_2 medium, in a number of respects: aryl hydrocarbon receptor (AhR)-regulated cytochrome P450 (CYP) expression, rates of benzo(*a*)pyrene-DNA adduct formation, E_2-dependent tumor growth as xenografts, and global gene expression and its modulation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a xenobiotic that elicits antiestrogenic effects [24]. This previous work revealed that LTEE MCF-7 cells had altered expression of a number of genes that had not previously been reported to be regulated by estrogen, and that LTEE cells exhibited biochemical changes that persisted after the removal of estrogen.

In the current study, we continued our investigation of gene expression in MCF-7 cells after 8–10 months of E_2 exposure. We investigated (i) the effects of RAL exposure on gene expression in LTEE and control cells, (ii) the persistence of certain changes in gene expression, and (iii) specific transcription factor regulation in LTEE and control cells. Our results revealed an up-regulation of *AHR* gene expression that persisted in the absence of added E_2, and a novel effect of extended E_2 exposure on the expression of STAT1 and a network of genes whose expression is regulated by STAT1.

2. Materials and methods

2.1. Cell culture

The MCF-7 human breast cancer cell line was the one used in our previous studies [24,25]. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. DC_5 medium, consisting of Dulbecco's Modified Eagle's Medium, without phenol red, supplemented with 5% (v/v) bovine calf serum (Cosmic calf serum, HyClone), 10 mM non-essential amino acids, 2 mM L-glutamine, 10 μg/L insulin, and 5 μg/mL plasmocin (Amaxa) was used, and cells were passaged in DC_5 medium containing either 1 nM 17β-estradiol (E_2) or dimethylsulfoxide (DMSO) vehicle at 0.01% (v/v); these are referred to as LTEE or control cultures, respectively. Cell cultures were maintained in these media for ≥23 passages; each passage was generally 10 days. Experiments were performed with cultures between passages 23 and 48. Tyrphostin AG1478 was purchased from LC Laboratories. For the study of the effects of RAL on gene expression, control and LTEE cultures were treated with 100 nM RAL (LY156758, Tocris) or DMSO vehicle for 48 h, and RNA and whole-cell lysates were isolated as described in following sections. LTEE (-E_2) refers to cultures derived from LTEE cultures that were further propagated for the specified intervals in DC_5 medium that did not contain added E_2.

2.2. RNA isolation and real-time PCR

RNA was isolated from confluent cultures using the *mir*Vana microRNA Isolation Kit (Ambion) for use in the RAL exposure experiments. For quantification of selected transcripts, 1 μg total RNA was reverse transcribed using SuperScript III RT (Invitrogen) after priming with oligo-dT, and cDNA was subjected to real-time PCR using the LightCycler FastStart DNA Master SYBR Green I system (Roche Applied Science). The primers used for amplification of specific cDNAs are listed in Supplementary Materials (Table S1). Quantification of RNA levels was carried out by comparison of amplification cycle number to standard curves prepared with known amounts of purified target cDNA [25].

2.3. cDNA microarray analysis

cDNA microarray analyses were performed to investigate the effects of RAL on gene expression in LTEE and control cells. For these determinations, 0.5 μg aliquots of total RNA from each sample were assessed for integrity using the Agilent 2100 Bioanalyzer, and cDNA microarray analyses were performed by the Wadsworth Center Applied Genomic Technologies Core using Affymetrix GeneChip Human Gene 1.0 ST arrays. Gene expression data were analyzed using GeneSpring GX 10.0 software (Agilent Technologies). Expression values for each probe set were log-summarized using the RMA16 algorithm, with subsequent baseline transformation to the median log-summarized values of the vehicle control-exposed samples. Probe set data were then filtered by expression to remove background-level signal intensities that were in the lowest 20th percentile of all signal intensities. The resulting expression data were subjected to analysis of variance (ANOVA) testing, which incorporated the Benjamini-Hochberg FDR multiple testing correction ($P < 0.05$). A fold change cut-off of 2.0 was applied to the list of filtered probe sets for determination of expression differences among the treatment groups, and a heatmap of the resulting gene expression signal intensities was generated with probe sets clustered hierarchically.

2.4. Western immunoblots

Confluent LTEE and control cultures in 6-well plates were treated with 100 nM RAL or DMSO vehicle for 48 h, or 10 ng/mL interferon (IFN)-γ (Thermo Scientific) or its vehicle, 0.1% BSA in PBS, for 20 min. Cells were then lysed in 500 μL sample lysis buffer, and total protein concentrations were determined by the bicinchoninic acid (BCA) assay (Thermo Scientific). Samples containing equal amounts of protein were subjected to denaturing gel electrophoresis in 10% acrylamide NuPAGE Bis-Tris gels (Invitrogen) and proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore). Nonspecific adsorptive sites were blocked with Blotto B (1% bovine serum albumin, 1% non-fat dry milk, 0.05% Tween 20 in PBS), and the membranes were probed with rabbit polyclonal primary antibodies from Santa Cruz Biotechnology directed against human AhR (sc-5579, diluted 1:600), GAPDH (sc-25778, diluted 1:3000), phospho-EGFR (Tyr1173, sc-12351, diluted 1:1000), and the following antibodies from Cell Signaling Technology: human STAT1 (#9172, diluted 1:800), phospho-STAT1 (Tyr701, #58D6, diluted 1:800), phospho-AKT (Ser473, #4058, diluted 1:800), AKT (#9272, diluted 1:1000), phospho-ERK1/2 (Thr202/Tyr204, #4376, diluted 1:1000), ERK1/2 (#4695, diluted 1:1000), EGFR (#2232, diluted 1:1000), or β-actin as previously described [25]. Horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:15,000) was used as the secondary antibody. Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific).

2.5. Ethoxyresorufin-O-deethylase (EROD) assay

EROD assays were carried out in 96-well plates with confluent control and LTEE MCF-7 cells in DC_5 medium. After seeding, cultures were treated with 10 nM TCDD or DMSO vehicle for 72 h, after which the EROD activity was determined with 4 μM ethoxyresorufin as substrate; the resorufin formed was quantified by fluorescence detection with 535 nm excitation and 590 nm emission wavelengths as described [25]. Activities were normalized to protein content as determined by the BCA assay.

2.6. Assay of the effects of growth factors and MCF-7 conditioned medium on MCF-10A cell proliferation

The effects of growth factors and MCF-7 conditioned medium on the proliferation of MCF-10A cells (ATCC) were determined as described by Martinez-Lacaci et al. [26] with modifications. MCF-10A cell proliferation was determined in a medium consisting of 1:1 DMEM/Ham's F12 supplemented with 10 mM non-essential amino acids, 2 mM L-glutamine, 1 μg/mL insulin, 10 μg/mL bovine pituitary extract, 1% bovine calf serum, and 0.5 μg/mL hydrocortisone as described [27] but without the addition of epidermal growth factor (EGF). MCF-10A cells were seeded at 6000 cells/well in a 96-well dish and treated every three days with the above medium containing 0.01% DMSO vehicle, 20 ng/mL murine EGF (Invitrogen), 20 ng/mL recombinant human amphiregulin (AREG; R&D Systems), or 1 nM E_2.

