Differential regulation and targeting of estrogen receptor α turnover in invasive lobular breast carcinoma

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Abstract

Invasive lobular breast carcinoma (ILC) accounts for 10-15% of breast cancers diagnosed annually. Evidence suggests that some aspects of endocrine treatment response might differ between invasive ductal carcinoma (IDC) and ILC, and that patients with ILC have worse long-term survival. We analyzed The Cancer Genome Atlas dataset and observed lower levels of ESR1 mRNA ($P=0.002$) and ERα protein ($P=0.038$) in ER+ ILC ($n=137$) compared to IDC ($n=554$) and further confirmed the mRNA difference in a local UPMC cohort (ILC, $n=143$; IDC, $n=877$; $P<0.005$). In both datasets, the correlation between ESR1 mRNA and ERα protein was weaker in ILC suggesting differential post-transcriptional regulation of ERα.

In vitro, 17β-estradiol (E2) decreased the rate of degradation and increased the half-life of ERα in ILC cell lines, whereas the opposite was observed in IDC cell lines. Further, E2 failed to induce robust ubiquitination of ERα in ILC cells. To determine the potential clinical relevance of these findings, we evaluated the effect of two selective estrogen receptor down-regulators (SERDs), ICI182,780 and AZD9496, on ERα turnover and cell growth. While ICI182,780 and AZD9496 showed similar effects in IDC cells, in ILC cell lines, AZD9496 was not as effective as ICI182,780 in decreasing ERα stability and E2 induced proliferation. Furthermore, AZD9496 exhibited partial agonist activity in growth assays in ILC cell lines.

Our study provides evidence for a distinct ERα regulation by SERDs in ILC cell lines and therefore it is important to include ILC models into preclinical and clinical testing of novel SERDs.

Keywords: Estrogen Receptor, breast cancer, Invasive lobular carcinoma, endocrine response
Background

Invasive lobular breast carcinoma (ILC) accounts for 10-15% of breast cancers diagnosed annually, making it the second most common histological subtype of invasive breast cancer diagnosed in women. While most ILCs cluster with luminal A (LumA) like tumors, they have clinical, pathological and molecular characteristics that are distinct from the more common subtype invasive ductal breast carcinoma (IDC). The hallmark of ILC tumors is loss of the adherens junction protein E-Cadherin, leading to a ‘single-file pattern’ of tumor growth. The majority of ILC tumors display favorable prognostic markers including estrogen receptor (ER) positivity and low Ki67 levels. Nevertheless, a subset of ILC tumors have been reported to be less responsive to endocrine therapy compared to stage-matched IDC tumors, and patients suffer from recurrence, often many years after the original diagnosis. Previous studies from our group have uncovered a unique estrogen response and de novo endocrine therapy resistance in human ILC cell lines.

Very few studies have compared ERα protein levels between IDC and ILC, with little concordance between the existing data. Some studies have detected similar ERα levels, while an analysis as part of a larger TCGA study reported decreased ERα levels in LumA ILC compared to LumA IDC tumors. In contrast, an additional study reported increased levels of ERα in ILC compared to IDC. However, the levels of ESR1 mRNA and the correlation between ERα mRNA expression and its protein abundance in IDC and ILC tumor samples have not previously been compared.

Regulation of ERα levels is fundamental in determining the receptor’s activity and response to therapy. ERα protein turnover is suggested to play a role in the development and progression of breast cancer (reviewed in ). 17β-estradiol (E2), the natural ligand of ERα, induces the degradation of ER via the ubiquitin-proteasomal pathway in breast cancer cells. Over-expression of various proteins and altered post-translational modifications of ERα can stabilize the receptor by inhibiting its degradation. There is evidence that increased stability of ERα results in increased ER activity contributing to E2-induced proliferation and hormonal resistance in breast cancer cells. However, there is also evidence that ER degradation is uncoupled from transcriptional activity, and prior and recent studies showing that full ER degradation does not ensure complete ER antagonism.

Selective estrogen receptor down regulators (SERDs) induce degradation of ERα via the proteasomal machinery. Fulvestrant (ICI 182, 780, ICI) is the only SERD currently
approved for the treatment of metastatic breast cancer. Owing to its present limitations of dosing and intramuscular route of administration, there is a need for oral SERDs with improved bioavailability. AZD9496 is a newly developed orally bioavailable, non-steroidal SERD. AZD9496 displayed significant tumor growth inhibition in \textit{in vitro} and \textit{in vivo}, including in a series of endocrine resistant models. Recent results from a Phase 1 study in women with ER+/HER2– advanced breast cancer show that AZD9496 is well tolerated and has an acceptable safety profile. Since ILC is a distinct subtype of breast cancer displaying unique ER biology, evaluating the efficacy of AZD9496 in inducing ERα degradation and growth inhibition of ILC tumor models is critical to assessing the potential utility of this compound in the clinical management of patients with ILC.

