Invasive Lobular Carcinoma Cell Lines Are Characterized by Unique Estrogen-Mediated Gene Expression Patterns and Altered Tamoxifen Response

Matthew J. Sikora1,3, Kristine L. Cooper4, Amir Bahreini5, Soumya Luthra2, Guoying Wang7, Uma R. Chandran2, Nancy E. Davidson1,3, David J. Dabbs6, Alana L. Welm7, and Steffi Oesterreich1,3

Abstract

Invasive lobular carcinoma (ILC) is a histologic subtype of breast cancer that is frequently associated with favorable outcomes, as approximately 90% of ILC express the estrogen receptor (ER). However, recent retrospective analyses suggest that patients with ILC receiving adjuvant endocrine therapy may not benefit as much as patients with invasive ductal carcinoma. On the basis of these observations, we characterized ER function and endocrine response in ILC models. The ER-positive ILC cell lines MDA MB 134VI (MM134) and SUM144PE were used to examine the ER-regulated transcriptome via gene expression microarray analyses and ER ChIP-Seq, and to examine response to endocrine therapy. In parallel, estrogen response was assessed in vivo in the patient-derived ILC xenograft HCI-013. We identified 915 genes that were uniquely E2 regulated in ILC cell lines versus other breast cancer cell lines, and a subset of these genes were also E2 regulated in vivo in HCI-013. MM134 cells were de novo tamoxifen resistant and were induced to grow by 4-hydroxytamoxifen, as well as other antiestrogens, as partial agonists. Growth was accompanied by agonist activity of tamoxifen on ER-mediated gene expression. Though tamoxifen induced cell growth, MM134 cells required antiestrogens, as partial agonists. Growth was accompanied by agonist activity of tamoxifen on ER-mediated gene expression. Though tamoxifen induced cell growth, MM134 cells required fibroblast growth factor receptor (FGFR)-1 signaling to maintain viability and were sensitive to combined endocrine therapy and FGFR1 inhibition. Our observation that ER drives a unique program of gene expression in ILC cells correlates with the ability of tamoxifen to induce growth in these cells. Targeting growth factors using FGFR1 inhibitors may block survival pathways required by ILC and reverse tamoxifen resistance. Cancer Res; 74(5); 1463–74. ©2014 AACR.

Introduction

Though approximately 80% of newly diagnosed breast cancers are considered invasive ductal carcinomas (IDC), less common subtypes are routinely identified. Invasive lobular carcinoma (ILC) is the second most common histologic subtype, accounting for 10% to 15% of breast cancer (1–4). ILC presents with unique clinicopathological features, including distinct issues with surgery and chemotherapy (reviewed by us; ref. 5). ILCs are frequently estrogen receptor (ER)- and progesterone receptor (PR) positive; approximately 90% of ILC express ER, versus 60% to 70% of IDC (4, 6, 7). ILC are rarely HER2 positive and are less proliferative than IDC. On the basis of these biomarkers, patients with ILC are excellent candidates for endocrine therapy, and should benefit from improved outcomes versus patients with IDC. However, recent retrospective analyses suggest that patients with ILC may have equivalent or worse outcomes than patients with IDC, despite higher proportions of ER-positive patients that receive endocrine therapy (3, 8–11).

Outcomes for patients with ILC receiving adjuvant endocrine therapy are poorly understood, as data currently available are limited to retrospective analyses. Low numbers hinder analyses due to the lack of prospective inclusion of patients with ILC. However, in a recent retrospective analysis of the adjuvant BIG 1–98 trial, Metzger-Filho and colleagues compared outcomes in IDC with ILC patients (12). Among patients with IDC (n = 2,599), the 8-year DFS confirmed the increased benefit for letrozole versus tamoxifen (HR = 0.8) as previously reported. However, patients with ILC (n = 324) that received tamoxifen were much more likely to experience recurrence (HR = 0.48). These data strongly suggest that patients with ILC receive less benefit from adjuvant tamoxifen than patients with IDC, and highlight the need for investigation in endocrine response and resistance specifically in ILC.