To determine the effects of conditioned medium from MCF-7 cells on MCF-10A cell proliferation, control and LTEE MCF-7 cells were exposed to serum-free medium containing 0.01% DMSO vehicle or 1 nM E_2, respectively, for 24 h. Serum-free medium was composed of DMEM/Ham's F12 supplemented with 1 μg/mL insulin (Invitrogen), 5 μg/mL human transferrin (Sigma), 5 ng/mL sodium selenite (BDH Chemicals), L-glutamine, and non-essential amino acids. The conditioned media from the control and LTEE cultures were collected, filtered, and phenylmethylsulfonyl fluoride (Sigma) was added to a final concentration of 0.5 mM. The conditioned media were concentrated approximately 2.7-fold using an Amicon Centriplus centrifugal filter device (Millipore) and equalized with respect to protein concentration. Hydrocortisone, bovine pituitary extract, and bovine calf serum were added to the concentrated conditioned media at their respective concentrations used in the MCF-10A medium. MCF-10A cells were seeded as above and treated every three days with these conditioned media. Cell proliferation was determined by the sulforhodamine B assay.

2.7. Statistical analysis

Data for EROD assays and RNA levels were subjected to ANOVA followed by the Bonferroni test for multiple comparisons, and presented as the mean ± standard error (SE), as indicated in the figure legends.

3. Results

3.1. RAL inhibits and induces gene expression in LTEE cells

The direct involvement of ER in the observed changes in gene expression was investigated by treatment with the SERM, RAL. After exposure of control and LTEE cells to 100 nM RAL or vehicle for 48 h, effects on gene expression were determined using cDNA microarrays. Analysis of the resulting data led to the identification of 493 genes whose expression was significantly increased or reduced ≥2-fold in at least one of the pairwise conditions between control, control plus RAL, LTEE, and LTEE plus RAL. This subset of genes in which expression was significantly altered was hierarchically clustered based on $\log_2$-normalized signal intensity values and the result was presented as a heatmap (Supplementary Materials Fig. S1). Of the 493 genes, 204 (41.4%) were up-regulated in LTEE cells ≥2-fold, whereas 142 (28.8%) genes were down-regulated ≥2-fold in LTEE cells (Supplementary Materials Table S2). Of the 493 genes, 28 (5.7%) were up-regulated by ≥2-fold by the exposure to RAL in LTEE cells; the largest induction observed was 4.4-fold for *PMP22*. This RAL-induced group included genes whose mRNA levels were significantly increased or repressed in expression in LTEE cells, relative to expression in control cells. Twenty-two of the 493 (4.5%) genes showed enhanced expression in LTEE cells and were sensitive to RAL exposure, as defined by a ≥2-fold reduction in mRNA expression levels (Supplementary Materials Table S3). Several examples of genes exhibiting this pattern of regulation are shown in Fig. 1A and include *PGR*, *PDZK1*, and *GREB1*, which are well characterized as estrogen-regulated genes.

In contrast to the RAL-sensitive genes, there were numerous genes, including those encoding AhR and CYP1B1, for which mRNA expression was increased in LTEE cells, but the levels were not affected by the addition of RAL (Fig. 1B). Of the 493 genes that were selected on the basis of differential expression between LTEE and control cells or in response to exposure to RAL, 443 (89.9%) showed only slight or no regulation by RAL exposure (<2-fold). In control cultures, 489 of the 493 (99.2%) genes showed <2-fold change in expression in response to RAL exposure. Several of the most highly up-regulated genes in LTEE cells that were RAL-insensitive are shown in Fig. 1B, and include *IFI27*, *IFI44L*, *OAS2*, *IFIT1*, *SAMD9*, and *MUCL1*, the first four of which are known to be IFN-inducible and to have roles in immune responses.

3.2. AhR expression and responsiveness are up-regulated in LTEE cells

Real-time PCR results revealed a significant 7.5-fold increase in AhR mRNA abundance in LTEE cells relative to control cells (Fig. 2A). Exposure of control or LTEE cells to RAL did not affect AhR mRNA expression. Western immunoblots showed a pattern of AhR protein expression that closely paralleled the pattern of AhR mRNA expression; the AhR protein level was highly increased in LTEE cells compared with the level in control cells, and AhR protein expression was not affected by exposure to RAL in either control or LTEE cells (Fig. 2B). Given that genes encoding cytochromes P450 of the CYP1 family are under transcriptional control of the AhR, we evaluated CYP1-catalyzed EROD activity in control and LTEE cells, with and without exposure to the AhR ligand, TCDD. The results revealed a 4.7-fold increase in TCDD-induced EROD activity in LTEE cells compared to the level of EROD activity in control cells treated with TCDD (Fig. 2C).

3.3. STAT1 expression is enhanced in LTEE cells

The expression of STAT1 mRNA was enhanced >5-fold in LTEE cells relative to the expression in control cells as determined by cDNA microarray. This transcriptional activation of STAT1 was reflected in increased STAT1 protein expression as determined by Western blot (Fig. 3A). The STAT1 α isoform (91 kDa) was more abundant than the β isoform (84 kDa) in control cultures, but the two could not be distinguished in LTEE cultures because of band saturation arising from the 20-min film exposure required to detect control levels of STAT1. Upon shorter exposures, more prominent bands were observed for STAT1α than for STAT1β in LTEE cultures (data not shown). Exposure to RAL had no effect on the level of STAT1 protein. Activated STAT1 (phospho-tyrosine 701,