In this study, we compared the levels of ESR1 mRNA and ERα protein, as well as the ERα mRNA-protein correlation, in IDC and ILC tumors from the TCGA dataset and in a local patient tumor tissue cohort. Our studies revealed significantly weaker correlation between ESR1 mRNA and ERα protein levels in ILC compared to IDC. Extensive analyses of ERα protein levels, E2-induced ERα protein expression and turn-over in ER+ estrogen responsive human IDC (MCF-7, T47D, ZR-75-1) and ILC (BCK4, MDA-MB-134-VI, SUM44PE) cell lines showed that E2 decreased the rate of degradation and increased half-life of ERα uniquely in the ILC models. Our studies suggest that E2 treatment failed to induce robust ubiquitination of ERα in the ILC cell line MDA-MB-134-VI, likely contributing to the extended ERα half-life. Furthermore, we found that the novel orally available SERD AZD9496 is less effective than ICI 182,780 in degrading ERα in ILC cell lines, and that it acts as a partial agonist in ILC but not in IDC models. In addition to pinpointing unique aspects of ERα biology in ILC, our data also underline the necessity of including models representing different histological breast cancer subtypes into development of novel SERDs.
Materials and Methods

TCGA Dataset Analysis

We utilized publicly available data from The Cancer Genome Atlas (TCGA, RRID:SCR_003193)\(^{39,40}\) to perform \textit{in silico} analyses of \textit{ESR1} and \textit{PGR} mRNA and ER\(\alpha\) and PGR protein expression between ER+ ILC (n = 137) and IDC (n = 554). TCGA RNA-Seq expression data (transcripts per million, TPM) were downloaded from the Gene Expression Omnibus database [GEO:GSE62944]\(^{41}\). For protein expression, reverse-phase protein array (RPPA) data were downloaded as median-normalized, batch-corrected expression values from the TCGA website (Level 4, version 4.0)\(^{42}\). The correlation between \textit{ESR1} mRNA and ER\(\alpha\) protein levels were calculated as Pearson (r) correlations and comparisons of correlations was performed using Fisher r-to-z transformation, followed by a two-tailed test using R (version 3.5.1).

Analysis of UPMC Study Population and Design

ER immunohistochemistry H-scores and \textit{ESR1} and \textit{PGR} mRNA levels were analyzed from tumor samples collected from patients with ER+ ILC (n = 143) and IDC (n = 877) treated at UPMC Magee Women’s Hospital, Pittsburgh (hereafter referred to as the “UPMC” dataset). \textit{ESR1} and \textit{PGR} mRNA levels were quantified using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as part of the Oncotype DX\textregistered{} (Genomic Health, Redwood City, CA, USA) analysis as described previously\(^{43}\). Immunohistochemical (IHC) detection of ER was performed using the clone SP1 ER antibody (Ventana Medical Systems, Tucson, AZ, USA, RRID:AB\_2857956)\(^{44}\), and PR IHC was performed using the anti-PR (1E2) antibody (Ventana Medical Systems, Tucson, AZ, USA, RRID:AB\_2335976)\(^{45}\). The protein levels of the receptors were scored using a semi-quantitative modified H-score method reported previously\(^{46–48}\). The \textit{ESR1} mRNA and ER\(\alpha\) protein correlation was calculated using Pearson correlation analysis as described above.