Studies of endocrine response in IDC benefit from the availability of extensively studied cell lines (e.g., MCF-7, T47D). However, only two ER-positive ILC cell lines have been reported in the literature: MDA MB 134VI (MM134) and...
SUM44PE (SUM44; ref. 5). Minimal data exist about endocrine response in these models (13–15). Thus, we characterized ER function and endocrine response in both ILC cell lines and a unique in vivo model of ILC. We observed that ER regulates a unique gene set in ILC cells. Furthermore, MM134 presents with de novo tamoxifen resistance via recognition of tamoxifen as an agonist. However, fibroblast growth factor receptor (FGFR)-1 may be necessary to maintain cell viability in the presence of tamoxifen. On the basis of recent clinical observations, improved understanding of endocrine response in ILC models is necessary to improve patient outcomes.

Materials and Methods

Cell culture

MCF-7 and T47D (American Type Culture Collection; ATCC) were maintained as described (16). MM134 (ATCC) was maintained in 1:1 DMEM-L-15 (Life Technologies) +10% FBS. SUM44PE (Asterand) was maintained as described (17) +2% charcoal stripped serum (CSS). Cell lines are authenticated annually by PCR RFLP analyses at the University of Pittsburgh Cell Culture and Cytogenetics Facility (Pittsburgh, PA), and confirmed to be mycoplasma negative.Authenticated cells are in continuous culture for <6 months. Cells were hormone deprived as described (16) in phenol red-free Improved Minimum Essential Medium with 5%, 10%, or 2% CSS for MCF-7, MM134, and SUM44, respectively.

17β-Estradiol (E2), tamoxifen-free base, 4-hydroxytamoxifen (4OHT), and endoxifen (Bx) were obtained from Sigma. Lapatinib and lasofoxifene were obtained from Santa Cruz Biotechnology. All other compounds were obtained from Tocris Biosciences. E2, tamoxifen, 4OHT, Bx, and ICI 182,780 (ICI) were dissolved in ethanol; all other compounds were dissolved in dimethyl sulfoxide.

Proliferation and viability assays

Cellular proliferation assays used the FluoReporter dsDNA quantitation kit (F2692; Life Technologies) according to the manufacturer’s instructions. Cellular viability assays used CellTox Green (Promega) according to instructions. Fluorescence was assessed on a VictorX4 plate reader (Perkin-Elmer). For proliferation/viability assays, points/bars represent the mean of six biologic replicates ± SEM.

RNA extraction and quantitative PCR

RNA extractions used the Illustra RNAspin Mini kit (GE Health); cDNA conversion used iScript master mix (Bio-Rad), and quantitative PCR (qPCR) reactions used Ssoadvanced SYBR green (Bio-Rad) on a CFX384 thermocycler (Bio-Rad), according to manufacturer’s instructions. Primer sequences are available in Supplementary Document S1.

Gene expression microarrays

Hormone-deprived cells were treated for 3 to 24 hours with 1 nmol/L E2 or 0.01% EtOH in biologic quadruplicate. DNA was harvested as above. cDNA synthesis labeling were performed using the MessageAmp Premier Kit (Life Technologies), and cDNA was hybridized to U133A 2.0 arrays (Affymetrix) by the University of Pittsburgh Cancer Biomarkers Facility. Data processing is described in Supplementary Document S1; data are available in Gene Expression Omnibus (GEO) as GSE50695.

MCF-7 data were obtained from the GEMS E2-regulation metasignature (18). Probe level expression values were condensed as above. Median expression values across datasets were used for individual genes. T47D and BT474 data were from GSE3834 (19) and processed as above. Venn analyses were performed using gene symbols.

Chromatin immunoprecipitation sequencing

Chromatin immunoprecipitation (ChIP) experiments were performed as described (20) with minor modifications. Hormone-deprived MM134 cells were treated with 1 nmol/L E2 or 0.01% EtOH for 45 minutes. Immunoprecipitation used ER-α (HC-20) and rabbit immunoglobulin G (sc2027) antibodies (Santa Cruz Biotechnology). DNA from six independent ChIPs was pooled for sequencing at Genome Quebec Innovation Center (McGill University, Montreal, Canada). 50bp DNA sequencing on the Illumina HiSeq 2000 platform allocated >6 × 10^7 single-end reads per sample.