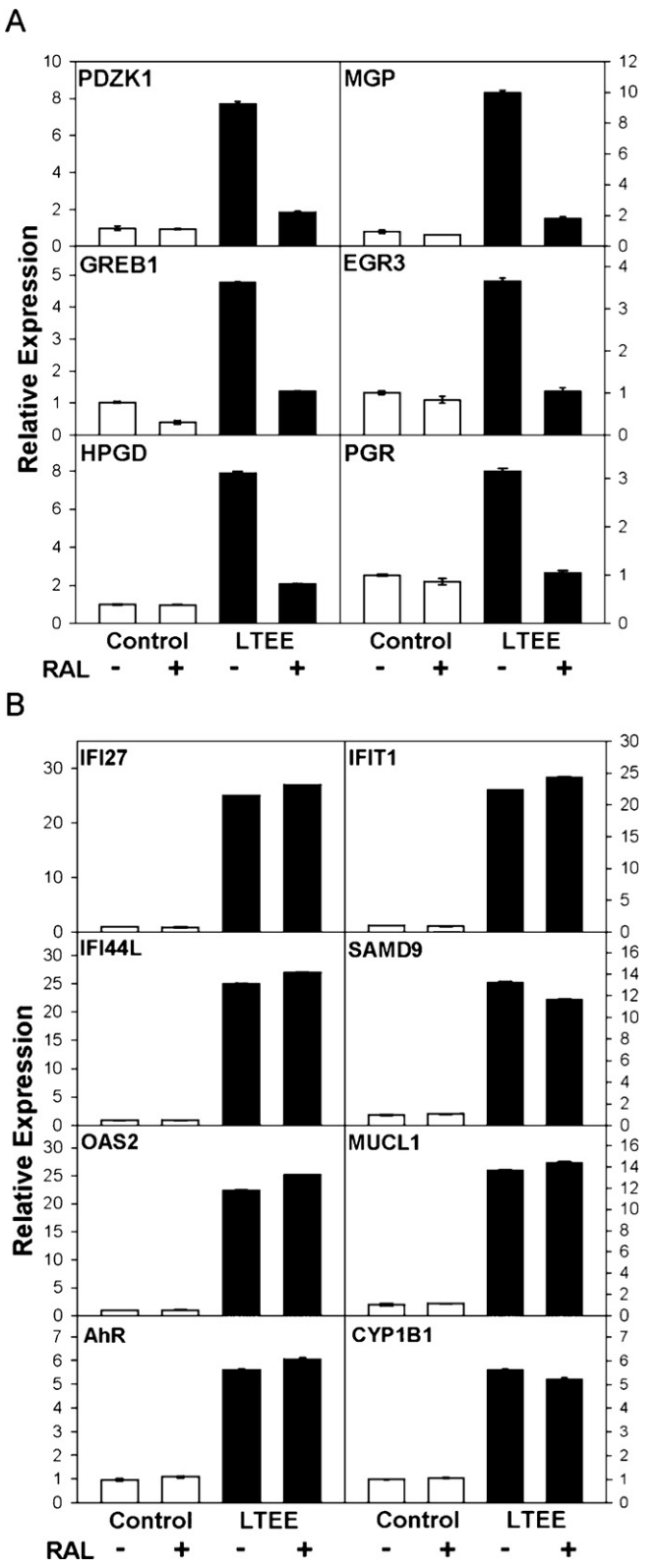

Fig. 1. Effects of RAL exposure on gene expression in control and LTEE cells. Control (white columns) and LTEE cultures (black columns) at passage 30 were treated in triplicate with 100 nM RAL or DMSO vehicle for 48 h, and mRNA expression was analyzed by cDNA microarray. Shown are representative genes (A) whose increased expression in LTEE cells was abrogated by exposure to RAL and (B) whose increased expression in LTEE cells was insensitive to RAL exposure. Data are expressed relative to the mean of the control values, and are presented as mean ± SE; $n = 3$.

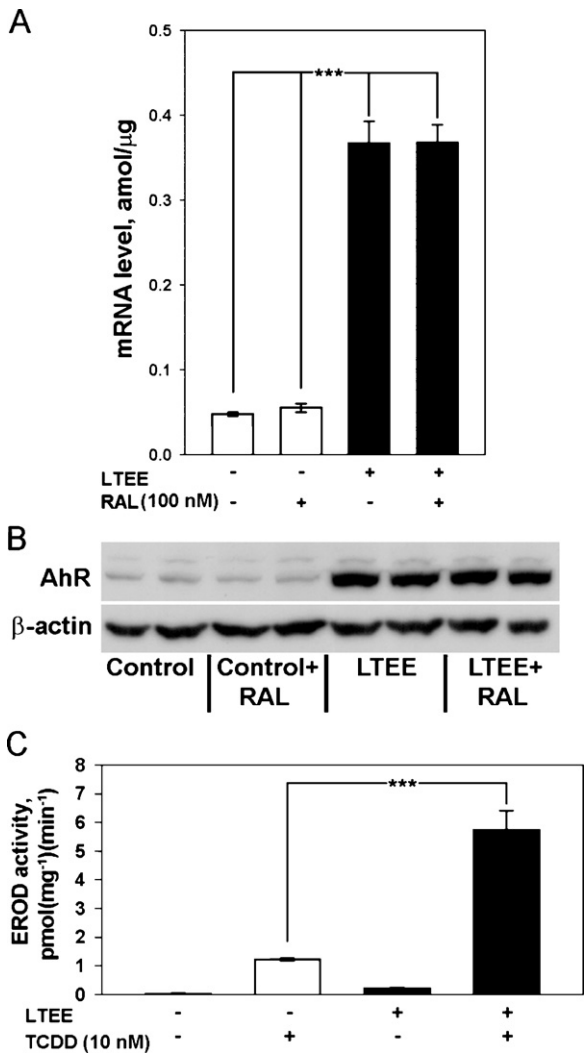

Fig. 2. AhR expression and AhR-regulated EROD activity are increased in LTEE cells. (A) Control (white columns) and LTEE cultures (black columns) were exposed for 48 h to 100 nM RAL or DMSO vehicle, as indicated. RNA was then isolated, and AhR mRNA levels were determined by real-time PCR; data are expressed as amol/μg RNA; data are the means ± SE, $n = 3$. (B) Control and LTEE cultures were exposed for 48 h to 100 nM RAL or DMSO vehicle, and whole-cell lysates were analyzed by Western immunoblot for AhR and β-actin. (C) Passage 30 control (white columns) and LTEE cultures (black columns) were exposed to DMSO vehicle or 10 nM TCDD for 48 h, and CYP1 activity was measured by the EROD assay; $n = 5$; data are the means ± SE. $^{***}P < 0.001$.

pSTAT1), on the other hand, was present in the LTEE and LTEE plus RAL cultures, whereas no pSTAT1 was detected in control or control plus RAL cultures during a 20-min film exposure period. STAT1α and STAT1β show approximately equal phosphorylation levels. From these data, it cannot be determined whether the activation of STAT1 by phosphorylation was enhanced in LTEE cells, or whether a constitutive phosphorylase activity (i.e., activity that is not enhanced in LTEE cells) resulted in higher Y701 pSTAT1 levels in LTEE cells, simply because much more STAT1 was available as substrate. If the ratios of pSTAT1:STAT1 were the same in control and LTEE cells, then the level of pSTAT1 in control cells would have been below the detection limit in the Western blot. The activation of the JAK/STAT pathway by exposure of cells to 10 ng/mL IFN-γ was evidenced by higher levels of pSTAT1 in both control and LTEE cultures; the biochemical pathway(s) leading to pSTAT1 were thus fully responsive to cytokine stimulation in our cultures (Fig. 3B).

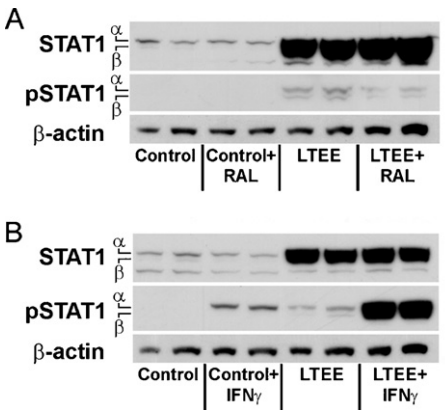

Fig. 3. Levels of STAT1 and pSTAT1 are increased in LTEE cells. (A) Control and LTEE cultures (passage 30) were exposed, in duplicate, to 100 nM RAL or DMSO vehicle for 48 h, and whole-cell lysates were prepared and analyzed for STAT1, pSTAT1, and β-actin by Western blotting. (B) Control and LTEE cultures (passage 35) were exposed to 10 ng/mL IFN-γ for 20 min, and whole-cell lysates were prepared and analyzed for STAT1, pSTAT1, and β-actin. The STAT1 α and β isoforms are indicated.