Cell culture and reagents

BT474 (RRID:CVCL\_0179)\(^{49}\), HCC1500 (RRID:CVCL\_1254)\(^{50}\), MCF-7 (RRID:CVCL\_0031)\(^{51}\), MDA-MB-231 (MM-231) (RRID:CVCL\_0062)\(^{52}\), MDA-MB-330 (MM-330) (RRID:CVCL\_0619)\(^{53}\), T47D (RRID:CVCL\_0553)\(^{54}\) and ZR-75-1 (RRID:CVCL\_0588)\(^{55}\) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). BT474, HCC1500, T47D and ZR-75-1 cells were maintained in RPMI-1640 (Product No: 11875119; Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS. MCF-7, MDA-MB-231 and MDA-MB-330 cells were maintained in DMEM (Product No: 11965; Life Technologies) supplemented with 10% FBS. MDA-MB-134-VI (MM134, ATCC,
RRID:CVCL_0617) 56 and SUM44PE (SUM44, Asterand Bioscience, Detroit, MI, USA, RRID:CVCL_3424) 57 cells were cultured as previously described 13. BCK4 cells kindly provided by Dr. Britta Jacobsen (Anschutz Medical Campus, Colorado) were cultured as detailed earlier 58. All cell lines were maintained in a 5% CO2 incubator at 37°C. Cell lines were authenticated by Arizona Research Laboratories, University of Arizona, Tucson, Arizona, and were tested to be mycoplasma negative. In hormone deprivation experiments, cells were grown in phenol red-free improved minimum essential medium (IMEM) supplemented with charcoal-stripped FBS (CSS) (12676, Life Technologies), as described earlier 13,59. The cell lines were maintained in IMEM with 2% (SUM44), 5% (MCF-7, T47D, ZR-75-1, BCK4) or 10% (MDA-MB-134-VI) CSS. 17β-Estradiol (E2), 4-hydroxytamoxifen (4-OHT), Cycloheximide and MG132 were obtained from Sigma-Aldrich (St. Louis, MO, USA). ICI 182,780 (Fulvestrant, ICI) was obtained from Tocris Bio-sciences (Bristol, UK). AZD9496 was provided by AstraZeneca, Macclesfield, United Kingdom. Additional experiments (Figure S4A, B) 60 were performed using commercially available AZD9496 (Cayman Chemical). The stock solutions of the compounds were prepared in DMSO and the final concentration of DMSO in the working solutions were kept at less than 0.01%.

**Immunoblotting**

Whole cell protein extracts were prepared in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.5% Na-deoxycholate, 50 mM NaF, 1% NP-40, and 0.1% sodium dodecyl sulfate (SDS)) containing protease-phosphatase inhibitors (Halt Phosphatase/Protease Inhibitor Cocktail, Thermo Fisher Scientific). The proteins were separated on SDS-PAGE and transferred to PVDF membrane. Primary antibodies used were: ERα (1:1000, 6F11, Novocastra/Leica, Germany, RRID:AB_876939) 61, E-cadherin (1:500, HEC-1, Life Technologies, RRID:AB_211510) 62 and β-Actin (1:5000, A5441, Sigma, RRID:AB_476744) 63. Goat-anti mouse (RRID:AB_621842) 64 or rabbit (RRID:AB_621843) 65 IRDye800CW secondary antibody was obtained from LI-COR Biosciences (Lincoln, NE). The blots were scanned using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and quantified using Image Studio Lite Version 2 (LI-COR Biosciences).
Immunocytochemical (ICC) staining for ERα

Cells grown in the above-mentioned standard culture conditions were pelleted and fixed with 10% neutral-buffered formalin and embedded in paraffin. Immunocytochemical detection of ER was performed on 5-μm sections using Clone SP1 ER antibody (RRID:AB_2857956) and the iVIEW detection on the Benchmark XT system (Ventana, Tuscon, AZ) at the UPMC Magee Womens Hospital Histology Core.

RNA isolation, qRT-PCR, and DNA Copy number analysis

Total RNA was prepared by illustra™ RNaSpin Mini Isolation Kit (GE Healthcare Bio-Sciences, Buckinghamshire, UK), according to the manufacturer's instructions. RNA was reverse-transcribed using iScript RT master mix (Bio-Rad, Hercules, CA, USA). The genes of interest were amplified by quantitative PCR (qPCR) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) using the following primer pairs:

- **ESR1** Forward, GAGTATGATCCTACCAGACCCTTC; **ESR1** Reverse, CCTGATCATGGAGGCTCAAATC; **RPLP0** Forward, TAAACCCTGCGTGGCAATC; **RPLP0** Reverse, TTGTCTGCTCCACAATGAAA; **GREB1** Forward, AAATCGAGGATGTGGAGTG; **GREB1** Reverse, TCTCACCAGACGAGGAG; **PGR** Forward TCGCCTTAGAAAGTGCTGTC; **PGR** Reverse, GCTTGGCTTCTTATTGGGAACG; **RNF148** Forward CCGAGGGCGAGTCAAATAA; **RNF148** Reverse, CCCTTGTACCTTGGGTAGAG; **UBE2E3** Forward, CGGGTTCTGTATATGAAGGTGG; **UBE2E3** Reverse, GATGACTCCCTGACTTGTTAGT.

qRT-PCR was performed on a CFX384 thermocycler (Bio-Rad, Hercules, CA, USA), according to manufacturer’s instructions. Relative mRNA levels were calculated using the comparative cycle threshold method (ΔΔCt). **ESR1** DNA copy number in MCF-7 and ILC cell lines were analyzed as previously reported using a NanoString based method.