Output was mapped to the Human Reference Genome (hg18) by BWA (21). Peaks were called using MACS v1.4.2 (22), with a P value cutoff of 10^-5. Features of ER-binding sites were mapped by Cis-regulatory Element Annotation System (23) and BedTools (24). MCF-7 consensus ER-binding sites represented the overlap of nine published datasets (25–29). Data are available in GEO as GSE51022.

Primary tumor xenograft HCl-013

Generation and maintenance of patient-derived xenografts (PDX) were previously described (30). HCl-013 was established from a pleural effusion from a 53-year-old woman with metastatic ER+/PR+/HER2-ILC (also see Supplementary Document S1).

To evaluate growth, nonobese diabetic/severe combined immunodeficient mice were ovarioctomized and/or supplemented with estradiol (1 mg/pellet; ref. 31) before tumor implantation; growth was assessed by caliper weekly. For estrogen deprivation studies, tumors were implanted in ovarioctomized mice with estradiol supplementation. Upon tumors reaching 600 to 800 mm^3, mice received sham or pellet removal surgery; tumors were harvested after 2 or 5 days. Mice from the growth experiment above were crossed over for pellet removal surgery; tumors were harvested after 10 days for this group. Upon tumor harvest, necrotic regions were excised and portions were formalin fixed, paraffin embedded, or flash frozen. Immunohistochemical (IHC) and Ki-67 analyses are described in Supplementary Document S1. For gene expression studies, RNA was extracted from 20 to 30 mg of pulverized frozen tissue using the RNeasy Mini Kit (Qiagen).

Nanostring gene expression analyses

Cells in culture were lysed using Buffer RLT + β-mercaptoethanol (Qiagen), with buffer volume normalized to total RNA. Xenograft tissue experiments used 100 mg of purified RNA per sample. Lysates/RNA were assessed using the mRNA expression protocol on the nCounter platform (Nanostring).
according to the manufacturer's instructions, using a custom codeset (see text). Cluster analyses utilized Multi Experiment Viewer v4.8.1 (32).

**Supplementary methods**

Additional methods and statistical considerations are described in Supplementary Document S1.

**Results**

**Estrogen induces growth and gene expression in ILC cells**

To assess endocrine responsiveness, MM134 and SUM44 cells were treated with E2 following hormone deprivation. E2 induces growth in both cell lines with similar potencies versus MCF-7 (EC50, 1–3 pmol/L; Fig. 1A; rescaled for SUM44 in 1B). Though growth seemed to be induced less in MM134 (2.5-fold) versus MCF-7 (6-fold), these data are consistent with slower basal growth for MM134 (data not shown).

We measured the expression of ten well-established E2-responsive genes following 1 nmol/L E2 treatment for 3 or 24 hours. Six of ten genes were similarly regulated across cell lines (Fig. 1C); for four genes, one ILC cell line showed differential regulation (Fig. 1D). All expression changes were reversed by ICI (Supplementary Fig. S1A), and were dose responsive (Supplementary Fig. S1B), suggesting that these effects are mediated by ER. PR mRNA was E2 induced in both ILC cell lines but was at detection limits (data not shown), consistent with their PR statuses (5). Taken together, the ER-positive ILC cell lines MM134 and SUM44 are endocrine responsive at both the growth and transcriptional levels. Differential regulation of some target genes (e.g., TFF1/pS2) suggests that ER-regulated gene expression may be unique in ILC cells.

**ER regulates unique genes in ILC cells**

We performed microarray analyses with MM134 and SUM44 cells following treatment with 1 nmol/L E2 for 3 or 24 hours to assess global E2-mediated gene expression. A total of 6,241 and 5,364 genes were differentially expressed versus vehicle (fdr < 0.05) at either timepoint in SUM44 or MM134, respectively (Supplementary Table S1); the majority were regulated in both cell lines (Fig. 2A).