3.4. Major histocompatibility complex (MHC) class I genes are up-regulated in LTEE cells

cDNA microarray analyses of gene expression revealed a marked difference in the expression of MHC genes between LTEE and control cells. MHC class I genes, which are known to regulated by STAT1 [28], were more highly expressed in LTEE cells than in control cells, whereas MHC class II transcripts showed comparable expression in LTEE and control cells (Fig. 4). The levels of the HLA-A, -B, -C, -E, -F, -G, and -H mRNAs were up-regulated ≥2-fold in LTEE cells, and no effect on expression was seen in cells exposed to RAL. Exposure to RAL likewise had no effect on the expression of MHC class II genes in either LTEE or control cells. mRNA levels for β-2-microglobulin (B2M), the dimerization partner for MHC class I molecules, showed the same pattern of expression as was observed for the MHC class I genes (Fig. 4, *inset*).

3.5. AhR and STAT1 differ in persistence of expression after E_2 withdrawal

The AhR and STAT1 mRNAs showed similar enhancement of expression in LTEE cells, and the expression of both was insensitive to RAL exposure. We therefore investigated whether the genes encoding the two transcription factors were under direct control of E_2 action but were not sensitive to RAL inhibition at the level of the ER, or whether they were persistently activated, possibly through an estrogen-dependent gene-imprinting mechanism. To differentiate between these two possibilities, LTEE (-E_2) cells were obtained from LTEE cells by culturing them without added E_2 for 25 days, during which they were subcultured three times. RNA was then isolated from control, LTEE, and LTEE (-E_2) cultures, all at the same passage, and was analyzed by real-time PCR. The results showed that AhR expression did not require the continued presence of E_2; AhR mRNA was persistently up-regulated in LTEE (-E_2) cells relative to its level in control cells and was comparable to its level in LTEE cells (Fig. 5A). The level of the mRNA encoding CYP1B1, which is under the regulatory control of the AhR, was also significantly higher in LTEE (-E_2) cells compared with its level in control cells. Functional differences in AhR activity were observed in control, LTEE, and LTEE (-E_2) cells (Fig. 5B) that were consistent with the observed levels of AhR mRNA (Fig. 5A) and protein (Fig. 5C) in these cultures. In control cultures that had been maintained in medium without E_2 supplementation, AhR-mediated, CYP1-catalyzed EROD activity was not inducible by TCDD exposure. In LTEE and LTEE (-E_2) cells, EROD activity was induced 13.3- and 14.6-fold by TCDD, respectively.

In contrast to the persistence of the increased AhR expression in LTEE (-E_2) cells, STAT1 mRNA expression showed dependence on the continued presence of E_2. STAT1 mRNA levels remained elevated after 5 days of E_2 withdrawal (data not shown); however, in LTEE (-E_2) cells after 25 days of E_2 withdrawal, levels of STAT1 mRNA (Fig. 5A) and protein (Fig. 5C) had decreased such that they were comparable to those of control cells. ISG15, a downstream target of STAT1, also showed dependence on the presence of E_2; expression levels of ISG15 mRNA in LTEE (-E_2) cells were reduced to those of control cells. These results indicate that the maintenance of increased STAT1 expression levels requires the continual presence of E_2 in MCF-7 cells.

3.6. Activation of the MAP kinase and AKT pathways in LTEE and LTEE (-E_2) cells

Since E_2 is known to activate cellular signaling pathways, including those involving MAP kinase and AKT, and since STAT1 activation is thought to be regulated by activated MAPK signaling through ERK1/2 phosphorylation [29], we investigated the phosphorylation

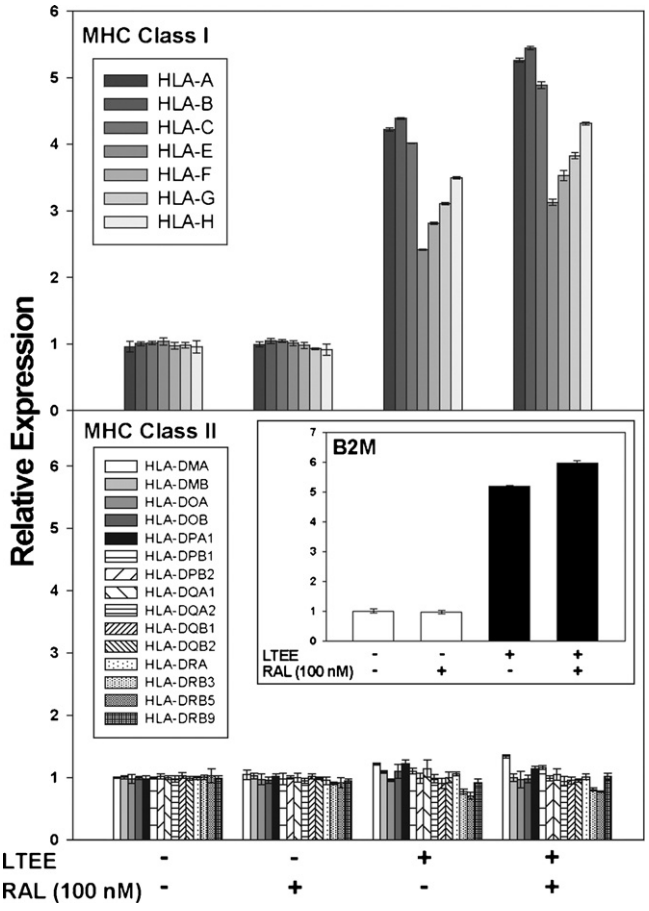

Fig. 4. Enhanced expression of MHC class I, but not MHC class II, genes in LTEE cells. LTEE and control cultures were exposed, in triplicate, to 100 nM RAL or DMSO vehicle for 48 h, and mRNA levels were analyzed by cDNA microarray. mRNA levels of MHC class I (upper panel) and MHC class II genes (lower panel) in control and LTEE cells are shown. MHC class II genes are represented in the figure legend as: HLA-DMA, white; HLA-DMB, light grey; HLA-DOA, grey; HLA-DOB, dark grey; HLA-DPA1, black; HLA-DPB1, horizontal lines; HLA-DPB2, right diagonal lines; HLA-DQA1, left diagonal lines; HLA-DQA2, fine horizontal lines; HLA-DQB1, fine right diagonal lines; HLA-DQB2, fine left diagonal lines; HLA-DRA, light dots; HLA-DRB3, dots; HLA-DRB5, heavy dots; HLA-DRB9, crossed lines. The inset shows B2 M mRNA levels in control and LTEE cells exposed to RAL. Expression levels are depicted relative to levels in control cells.