Proliferation Assays

Cells were hormone deprived and plated in 96-well plates (IDC- 5000-8000 cells/well; ILC 15000-18000 cells/well), allowed to attach overnight and were treated with the specified ligands. After the indicated duration of treatment, cells were harvested, and cell proliferation was measured using FluoReporter Blue Fluorometric dsDNA Quantitation Kit (F2692; Life Technologies) according to the manufacturer’s instructions. Fluorescence was assessed using a VICTOR X4 plate reader (PerkinElmer, Waltham, MA, USA). Data are presented as
mean of six biological replicates ± SD. Each experiment was repeated two to three independent times with similar trends of results.

**Cycloheximide (CHX) chase experiments**

The half-life of ERα in cell lines was determined by Cycloheximide (CHX) chase assay. IDC and ILC cell lines were maintained in complete growth medium or treated with vehicle (0.001% DMSO), 1 nM E2, 100 nM ICI 182,780 or 100 nM AZD9496 after hormone deprivation for 3 days. Cells were treated with cycloheximide (CHX, 50 μg/ml), with addition of CHX considered as time 0 (T= 0). Following addition of cycloheximide, cells were lysed at the times of 3, 6, 12, 24 and 48h. Lysates were separated by SDS-PAGE, and ERα levels were measured by immunoblotting as described above. ERα protein levels relative to β-actin were quantitated by densitometry using Odyssey imaging system (Licor, Lincoln, NE). Half-life of the protein was calculated using one-phase exponential decay curve using GraphPad Prism software.

**Ubiquitination pathway PCR Array**

After hormone depriving for 3 days, MCF-7 and MDA-MB-134-VI cells were treated with 1 nM E2 for 6h. Total RNA was isolated with RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA was reversed transcribed using the RT² First Strand kit (SA Biosciences, Frederick, MD, USA). The expression of 84 Ubiquitin pathway related genes was analyzed using RT² profiler PCR array PAHZ-079ZD (Human Ubiquitination Pathway Array, SA Biosciences) on the CFX96 thermocycler (BioRad, Hercules, CA, USA) as per the manufacturer’s recommendations. The array was performed at three independent times and the data analyzed represents these biological repeats. Analysis for relative changes in gene expression was performed with SA Biosciences RT² Profiler PCR Array Data Analysis software v3.5 using the comparative threshold cycle (ΔΔCt) method. Heat maps were generated using Multiple Experiment Viewer (MeV).

**Ubiquitination Assay**

Ubiquitination Assay was performed as described previously, with modifications. Briefly MCF-7 and MDA-MB-134-VI cell lines were hormone deprived for 4 days and pretreated with proteasomal inhibitor MG132 (10 mM, Calbiochem, La Jolla, CA) for 30 min. Cells were then treated with Vehicle (0.01% DMSO) or 1 nM E2 for 5 h. Following treatment, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 50 mM NaF, 1% NP-40. Lysates were pre-cleared by incubation with normal rabbit-IgG (sc-2027) and Protein G-Plus Agarose beads (sc-2002, Santa Cruz Biotechnology, TX, USA) for 2h at 4°C. Immunoprecipitations were carried out using anti-ERα antibody (HC-20;
Santa Cruz Biotechnology, RRID:AB_631471) and Protein G-Plus Agarose beads. Beads were washed four times in lysis buffer and boiled in 2x SDS sample buffer. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were boiled in transfer buffer for 10 min and probed for ubiquitinated ERα using anti-Ubiquitin antibody (P4D1; Santa Cruz Biotechnology, TX, USA, RRID:AB_628423), followed by goat-anti mouse IRDye800CW antibody (Licol, Lincoln, NE, RRID:AB_621842). The bands were visualized using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

**Statistical Analysis**

Unless otherwise specified, statistical analyses were performed using GraphPad Prism 7 (San Diego, CA, USA) software. The experiments were repeated a minimum of two independent times. Each figure is from a representative experiment and number of independent repeats indicated in the figure legend. In all experiments, statistical comparisons were made between control and treatment groups using unpaired t-test (two groups), one-way/two-way ANOVA (three or more groups) with Bonferroni or Dunnett’s multiple comparison test, or Wilcoxon rank-sum tests, as indicated. \( P \) values <0.05 were considered as significant and are indicated by asterisks in figures (****, \( P < 0.0001 \); ***, \( P < 0.001 \); **, \( P < 0.01 \); *, \( P < 0.05 \)).
Results

ILC tumors display discordant ESR1 mRNA levels and ERα protein expression compared to IDC tumors

We utilized the TCGA dataset to compare ESR1 mRNA and ERα protein levels between ER+ ILC (n = 137) and ER+ IDC (n = 417) tumor samples. Tumor ESR1 mRNA and ERα protein levels had been assessed by RNA-Seq and RPPA, respectively. ER+ ILC tumors displayed significantly lower levels of ESR1 mRNA (P = 0.0021) (Fig 1A) and ERα protein (P = 0.038) (Fig. 1B) compared to ER+ IDC tumors. Despite lower ESR1 mRNA and ERα protein levels in ILC, we did not detect significant decreases in PGR mRNA expression (Fig. 1C) and even slightly higher PGR protein level (Fig. 1D). Given the lower expression if ESR1 in ILC relative to IDC we assessed the correlation between ESR1 mRNA and protein levels and observed that this was also significantly weaker in ER+ ILC (r = 0.58) compared to IDC (r = 0.80) (P = 5.9e-6) (Fig. 1E).