These data were compared with public data for three ER-positive IDC cell lines, MCF-7, T47D, and BT474. A total of 7,369 genes were E2 regulated in any IDC cell line (i.e., the union of differentially expressed genes), whereas only 383 genes were E2 regulated in any ILC cell line (i.e., the union of differentially expressed genes), whereas only 383 genes were

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**Figure 1.** E2 induces growth and gene expression in ILC cells. A, proliferation at indicated time after treatment. B, zoomed scale for SUM44. C and D, qPCR-based gene expression; mean log2 fold versus vehicle of three biologic replicates. Red, increased expression; green, decreased expression. Genes similarly regulated (C) and genes differentially regulated (D) in ILC cells versus MCF-7.
commonly regulated across all IDC cell lines (i.e., intersect; Fig. 2B). E2-regulated genes in ILC cells were compared with this union or intersect; a total of 915 genes were regulated by E2 specifically in the ILC cells (versus union, Fig. 2C), including WNT4, SNAI1, and FOXO1. Of note, approximately 44% of ILC-specific genes were repressed versus <25% among other pairwise comparisons (Supplementary Table S1). A total of 254 genes were E2 regulated in all five cell lines (vs. intersect, Fig. 2D), including GREB1, IGFBP4, and CCNG2, and only 31 genes were IDC specific, including PLK1 (Fig. 2D; Supplementary Table S1).

To evaluate ILC-specific E2-regulated genes, we selected the most induced and repressed ILC-specific genes, in addition to eight commonly regulated genes, to yield 107 target genes ("ILC-E2 geneset"; Supplementary Table S2). E2 regulation and ER dependency of these target genes were validated in MM134 (Fig. 2E). E2 regulation was confirmed for 88 of 107 genes (82%), and subsequently reversed by ICI for
85 of 88 confirmed genes (97%), demonstrating ER-dependent regulation.

**ILC-specific ER binding is primarily in distal intergenic regions**

Genome-wide ER binding in MM134 was assessed by ChIP-seq. A total of 2556 E2-induced ER-binding sites were observed. Overall, sites were enriched within 10 kb of gene bodies (Fig. 3A), consistent with IDC cell lines, for example, MCF-7 (33). We confirmed ER binding in MM134 cells at sites associated with E2-regulated genes (Supplementary Document 1). E2 induced binding at commonly regulated genes and ILC-specific genes including WNT4 (Fig. 3B).

MM134 ER-binding sites were compared with a consensus of MCF-7 ER-binding sites. Roughly half of MM134-binding sites were shared with MCF-7 (1,384/2,556, 54%; Fig. 3C). MCF-7–specific sites (n = 3,855) and those common to both (n = 1384) were similarly enriched in promoters within 10 kb (13.0% and 11.1%, respectively, vs. 6.0% expected; Fig. 3D, top left). However, MM134–specific ER-binding sites (n = 1,172) were not

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Figure 3. E2 induces unique distal ER binding in MM134 cells. A, localization of ER-binding sites in MM134. Bolded/asterisk indicate q value versus genome < 0.05. B, ChIP-qPCR of ER-binding sites in independent samples. NFERE, nonfunctional estrogen-response element. P value for vehicle versus E2 < 0.05 for all sites except NFERE. Represents repeat experiments, ± SD of technical replicates. C, MM134-binding sites versus consensus MCF-7 sites. D, localization of ER-binding sites for specific/shared sites between MCF-7/MM134. Bolded/asterisk indicate q value versus genome < 0.05.
enriched in these regions, trending toward depletion (5.3%; Fig. 3D, top left). A similar pattern emerged in downstream regions (Fig. 3D, top right); MM134-specific sites were not enriched within 10 kb downstream, whereas both common and MCF-7–specific sites were enriched. Furthermore, although common and MCF-7–specific sites were enriched in intragenic regions (Fig. 3D, bottom: 48.5% and 48.1% observed, respectively, vs. 46.3% expected), MM134-specific sites were significantly depleted (43.1%).