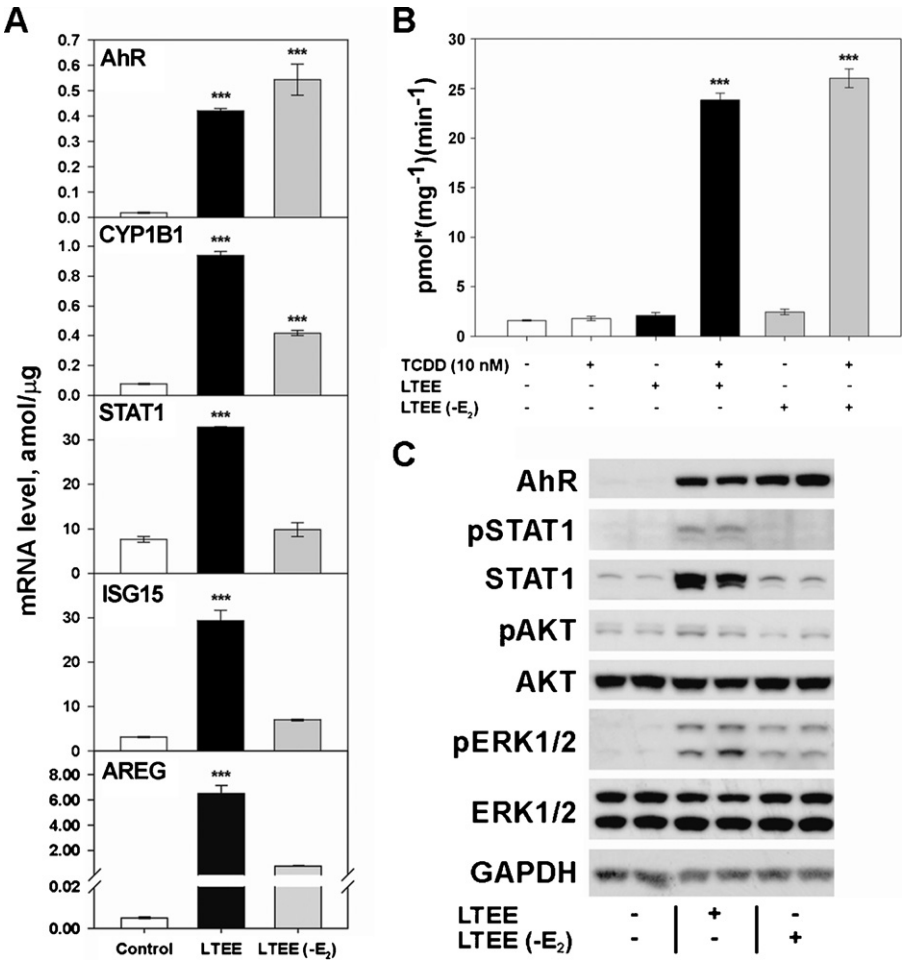

Fig. 5. STAT1 and AhR are differentially affected by E_2 withdrawal from LTEE cultures. (A) Passage 48 control (white columns), LTEE (black columns), and LTEE cells that were propagated in DC_5 medium without added E_2 for an additional 25 days (LTEE (-E_2), grey columns) were cultured to confluence in 6-well plates. RNA was then isolated, and real-time PCR of AhR, CYP1B1, STAT1, ISG15, and AREG was performed; data are the means ± SE, $n = 3$. Significant differences from control are shown; $^{***}P < 0.001$. (B) Passage 45 control, LTEE, and LTEE (-E_2) cells that had been deprived of E_2 for 8 days were seeded in DC_5 medium without added E_2. The following day, media were changed to DC_5 containing 10 nM TCDD or 0.01% DMSO vehicle. After 72 h, EROD activity was determined as described in Materials and Methods; $n = 5$; data are the means ± SE. $^{***}P < 0.001$. (C) Control, LTEE, and LTEE (-E_2) cells that had been deprived of E_2 for 25 days were seeded near confluence in 6-well plates, in duplicate. After 24 h, whole-cell lysates were prepared and analyzed for the expression of AhR, pSTAT1, STAT1, pAKT, AKT, pERK1/2, ERK1/2, and GAPDH by Western immunoblot.

of these signal transducers in control, LTEE, and LTEE (-E_2) cells. In LTEE MCF-7 cells, we observed the elevated expression of AhR, total STAT1, pSTAT1, pAKT, and pERK1/2 in comparison with their levels in control cells (Fig. 5C). In LTEE (-E_2) cells, a persistence of AhR protein expression was observed (Fig. 5C) that was consistent with the observed AhR mRNA levels (Fig. 5A). Both total STAT1 and pSTAT1 were reduced in expression in LTEE (-E_2) cells in comparison with their levels in LTEE cells, which parallels STAT1 mRNA levels in these cultures (Fig. 5A). Interestingly, the levels of pERK1/2, which may act as a STAT1 kinase in MCF-7 cells, remained elevated in comparison with its levels in control cells after 25 days of E_2 withdrawal; pAKT showed a decrease in LTEE (-E_2) cells in comparison with its level in LTEE cells. No changes in expression were detected for total AKT, total ERK1/2, or GAPDH among the control, LTEE and LTEE (-E_2) cultures.

3.7. Growth factors and conditioned medium from LTEE MCF-7 cells stimulate MCF-10A cell proliferation

The observed phosphorylation of ERK1/2 suggests that epidermal growth factor receptor (EGFR) is activated in LTEE cells, possibly through an autocrine loop mediated by the E_2-induced production of the EGFR ligand, AREG. The mRNA encoding AREG is highly elevated in LTEE and LTEE (-E_2) cells in comparison with its level in control cells as determined by real-time PCR (Fig. 5A); if LTEE cells produce and respond to AREG or other EGFR ligands in an autocrine loop, then conditioned medium from LTEE MCF-7 cells should contain EGFR ligands. To investigate this possibility, MCF-10A cells, which are highly responsive to growth factors that activate EGFR, were used to assay for growth factors secreted by MCF-7 cells. In Fig. 6A, the effects of the addition of E_2, AREG, and EGF to MCF-10A minimal media on MCF-10A cell proliferation were determined. MCF-10A cells proliferated in response to AREG and EGF, whereas the addition of E_2 to the media had no effect on proliferation in comparison with the vehicle control in this ER-negative cell line.