Next, we analyzed ERα and PGR IHC H-scores and mRNA levels in tissue specimens from patients with ER+ ILC (n = 143) and IDC (n = 876) treated at UPMC Magee Women’s Hospital. Despite having significantly (P < 0.0037) lower ESR1 mRNA levels (Fig 1F), ILC tumors displayed similar ERα IHC H-scores (ILC, H-score = 246) compared to IDC tumors (IDC, H Score = 252, P = 0.056) (Fig. 1G). Similar to our results from the TCGA analyses, PGR levels were not significantly different between ILC and IDC (Fig. 1H-I). In further concordance with the observations from the TCGA dataset, the correlation between ERα mRNA and protein levels was found to be significantly (P = 0.0017) weaker in ER+ ILC (r = 0.34) compared to ER+ IDC (r = 0.57) tumors (Fig. 1J). The consistently weaker ERα mRNA-protein correlation in ILC compared to IDC samples suggests differences in post-transcriptional regulation of ERα, potentially due to increased protein synthesis, decreased protein degradation, or increased protein stability.
ILC cell lines are estrogen responsive and demonstrate higher ERα levels than IDC cell lines

To assess any potential differences in the post-transcriptional regulation of ERα between ILC and IDC, we measured ERα protein levels, ESR1 mRNA expression and DNA copy number in a series of breast cancer cell lines. We included the four most commonly used ER+ IDC cell lines MCF-7, T47D, BT474, and ZR-75-1. We also included HCC1500 since it showed the highest ESR1 mRNA expression in the Cancer Cell Line Encyclopedia (CCLE) data set (Fig. S1A) 60. MDA-MB-231 served as an ER-negative IDC cell line control. For ILC, we used all currently available ER+ cell lines: MDA-MB-134-VI and SUM44 69–71, MDA-MB-330 with disrupted adherens junctions through α-catenin (CTNNA1) mutations 72 and BCK4, a recently described model of lobular disease of mucinous origin 58.

ESR1 mRNA analysis showed the lowest levels in BT474 and ZR-75-1 cells, and the highest levels in MDA-MB-134-VI, SUM44 and HCC1500 (Fig. 2A). These findings are consistent with publicly available mRNA expression data from the CCLE and Marcotte et al (Fig. S1A, S1B) 60,73. The observed higher mRNA expression was not a result of altered ESR1 DNA copy number (Fig. S1C) 60. In general, mRNA expression correlated well with ERα protein expression as measured by immunoblotting (Fig. 2B). We also performed immunohistochemical staining of ERα in cell line pellets and observed intense and mostly nuclear ERα staining in MDA-MB-134-VI and SUM44 cells with less heterogeneity compared to IDC cell line pellets (Fig. 2C).

To assess endocrine response in selected IDC and ILC cell lines, cells were hormone deprived and stimulated with 17β-estradiol (E2). E2 induced the proliferation of all cell lines with EC50 values ranging between 1 – 30 pmol/L (Fig. 2D). In general, the magnitude of the E2 response is weaker in ILC lines despite the higher ERα levels, as we have previously described 8,13. The expression of the classical E2-induced genes GREB1 and PGR was analyzed after treatment with 1nM E2 ± 1µM ICI 182,780 for 6h. In all cell lines, E2 induced the expression of GREB1, which was antagonized by the SERD, ICI 182,780 (Fig. 2E), confirming the ER-dependency of the effects. Thus, both the ILC and IDC cell lines used in our study were demonstrated to be ER responsive.
Differential estradiol induced turnover of ERα in ILC and IDC cell lines

E2 stimulation has been reported to induce ERα turnover in breast cancer cells. We examined the effect of E2 stimulation (1 nM) spanning several hours in IDC (MCF-7, T47D, and ZR-75-1) and ILC cell lines (BCK4, MDA-MB-134-VI, SUM44) and noted a differential response. Treatment with E2 decreased ERα protein levels in all three IDC cell lines. A 40-60% reduction in ERα protein levels in IDC cells was detected in time course experiments up to 24 h after E2 treatment. 48 h after treatment with E2, the reduction in ERα levels continued at significant levels in T47D and at nonsignificant levels in MCF-7 and ZR-75-1 (Fig. 3A). In the ILC cell lines, ERα protein levels transiently decreased in the BCK4 (significantly) at 3h (29%) and 6h (43%), and SUM44 (non-significant trend) at 3h (34%) post E2 treatment. In stark contrast, ERα levels paradoxically increased in MDA-MB-134-VI ILC cells upon E2 treatment (Fig. 3A). Furthermore, the increase in ERα protein levels in MDA-MB-134-VI cells was dose-dependent (Fig. 3B).