Motif analysis showed that MM134-specific, MCF-7–specific, and common binding sites were enriched for estrogen response elements (ERE) and forkhead (FOXA1) sites (Supplementary Fig. S2A). Cofactor motifs, including GATA1 and AP-2, were only enriched in common and MCF-7–specific sites and limited to ERE and forkhead sites in MM134-specific sites. Motif analyses were repeated using only distal ER-binding sites, that is, >20 kb from genes (Supplementary Fig. S2B). Cofactor sites were largely lost, and identifiable motifs were limited to ERE/forkhead; only EREs were enriched in distal MM134-specific sites. These observations suggest that ILC-specific E2-regulated gene expression may be modulated by distal sites regulated by unique cofactors in MM134 cells.

A unique in vivo model of ILC is E2 responsive

We evaluated two ER-positive PDXs: HCl-005, derived from a mixed ILC/IDC (30), and HCI-013, derived from an ILC. HCl-005 was found to be purely IDC, whereas HCI-013 was found to mimic clinical ILC (Fig. 4A). We assessed HCl-013 growth in mice ±ovariectomy and ±E2 supplementation. E2 supplementation significantly decreased time to tumor detection and increased growth rate (Fig. 4B); ovariectomy had no effect against E2 supplementation. Without E2 supplementation, ovarian estrogens promoted faster tumor formation, but resulting tumors grew slower than those in the absence of estrogens. These data demonstrate that HCl-013 tumors are an ER-positive, E2-responsive, in vivo ILC model.

HCl-013 proliferation (Ki-67 IHC) and gene expression (E2 E2 geneset) were measured following estrogen deprivation. Following initial establishment +E2, tumors were harvested following E2 pellet removal or sham surgery. No significant proliferation changes were observed following 2 or 5 days of estrogen deprivation; however, following 10 days of estrogen deprivation, few cells are Ki-67 positive (8% ± 4%; vs. d5 +E2; P = 0.0016). Representative images of Ki-67 staining are included (Fig. 4C). Expression of 32 of 107 genes (30%; Fig. 4D and Supplementary Table S3) was significantly different in tumors ±E2, including 27 of 99 ILC-specific genes and five of eight common genes (examples shown in Fig. 4E). These results demonstrate that HCl-013 proliferation is E2 regulated, and that ILC-specific genes identified in vitro are also regulated in vivo in an independent ILC model.

ILC cells are de novo tamoxifen resistant

We then assessed the efficacy of tamoxifen in MM134 and SUM44. E2-induced growth is completely blocked by both 4OHT and ICI in MCF-7 (Fig. 5A). In SUM44 cells, 4OHT blocks E2-induced growth as previously described (data not shown; ref. 13); 4OHT alone does not induce growth. However, ICI alone strongly suppressed growth (Fig. 5B), suggesting that ligand-independent, ER-dependent pathways may regulate growth. In MM134 cells, ICI suppresses E2-induced growth. However, 4OHT only partially reverses E2-induced growth, and induces approximately 30% growth itself as a partial agonist (Fig. 5C). This de novo tamoxifen resistance in MM134 cells may mirror clinical resistance to tamoxifen in some ILC tumors.

We further investigated the partial agonist activity of 4OHT in MM134 cells. MCF-7 and MM134 cells were treated with ER ligands ±ICI. ICI blocked E2-induced growth in MCF-7, and no tamoxifen compound induced growth (Fig. 5D, left). However, in MM134, E2, tamoxifen, 4OHT, and endoxifen induced growth that was reversed by ICI (Fig. 5D, right), demonstrating that tamoxifen-induced growth is via activation of ER.

Additional selective estrogen receptor modulators (SERM) raloxifene and lasofoxifene, which belong to unique chemical classes versus tamoxifen (34), were also tested. Both SERMs blocked E2-induced growth in MCF-7; neither alone induced growth (Fig. 5E, top). MM134 cells were growth induced by both raloxifene and lasofoxifene, neither of which completely suppressed E2-induced growth, consistent with partial agonist activity (Fig. 5E, bottom). Toremifene, a tamoxifen analog, induced growth only in MM134 (data not shown). Conversely, the ICI analog RU 58668 completely reversed E2-induced growth and had no agonist activity (data not shown). Thus agonist activity may be a class effect of SERMs, but does not extend to selective estrogen receptor down-regulators.