To determine whether LTEE MCF-7 cells secrete growth factors that affect MCF-10A proliferation, control and LTEE MCF-7 cells were exposed for 24 h to serum-free media containing either the DMSO vehicle or E_2, respectively. The results showed that conditioned medium from LTEE MCF-7 cells supported MCF-10A cell proliferation to a greater extent than conditioned medium from control MCF-7 cells (Fig. 6B). Enhanced autocrine signaling due to increased production of growth factors would be expected to result in the activation of EGFR through its phosphorylation. To further investigate this putative auotcrine loop, EGFR expression and activation of EGFR by phosphorylation of tyrosine 1173 were assayed by Western immunoblot in control and LTEE MCF-7 cells exposed to

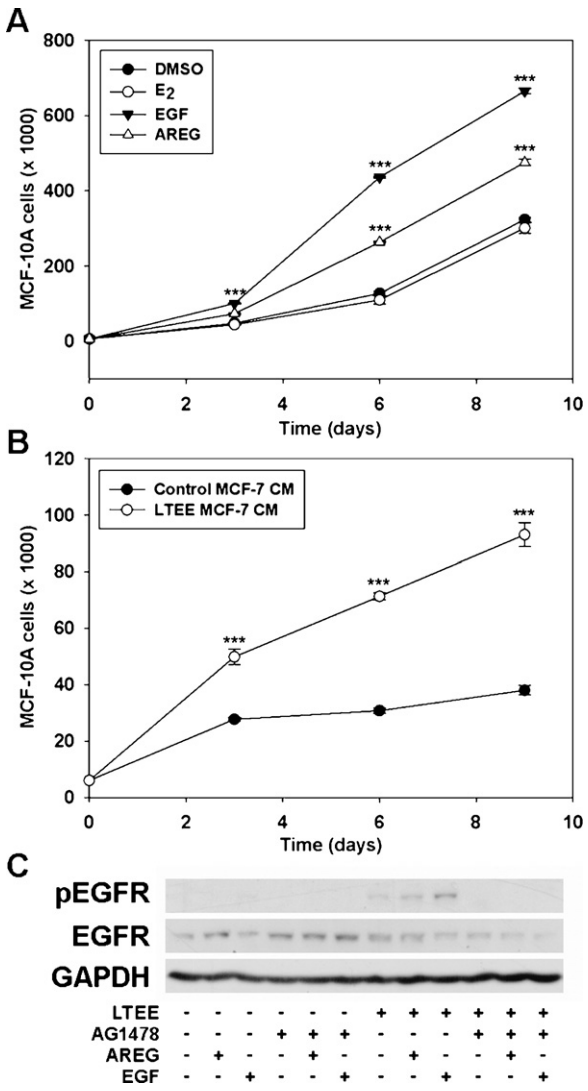

Fig. 6. Conditioned medium from LTEE MCF-7 cells enhances the proliferation of MCF-10A breast epithelial cells. (A) The effects of 1 nM E_2, 20 ng/mL EGF, and 20 ng/mL AREG on the proliferation of MCF-10A cells relative to the DMSO-vehicle controls after 3, 6, and 9 days of incubation are shown. (B) MCF-10A cells were incubated in serum-free conditioned medium (CM) from control (black circles) and LTEE cell cultures (white circles). MCF-10A cell proliferation was determined by sulforhodamine B assay. Data are the means ± SE; $n = 6$. Significant differences from the cells exposed to DMSO are shown; $^{***}P < 0.001$. (C) Western blot analysis of phospho-EGFR, total EGFR, and GAPDH levels in control and LTEE cells exposed to 20 ng/mL EGF, 20 ng/mL AREG, with or without pretreatment with 1 μM tyrphostin AG1478 for 1 h.

20 ng/mL EGF or AREG, with and without pretreatment with 1 μM of the EGFR-specific tyrosine kinase inhibitor, tyrphostin AG1478. Activation of EGFR was observed in LTEE cells and by further exposure to EGF; phospho-EGFR was only observed after exposure to EGF in control cells, resulting in a very faint band (Fig. 6C). EGFR activation was completely inhibited by pretreatment of cultures with AG1478 for 1 h.

3.8. STAT1-associated genes are differentially expressed in LTEE cells

Various target genes known to be downstream of STAT1 in the cytokine signaling pathway are up-regulated in LTEE cells, even without the addition of cytokines to the culture medium (Fig. 7). Examination of the proximal promoters for transcription factor binding sites [30] revealed that at least one putative gamma-interferon activation site (GAS) or interferon-stimulated response element (ISRE) was present in each of these genes [31]. The mRNA levels of ISG15, IFI6, PSMB9 and STAT1 were up-regulated by 29.9-, 20.0-, 58.0- and 8.6-fold, respectively, in LTEE cells; these mRNA expression levels were also refractory to RAL exposure in LTEE cells.

cDNA microarray data indicated that the levels of mRNAs encoding proteins involved in the activation of STAT1 generally did not differ or were repressed in LTEE in comparison with control cells. Only the cytokine receptor IL27RA and cofactor IL6ST transcripts were up-regulated ≥2-fold in LTEE cells (2.1- and 2.6-fold, respectively). The levels of mRNAs encoding kinases that are known to catalyze the phosphorylation of STATs (JAK1, JAK2, JAK3, TYK2) were not increased in LTEE cells compared with their levels in control cells as determined by cDNA microarray. mRNAs encoding two STAT1 phosphatases (PIAS1, PTPN11) were not considerably down-regulated in LTEE cells relative to control cells, although array data did suggest reduced mRNA expression of STAT1 phosphatases PTPN1 and PTPN6 in LTEE cells. While STAT3 and STAT5 have shown persistent activation in various human tumors and tumor cell lines [32,33], their expression levels were not appreciably up-regulated (<2-fold) in LTEE cells relative to control cells.

3.9. Genes associated with resistance to radiation and chemotherapy are up-regulated in LTEE cells

A specific gene signature, in tumors, that is associated with cross-resistance to radiation and chemotherapy has been experimentally derived by Weichselbaum and coworkers [34]. This concerted expression of genes, termed the "interferon-related DNA damage resistance signature" (IRDS), is associated with the IFN-signaling pathway, and the expression of the genes of the IRDS can segregate a number of human cancers into IRDS(+) and IRDS(−) states. Genes included in the signature, *STAT1*, *ISG15*, and *IFIT1*, are thought to be key mediators of experimental cross-resistance, and their up-regulation has been observed in human cancers. In LTEE cells, we observed the up-regulation of 24 of the 49 (48.9%) genes that make up the IRDS by ≥2-fold (Table 1). Many of these genes are down-stream targets in the IFN signaling pathway. The expression of all the genes in the IRDS also showed insensitivity to RAL exposure in LTEE cells. In contrast to the 24 up-regulated genes, the expression of 9 of the 49 (18.4%) genes of the IRDS showed down-regulation in LTEE cells, with 6 of the 9 genes down-regulated <2-fold. *GALC*, which encodes a lysosomal protein, *THBS1*, which encodes an adhesive glycoprotein that mediates cell–cell and cell–matrix interactions, and *TIMP3*, which encodes an inhibitor of extracellular matrix metalloproteinases, whose low expression is associated with poor success of endocrine therapies in breast cancer patients [35,36], were the only IRDS genes that were down-regulated ≥2-fold in LTEE cells.