We next conducted cycloheximide chase studies to determine the contribution of protein degradation in regulating ERα protein levels in these cell lines. Consistent with the differential effects of E2 on ERα levels in IDC and ILC cells, E2 decreased the half-life of ERα protein in IDC cells lines and caused an increase in the half-life of ERα protein in both SUM44 and MDA-MB-134-VI cell lines following hormone deprivation (Fig. 3C).

Since E2-induced proteasomal degradation of ERα is preceded by poly-ubiquitination of the receptor, we assessed the ubiquitination of ERα in the presence of MG132, a proteasome inhibitor. We performed these studies in MDA-MB-134-VI cells, in which the observed effects of E2 on protein stability were the strongest among the ILC cells tested. In contrast to MCF-7 cells, we did not detect ubiquitination of ERα in MDA-MB-134-VI cells after treatment with E2 (Fig. 3D). Since this data suggested a potential deregulation of the ubiquitin pathway in MDA-MB-134-VI cells, we treated MCF-7 and MDA-MB-134-VI cells with E2 and analyzed the expression of 84 genes involved in the ubiquitin-proteasomal degradation pathway using an RT-PCR Array (Fig. S2A). In MCF-7 cells, more than 70% of the ubiquitin pathway genes were found to be upregulated/unaltered after exposure to E2 though the fold-change induction of these genes was small. In contrast, in MDA-MB-134-VI cells, E2 treatment did not cause the increase in gene expression observed in MCF-7 cells, but instead elicited moderate repression of many ubiquitin pathway genes but again with small fold-changes (Fig. S2 A-C). These data suggest that in MDA-MB-134-VI cells, ERα protein has an extended half-life potentially in part due to decreased ubiquitination resulting in decreased protein degradation. Further, we compared expression of the top 20 E2 regulated ubiquitination pathway genes in MCF7 (Fig. S2B) between ER+ IDC and ILC in TCGA. We observed that the majority (13/20) of these genes are significantly
downregulated in ILC relative to IDC, suggesting that protein turnover may indeed be altered in these tumors (Fig. S3)\(^{60}\), however, additional studies are needed to confirm a role for ubiquitination pathway in differential mRNA and protein correlation between IDC and ILC\(^{60}\).

**Oral SERD AZD9496 is less effective than ICI 182,780 in degrading ER\(\alpha\) in ILC cells relative to IDC cell lines and displays agonist activity in cell proliferation assays**

In addition to the subtype specific effects in growth and ER\(\alpha\) protein levels in response to E2, we next sought to evaluate any differences to SERDs comparing ILC and IDC cells. To this end, we determined the ability of the novel oral SERD AZD9496 in modulating ER\(\alpha\) levels and inhibiting the growth of ILC (MDA-MB-134-VI, SUM44) and IDC (MCF-7, T47D) cell lines in comparison to ICI 182,780 (ICI). Hormone deprived cells were treated with increasing doses of SERDs for 24h and ER\(\alpha\) protein levels assessed by immunoblotting. Both ICI 182,780 and AZD9496 (10\(^{-12}\) to 10\(^{-8}\) M) were equally efficient in decreasing ER\(\alpha\) levels in MCF-7 cells (IC\(_{50}\) ICI 182,780 = 4.4 \times 10\(^{-10}\) M; AZD9496 = 3.5 \times 10\(^{-10}\) M; Fig 4A). In T47D cells, ICI 182,780 (10\(^{-10}\) to 10\(^{-6}\) M) induced a 15-80% reduction in ER\(\alpha\) levels (IC\(_{50}\) = 3.7 \times 10\(^{-10}\) M) while AZD9496 (10\(^{-11}\) to 10\(^{-6}\) M) induced a dose-dependent 15-50% reduction (IC\(_{50}\) = 6.3 \times 10\(^{-10}\) M) (Fig 4A). Similar to IDC cell lines, ICI 182,780 (10\(^{-11}\) to 10\(^{-6}\) M) robustly reduced ER\(\alpha\) protein levels in both MDA-MB-134-VI and SUM44 cell lines (IC\(_{50}\) ICI 182,780 = 1.3 \times 10\(^{-10}\) M and 4.4 \times 10\(^{-11}\) M respectively). Importantly, in striking contrast to IDC cell lines, AZD9496 was ineffective in down-regulating ER\(\alpha\) proteins levels in both MDA-MB-134-VI and SUM44 cells (IC\(_{50}\) = 1.5 \times 10\(^{-8}\) M and 1.0 \times 10\(^{-7}\) M respectively) except at the highest dose tested (10\(^{-6}\) M, 40% reduction) for SUM44 (Fig 4A). Furthermore, in SUM44 cells, at lower doses of AZD9496, ER\(\alpha\) protein levels were found to be stabilized, an effect similar to that evoked by 4-OHT\(^{74}\).