Tamoxifen induces gene expression as an agonist in ILC cells

We hypothesized that tamoxifen would also regulate gene expression as an agonist in MM134 cells, and assessed the ILC-E2 geneset following treatment with E2, 4OHT, or ICI. Gene clustering revealed two dominant groups, strongly E2-induced genes (Fig. 6A, red bar; n = 22) and E2-repressed genes (light green bar; n = 34). 4OHT-induced genes could be divided in to two subclusters. In the larger subcluster (orange bar, n = 17) 4OHT acted as an antagonist, similar to ICI, and this included classical ER targets (e.g., GREB1). However, for the smaller subcluster, 4OHT acted as an agonist and induced gene expression (blue bar, n = 5; e.g., SNAI1); ICI reversed induction by both ligands (Fig. 6B). E2-repressed genes were also subdivided; both subclusters 4OHT acted as an agonist and repressed gene expression. Among weakly E2-repressed genes (yellow bar, n = 11) and strongly E2-repressed genes (dark green bar, n = 31), 4OHT displayed greater agonist activity (stronger repression) among the latter (e.g., FOXO1). ICI reversed 4OHT-induced repression (Fig. 6B). Upon sample clustering, all 24 and 72 hour ICI-treated samples clustered independently from E2 and 4OHT-treated samples (data not shown). 4OHT-regulated expression more closely mirrors the agonist E2 versus the antagonist ICI, consistent with the ability of 4OHT to induce growth in MM134 cells.

Tamoxifen-induced gene expression was also assessed in full serum in MCF-7 and MM134. In MCF-7, expression changes for all tamoxifen compounds paralleled those with ICI (Supplementary Fig. S3A). However, in MM134, tamoxifen-
Figure 4. Novel PDX HCI-013 is estrogen responsive. A, 5 μmol/L sections of FFPE blocks from HCI-013 and HCI-005 stained as indicated. E-Cadherin/p120 staining was previously described (40). B, PDX growth in defined estrogen conditions. Lines represent individual tumors. Growth rates ± SD shown.

C, Ki-67 under indicated estrogen conditions. D, differential gene expression; d5 sham surgery (+E2) versus d10 pellet removal (–E2; q < 0.05) shown as log2 fold change (–E2 vs. +E2). E, example genes identified in D. Points, mean ± SD.
induced changes were opposite to those with ICI for 34/107 genes (Supplementary Fig. S3B). These genes all were ICI induced, but tamoxifen repressed (Supplementary Fig. S3C), including cell-cycle regulators (CDKN1C, MAP3K8) and growth factor receptors (ACVR1B, ACVR2A). These data are consistent with the agonist activity of 4OHT being specific to MM134 versus MCF-7.

**FGFR1 is necessary for survival in the presence of tamoxifen in ILC cells**

Overexpression of FGFR1 has been implicated in endocrine resistance and sensitivity to FGFR inhibitors (5, 14, 35). Both MM134 and SUM44 carry high-level amplification of this locus at 8p12, and overexpress FGFR1 (Supplementary Fig. S4A). MM134 cell growth was assessed following treatment with the FGFR1 inhibitor PD173074 in the absence of E2 (−E2) or presence of 4OHT (+4OHT) or E2 (+E2). PD173074 reduced cell growth +E2; however, growth was reduced below control −E2 or +4OHT (Fig. 7A). Similar results were observed using LY294002, which inhibits phosphoinositide 3-kinase, a downstream FGFR1 effector (Fig. 7B), as well as FGFR1 inhibitor SU5402 (Supplementary Fig. S4B). Lapatinib, an EGFR/HER2 inhibitor, caused no changes in cell growth (Supplementary Fig. S4C). Reduction of growth below controls suggests that FGFR1 inhibition induces cell death specifically −E2 or +4OHT, but not +E2.