4. Discussion

Lifelong exposure to estrogens is known to be a risk factor for the development and progression of breast cancer, yet the impact of extended exposure to estrogens on gene expression in breast cancer has not been fully elucidated. Our previous results [24] and those reported here show altered expression in LTEE cells of numerous genes, including *AHR* and *STAT1*, which have not been previously reported to be regulated by estrogen in MCF-7 cells. Therefore, in the context of these data, we have taken a broader view of estrogen regulation and evaluated mechanisms that might be responsible for the observed changes in gene expression in LTEE cells. The sensitivity to SERM inhibition, the necessity for the continued presence of estrogen, the potential for regulation down-stream of the initial

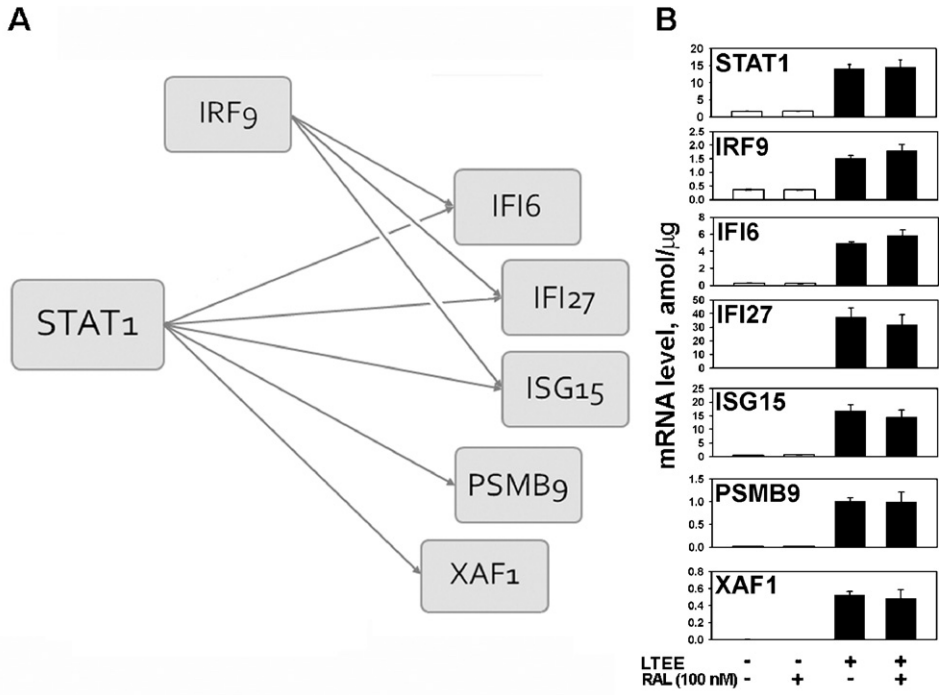

Fig. 7. STAT1 and its downstream target genes are up-regulated in LTEE cells. (A) As shown in the scheme, STAT1 is known to mediate gene expression independently of IRF9 for *PSMB9* and *XAF1*, or as a complex with IRF9 (which also includes STAT2), for *IFI6*, *IFI27*, and *ISG15*. (B) Control (white columns) and LTEE cultures (black columns) were exposed to 100 nM RAL or DMSO vehicle for 48 h, and mRNA levels were determined by real-time PCR. Expression levels are presented as amol/μg RNA; data are the means ± SE; $n = 3$. For each of the mRNAs, differences in expression between control and LTEE cells, with or without RAL exposure, were significant at $P < 0.01$.

estrogenic effect, and the possibility of estrogen imprinting, or reprogramming of gene expression, were considered in this study.

We investigated the effects of the SERM, RAL, to determine to what extent the genes that showed higher levels of expression in LTEE cells were directly dependent on ER function. RAL is generally an ER antagonist in breast cells; however, in some tissues, notably bone, RAL exhibits ER agonist activity [6]. Exposure of control and LTEE cells to RAL revealed subsets of genes that differed in response to the SERM. Only 20 genes that showed elevated expression in LTEE in comparison with control cells were inhibited >2-fold by RAL, which would be indicative of a direct antagonistic effect on ER-mediated transcriptional activation. In contrast, many of the genes that showed enhanced expression in LTEE cells, including *STAT1*, *AHR*, *CYP1B1*, and numerous IFN-inducible genes downstream of STAT1, genes that were not previously reported to be induced by estrogen exposure in the early (3–4 h) or late (24 h) metasignatures [37], were not affected by RAL exposure. For this subset of RAL-insensitive genes, there may not be direct involvement of ER in transcriptional regulation; the increased observed effects may be downstream of persistent, E_2-induced genetic changes.

One mechanism that may be involved in differential gene expression in LTEE in comparison with control cells is cross-talk between estrogen and growth factor signaling pathways. Our cDNA microarray study showed that AREG transcript levels were the most abundant in LTEE MCF-7 cells in comparison with control cells (>50-fold; Supplementary Materials Table S2). *AREG* is a known E_2-regulated gene; however, only four other (*MGP*, *NPY1R*, *PDZK1*, and *PRSS23*) of the 25 genes that showed the highest relative expression in LTEE cells were previously characterized as estrogen responsive [37]. On the other hand, 10 of the 25 genes showing the highest relative expression in LTEE cells are found in the IRDS and appear to be regulated by STAT1. The high transcript levels of AREG and STAT1 in LTEE cells may be indicative of the underlying gene-regulatory mechanism. AREG is an agonist of EGFR, and an AREG-EGFR autocrine loop in MCF-7-derived cells has been demonstrated [38]. Our results are suggestive of an autocrine loop, as we observed phosphorylation of EGFR in LTEE cells without addition of an EGFR ligand.

While AREG-mediated activation of EGFR may be responsible for the observed activation of the MAP kinase pathway, resulting in ERK1/2 phosphorylation in LTEE cells, there is another charac-

Table 1
Enhanced expression of genes of the IRDS in LTEE cells.

Gene symbol[a]	Fold change[b]	Gene symbol	Fold change
ALDH3A1	1.1	*IRF7*	1.4
BST2[c]	**12.4**	***ISG15***[c]	**2.5**
CA2	1.0	***LAMP3***	**3.2**
CCDC75	0.98	***LGALS3BP***	**6.7**
CCNA1	1.0	***LY6E***	**2.3**
CD59	0.61	*MCL1*	1.0
CXCL1	1.1	*MX1*[c]	1.3
CXCL10	1.8	***MX2***	**4.6**
DAZ1	0.98	***OAS1***[c]	**8.9**
DCN	1.0	***OAS3***[c]	**9.4**
DDX60[c]	**10.5**	***OASL***	**2.3**
GALC	0.38	***PLSCR1***[c]	**6.7**
HERC6[c]	**11.2**	*RAP2C*	1.1
HLA-B	**4.7**	*ROBO1*	1.0
HLA-G	**3.2**	*SERPINB2*	1.1
HSD17B1	0.95	*SH3YL1*	0.89
IFI27[c]	**35.4**	*SLC6A15*	1.1
IFI35[c]	**3.3**	***STAT1***[c]	**5.4**
IFI44[c]	**12.3**	*THBS1*	0.5
IFI44L[c]	**24.6**	*TIMP3*	0.22
IFI6	**5.7**	*TncRNA*[d]	NA
IFIT1[c]	**22.5**	*TRIM14*	1.9
IFIT3[c]	**5.1**	***USP18***	**3.4**
IFITM1[c]	**12.2**	*ZNF273*	0.88
IGF2	1.1		

[a] IRDS genes are those previously identified [34].
[b] Data are from the cDNA microarray experiment and are the ratios of the transcript expression values in LTEE cells to those of the corresponding values in control cells; genes up-regulated ≥2-fold are in bold.
[c] Gene reported to be induced by unphosphorylated STAT1 [46].
[d] NA, not analyzed.