In order to measure the AZD9496-induced turnover of ER\(\alpha\), CHX chase assays were performed and ER\(\alpha\) protein levels were measured after treatment with SERDs in the presence of cycloheximide. In MCF-7, the half-life of ER\(\alpha\) was decreased at a similar rate by ICI 182,780 (t1/2 = 4.6 h) and AZD9496 (t1/2 = 3.9 h). A moderately increased half-life of ER\(\alpha\) was observed in T47D after treatment with AZD9496 compared to ICI 182,780 (ICI 182,780 t1/2 = 1.4h, AZD9496 t1/2 = -3.5h) (Fig 4B), consistent with the less dramatic reduction in protein levels, relative to MCF-7 (Fig 4A). ER\(\alpha\) protein was more stable in ILC cells in the presence of AZD9496, with a four to ten-fold increase in half-life (SUM44 ICI 182,780 t1/2 = 1.5h, AZD9496 t1/2 = 4.6h; MDA-MB-134-VI, ICI 182,780 t1/2 = 1.1h, AZD9496 t1/2 = 13.5h) compared to ICI 182,780 (Fig 4B). This data supports a differential effect of AZD9496 between the IDC and ILC cell models.
Furthermore, we assessed the efficacy of AZD9496 in inhibiting the growth of ILC and IDC cell lines. E2-induced growth was completely blocked by both AZD9496 and ICI 182,780 in MCF-7 and T47D (Fig 4C). Neither drug had any agonist activity in these cell lines in the absence of E2 (Fig 4D). In MDA-MB-134-VI, ICI 182,780 completely repressed E2-induced growth (Fig 4C) while AZD9496 only partially repressed E2-induced growth, implying inhibition did not reach baseline growth conditions as seen in the IDC cell lines (Fig 4C). In the absence of E2, AZD9496 elicited more than 2-fold growth induction in MDA-MB-134-VI acting as a partial agonist (Fig 4D). These data were repeated in an external laboratory (MJS, Fig. S4A), where the unique partial agonist activity of AZD9496 (using a commercial source of the drug) was again observed in MDA-MB-134-VI. As previously reported by us, ICI 182,780 suppressed the growth of SUM44 in the presence and absence of E2, suggesting some ligand-independent ER activity (Fig 4D); however, AZD9496 was not able to block the ligand-independent growth in SUM44 cells in the presence of E2 (Fig 4C). In addition, similar to MDA-MB-134-VI cells, AZD9496 displayed agonist activities on growth in estrogen deprived SUM44 cells (Fig 4D). Cell proliferation experiments in hormone replete conditions performed in an independent laboratory (MJS) again using a commercial source of AZD9496 confirmed the decreased antagonist activity of AZD9496 in MDA-MB-134-VI and SUM44 cells compared to MCF-7 cells (Fig. S4B (MJS), C-E (SO))

Discussion

Resistance to endocrine therapy is a major problem in the management of ER+ breast cancer. Given that >90% of ILCs are ER+, and that a significant number of patients suffer from late recurrences due to resistance to endocrine therapy, it is critical to increase our understanding of the regulation of ERα expression and activity in ILC. In this study, we show that there is a weaker correlation between ESR1 mRNA transcripts and ERα protein levels in ILC compared to IDC patient samples, and we propose that altered E2-mediated degradation of ER proteins could potentially be playing a role in this observation. Clinical relevance is provided by our findings that the novel SERD AZD9496 demonstrates partial agonism in ILC cell lines, a surprising result that was not observed in the IDC cell lines tested in our study.