Parallel experiments assessed cell death compared with a positive control, staurosporine (STS), which induces extensive cell death. Modest cell death was accumulated −E2 or +4OHT versus +E2; STS induced extensive cell death (Fig. 7C). In +E2, PD173074 induced a moderate amount of cell death; however, in −E2 or +4OHT cell death was comparable with STS-induced death. Though LY294002 was less effective than PD173074, +E2 cells were similarly resistant to LY294002, but were sensitive −E2 or +4OHT (Fig. 7D). Lapatinib treatment did not change total cell death in any condition (Supplementary Fig. S4D). FGFR1 signaling may be required for cell growth and survival −E2, as tamoxifen alone cannot maintain viability when FGFR1 signaling is inhibited.

**Discussion**

Patients with ILC generally present with tumor biomarkers associated with benefit from adjuvant endocrine therapy (reviewed in ref. 5). However, recent observations suggest that patients with ILC may have equivalent or worse outcomes than patients with IDC. Retrospective analysis of the BIG 1–98 trial suggests that a subset of patients with ILC may not benefit from adjuvant tamoxifen (12). Understanding endocrine
Figure 6. Tamoxifen induces gene expression as an ER agonist. A, MM134 cells were hormone deprived and treated with 100 pmol/L E2, 1 μmol/L 4OHT, or 1 μmol/L ICI for 3, 24, or 72 hours. Heatmap represents log2 fold versus time-matched vehicle control. Genes clustered by Pearson complete correlation. +, commonly E2-regulated genes. Dashed white boxes highlight genes regulated by 4OHT as an agonist. B, gene expression (log2 fold vs. control) per treatment and time point for all genes in indicated clusters. Points represent mean of n genes ± SD. P values represent treatment effect by two-way ANOVA.
response and resistance in ILC is necessary to identify and treat endocrine resistance in ILC.

Despite extensive study in IDC, minimal data exist about the endocrine responsiveness of ILC. This report represents the largest study on ER function and endocrine response specifically in ILC cells. Although >90% of ILC are ER positive, most model systems for ILC, including a mouse model (36) and three cell lines (5), are ER negative. These models may recapitulate other aspects of ILC biology, but studies in endocrine response and resistance are critical. Our studies are limited to the only ER-positive ILC models available, MM134, SUM44, and PDXHCI-013. Our observations demonstrate the need for new models to further investigation and facilitate clinical translation.

Among several genomic studies of ILC tumors (5, 37–39), the only consistent finding is the loss of E-cadherin (CDH1), a hallmark of ILC (40). We previously observed that ER can regulate CDH1 expression (41), and hypothesized that E-cadherin loss may mediate tamoxifen resistance. We abrogated E-cadherin in MCF-7 using antibodies or siRNA-mediated knockdown, but these preliminary experiments did not induce endocrine resistance (data not shown). Thus, we further characterized ER function in ILC cell lines.

The partial agonist effect of tamoxifen in MM134 cells may have important implications for the treatment of patients with ILC. As a partial agonist, tumor growth may initially decrease versus pretreatment. Tamoxifen alone induces cell death versus estrogen, and coupled with decreased growth may initially produce a favorable response. However, the overall growth observed suggests that ILC tumors similar to MM134 will outgrow with tamoxifen. Modeling this in vivo will require a xenograft model; however, neither MM134 nor SUM44 have been used in vivo in the literature. PDX HCI-013 represents a potential model, in particular, given the E2 regulation of ILC-specific genes. Though the de novo agonist activity of tamoxifen was specifically observed in MM134, one other study has characterized tamoxifen resistance in ILC cells, and observed tamoxifen-induced growth in resistant SUM44 clones (13). Thus, this may be a common mechanism of endocrine resistance among a subset of ILC tumors. Although the expression of tamoxifen-regulated genes may help identify these tumors, FGFR1 amplification or overexpression may also identify these tumors.