terized pathway of E_2 signaling through the MAP kinase pathway. Numerous studies indicate that membrane-bound ERα mediates rapid signaling via activation of Src kinase and EGFR in response to estrogen [39], a pathway which could also lead to ERK1/2 activation in LTEE cells. The relative contributions of the AREG autocrine loop and the rapid signaling pathway mediated by plasma membrane ERα are currently unknown; however, both could contribute to ERK1/2-mediated signaling. ERK1/2 activation is associated with the regulation of gene expression [40], cancer-cell proliferation, and the phosphorylation of STAT1 [41–43], which involves the direct recruitment of STAT1 to the cytoplasmic C-tail of EGFR [44], and/or RAS-mediated MAP kinase activation [29]. Activated STAT1 is known to induce its own expression and that of other components of STAT signaling in a positive feedback loop [45]. Unphosphorylated exogenous STAT1 was also recently shown to induce STAT1 expression from the endogenous STAT1 gene [46]. Given the high levels of total STAT1 and increased levels of pSTAT1 in LTEE MCF-7 cells, a similar positive feedback loop may be active in LTEE cells, through which STAT1 regulates its own expression.

Because AhR and STAT1 were similar in their relative mRNA expression levels and their lack of sensitivity to RAL in LTEE cells, and because STAT1 and AhR have been shown to be binding partners [47], it was of interest to determine whether ER is directly involved in the regulation of these genes in LTEE cells. Genes that are up-regulated in expression in LTEE cells and are insensitive to RAL exposure may not be under the direct regulatory control of ER, but may be indicative of a persistent gene-regulatory effect of LTEE. The experiments with LTEE ($-E_2$) cells allowed investigation of the persistent changes in gene expression resulting from LTEE, for which ligand-bound ER was no longer required. Our results showed that STAT1, when up-regulated in LTEE cells, relied on the continued presence of estrogen for maintenance of enhanced expression, whereas AhR transcript levels did not. These results suggest that STAT1 is not involved in the persistent up-regulation of AhR expression. Conversely, treatment of control and LTEE cells with TCDD, which activates AhR-regulated gene transcription, did not affect STAT1 transcript levels in recent gene expression studies [24], suggesting that AhR is not involved in the regulation of STAT1 expression.

The fact that the increased levels of AhR mRNA in LTEE cells persist even when E_2 is no longer added to the culture medium suggests that an epigenetic mechanism is involved in the regulation of AhR expression. The ability of estrogens to elicit epigenetic changes in gene expression has been recognized for some time. For example, diethylstilbestrol is known to cause epigenetic reprogramming, or estrogen imprinting, in mice that had been exposed to this synthetic estrogen on postnatal days 1–5 [48,49]. While the underlying mechanism for the epigenetic regulation of AhR expression in LTEE cells observed in our study is unknown, recent reports suggest that estrogens may impact the regulation of gene expression at the point of histone methylation. Increased levels of phosphorylated AKT in response to estrogen exposure resulted in phosphorylation and inactivation of EZH2, a methyltransferase specific for lysine 27 of histone 3 [50]. Whether a mechanism involving EZH2 is responsible, or whether the persistent up-regulation of AhR expression relies on a different process, elevated levels of AhR determine the level of CYP1 induction [24,25] and thus the rate of CYP1-catalyzed metabolism of xenobiotics and endobiotics. Reactive intermediates resulting form CYP1-catalyzed metabolism, if not effectively inactivated, may initiate carcinogenesis through formation of mutagenic lesions in DNA.

There are also potential consequences of increased STAT1 expression in cancer. STAT1 is known to regulate the expression of genes encoding MHC class I antigens, antigen processing enzymes, and antigen transport proteins. The levels of MHC class I antigens on the surface of tumor cells may have differential consequences with regard to immune surveillance and the immune response against the tumor, dependent on the tissue, tumor type, and the relative roles of cytotoxic T-lymphocytes and natural killer cells in the immune response. For example, in colon cancer, high levels of STAT1-regulated MHC class I expression and high levels of intratumoral T-cells were associated with increased patient survival [51].

Conversely, in human breast cancer, high MHC class I expression in tumors appears to be unfavorable. HLA-G expression was positively correlated with tumor size, nodal status, and clinical disease stage [52], whereas the total loss of MHC class I expression correlated with good prognosis for breast cancer patients [53]. Our results showed elevated levels of MHC class I antigen-presenting molecules in LTEE cells, an effect that appears to be a result of the enhanced expression of STAT1. Similarly, in LTEE cells, transporter of antigenic peptides 1 and 2 (*TAP1*, *TAP2*), proteosome β-type subunits 8 and 9 (*PSMB8*, *PSMB9*), and *B2M*, IFN- and STAT1-inducible genes that are required for antigen transport and processing in the immune-response pathway [54–56], were up-regulated > 2-fold in LTEE cells. The up-regulation of specific MHC class I genes by estrogen has been previously reported [52,57]. If increased expression of these genes also occurs in estrogen-exposed breast tumors *in vivo*, this effect could lead to overexpression of cell-surface antigens, making the tumors less susceptible to recognition and elimination by natural killer cells, and a poorer clinical prognosis. In breast-tumor cells experiencing minimal levels of estrogen exposure, comparable to the control cells in our study, low levels of MHC class I may experience increased targeting by natural killer cells.

Some IFN-/STAT1-inducible genes are thought to act as mediators of cross-resistance to radiation and adjuvant chemotherapy; the effects of this cross-resistance can be reversed through inhibition of STAT1 [58]. Increased STAT1 expression has been documented in various human tumors and tumor cells exhibiting increased resistance to radiation, doxorubicin, and platinum drugs [59–62]. This resistance was proposed as a therapy-predictive marker for clinical outcome, based on the expression of a subset of 49 genes known collectively as the IRDS, most of which we have shown to be differentially expressed in LTEE cells (Table 1). Knowledge of the level of expression of the IRDS may aid in the determination of effective therapies for ER-positive breast cancer patients. Specifically, if E_2 persistently enhances expression of the IRDS genes in breast cancers, this effect may compromise efficacy of radiation and chemotherapy in cancer treatment.

In summary, the results reported here show that extended exposure to E_2 induces marked changes in gene expression in MCF-7 breast cancer cells. We have shown that (i) the expression of genes that were not previously known to be estrogen-responsive are up-regulated in LTEE cells; (ii) that mRNA levels of some genes that are up-regulated in LTEE cells, such as *AHR*, *CYP1B1*, and *STAT1*, are refractory to exposure to the SERM, RAL; and (iii) although both AhR and STAT1 show increased expression in LTEE cells, differences in the epigenetic regulation of the two are apparent. Induction of the STAT1 pathway in LTEE cells may have implications in resistance to breast cancer therapy, given that *STAT1* is a component of a gene signature that mediates cross-resistance to chemotherapy and radiation therapy. This is the first study to relate estrogen exposure to increased STAT1 expression in breast cancer cells, an effect that may represent an additional role of estrogen in the pathogenesis of breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.12.010.

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