Previous reports identified FGFR1 as a novel target for ILC and a driver of endocrine resistance (14, 35). Our observations suggest that either tamoxifen or FGFR1 inhibitor monotherapy may be suboptimal against tamoxifen-resistant ILC. Combination therapy produced maximum cell death in vitro and may be most effective by blocking both pathways required for growth and survival. FGFR1 amplification may be a common event in primary ILC (14); however, public data from the Cancer Genome Atlas suggest that FGFR family amplification occurs in approximately 10% of both ILC and IDC (data not shown). Recurrent or metastatic ILC may be unique versus primary tumors. A recent analysis of recurrent ILC did not observe FGFR1 amplification in primary tumors, but three of 11 recurrent tumors harbored amplification (42). Another
inhibitor with endocrine therapy. We have gained FGFR1 amplification and are therefore tamoxifen resistant. Tamoxifen-resistant recurrent ILCs that were observed primarily for repressed genes. This suggests that although MM134 cells have acquired FGFR1 amplification, they may benefit from an FGFR1 inhibitor with endocrine therapy.

One of the most strongly induced ILC-specific genes is WNT4. The mouse homolog Wnt4 is a critical mediator of ductal elongation and side-branching during mammary gland development (44). Though Briskin and colleagues demonstrated that Wnt4 expression is PR driven, ILC cells may hijack this pathway by placing it under the control of ER. Consistent with this, an ER-binding site near the WNT4 promoter is MM134 specific, and is not observed in IDC cells (33). Taken together, ILC cells may utilize a WNT4-driven developmental pathway to drive E2-induced growth.

Conversely, gene repression was enriched among ILC-specific genes, and the agonist effect of tamoxifen in the ILC-E2 geneset was observed primarily for repressed genes. This preferential repression may be sufficient to partially drive growth, but may explain why tamoxifen cannot fully induce growth or maintain viability versus E2. This suggests that in ILC cells, both estrogen and tamoxifen may recruit unique corepressor complexes. Though the role of ER cofactors in tamoxifen resistance is actively studied (45–47), no unique cofactors have been reported in ILC, making this an important future direction. Interestingly, forkhead motifs were not enriched in MM134-specific distal ER-binding sites, suggesting that alternative factors may be required for ER binding. Though not representative of distal sites, MM134 ER-binding sites within 20 kb of an ILC-specific gene were weakly enriched for Pax4 motifs (Supplementary Fig. S2C), consistent with the hypothesis that novel cofactors regulate ER binding in ILC. However, these represent subset analyses, and should be interpreted with caution. Of note, Hsu and colleagues characterized MM134 distal EREs in MCF-7; amplification of distal EREs may be permissive for tamoxifen resistance. Tamoxifen-resistant recurrent ILCs that have acquired FGFR1 amplification may benefit from an FGFR1 inhibitor with endocrine therapy.

Our characterization of ER function in ILC represents an important basis for future studies in endocrine response and resistance, as this has previously only been studied in IDC models. Further investigation in to ER-mediated gene expression in ILC, utilizing in vitro models and in vivo PDX models, may identify additional genes that can be targeted in conjunction with endocrine therapy to modulate ER activity in ILC tumors. Our observations that ER drives a unique program of gene expression in ILC cells correlates with the ability of tamoxifen to act as an agonist to induce growth in these cells. This phenotype mimics recent clinical observations that a subset of ILC tumors may be tamoxifen resistant. Genes regulated by tamoxifen as an agonist may serve as powerful biomarkers to identify de novo tamoxifen resistance, thus we envision that a neoadjuvant window trial may be an ideal setting to further examine tamoxifen resistance in patients with ILC.

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

Authors’ Contributions

Conception and design: M.J. Sikora, S. Oesterreich
Development of methodology: M.J. Sikora, A. Bahreini, G. Wang, A.L. Welm
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Sikora, G. Wang, A.L. Welm
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.J. Sikora, K.L. Cooper, A. Bahreini, S. Luthra, U.R. Chandran, N.E. Davidson
Writing, review, and/or revision of the manuscript: M.J. Sikora, A. Bahreini, U.R. Chandran, N.E. Davidson, D.J. Dubbs, S. Oesterreich

Study supervision: S. Oesterreich

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