Our TCGA analyses demonstrate that both ESR1 mRNA and ERα protein (RPPA) levels are significantly lower in ILC tumors compared to IDC tumors, consistent with the data
from the ILC TCGA working group. Determination of ERα expression levels using IHC in a local tumor cohort revealed similar ERα levels in both histological subtypes, consistent with previous reports using IHC. This discrepancy is likely, at least in part, a result of using IHC rather than RPPA, in that the latter is more affected by tumor cellularity and heterogeneity. Importantly, irrespective of the differences in total ER detected in the TCGA (RPPA) and UPMC (IHC) studies, we observed a significantly weaker ESR1 mRNA-ERα protein correlation in ILC tumors compared to IDC in both cohorts. Although a linear proportional relationship between ER protein and ESR1 mRNA in breast tumors has been identified in multiple studies, non-linear relationships have also been reported, for example in a subset of tumors expressing low ESR1 mRNA levels. The weaker correlation between ESR1 mRNA and ERα protein we observed in ILC tumors compared to IDC in both cohorts. Although a linear proportional relationship between ER protein and ESR1 mRNA in breast tumors has been identified in multiple studies, non-linear relationships have also been reported, for example in a subset of tumors expressing low ESR1 mRNA levels. The weaker correlation between ESR1 mRNA and ERα protein we observed in ILC tumors compared to IDC in both cohorts. Although a linear proportional relationship between ER protein and ESR1 mRNA in breast tumors has been identified in multiple studies, non-linear relationships have also been reported, for example in a subset of tumors expressing low ESR1 mRNA levels. The weaker correlation between ESR1 mRNA and ERα protein we observed in ILC tumors compared to IDC in both cohorts. Although a linear proportional relationship between ER protein and ESR1 mRNA in breast tumors has been identified in multiple studies, non-linear relationships have also been reported, for example in a subset of tumors expressing low ESR1 mRNA levels. The weaker correlation between ESR1 mRNA and ERα protein we observed in ILC may result from altered protein translation efficiency in ILC recently reported by us. Alternatively, or in parallel, there could be unique mechanisms of ERα degradation in place, which should be tested further in future studies.

The observed high expression of ERα in ILC cell lines is somewhat contradictory to our finding in human tumors. Potential explanations for this include the notorious difficulty of establishing ER+ cell lines, which is amplified by the slow growth of ILC cell lines. It is possible that currently available ILC models represent a minority of the heterogenous ILC cases with the ability to propagate in vitro and in which ER is highly expressed; additional work is needed to fully characterize these models within the context of ILC heterogeneity. Another possible explanation is that many ILC tumors have a lower tumor cellularity compared to IDC owing to their unique growth pattern, and in this study we did not control for tumor cellularity. Since it is well established that E2 regulates the steady state levels of ERα protein by inducing its degradation via the ubiquitin-proteasomal system, we characterized ERα expression in all currently available ER+ ILC cell line models, and three ER+ IDC cell lines in order to study ERα protein level changes in response to E2 stimulation. As expected, E2 caused downregulation of ERα protein levels in the IDC cell lines MCF-7, T47D and ZR-75-1 for up to 24-48 hrs; however, there was heterogeneity in response in the ILC cell lines, with BCK4 showing ERα downregulation limited to early timepoints, no significant downregulation in SUM44, and increased in ERα protein levels in MDA-MB-134-VI. These data, supported by measurement of ERα half-lives, suggested different mechanisms of ERα turnover in ILC vs IDC cells, however, this need to be studied in more detail. Our data also suggests a heterogeneity of response to E2 in ERα levels within ILC, which needs to be considered in further studies, and emphasizes the need for generation of additional ILC models. Given that lack of E-cadherin is the hallmark of ILC, it will be important in future...
studies to assess if E-cadherin plays a role in altering ERα half-life by assessing that effect of CDH1 overexpression and downregulation in ILC and IDC cell lines, respectively.

The lack of E2-mediated decrease of ERα in MDA-MB-134-VI was noted in a prior report \(^81\), and our studies now reveal mechanistic insights for this prior observation, suggesting that E2 stimulation fails to activate ubiquitination and the subsequent proteasomal degradation of ERα in this ILC cell line model. Lack of ERα polyubiquitination has been suggested to be associated with breast cancer progression and endocrine therapy resistance (Reviewed in \(^21\)). Along these same lines, E2-induced ERα degradation was also found to be absent in tamoxifen-resistant endometrial adenocarcinoma cell lines \(^82,83\). It is possible that the lack of ERα degradation could contribute to the previously reported tamoxifen-resistance in MDA-MB-134-VI \(^13\). Of note, despite stabilized ERα levels, MDA-MB-134-VI cells remain responsive to E2 and have transcriptionally active ER. This data is in contrast with the idea that degradation of ERα is essential for E2-induced transcriptional activation of ER \(^24\). We are currently leading a preoperative window trial of endocrine response in women with ILC (NCT02206984) where patients are randomized to receive either tamoxifen, fulvestrant or anastrozole and the change in Ki67 from baseline to post-treatment will be correlated with a number of other biomarker including ER levels, and downstream signaling.

To potentially link changes in ERα degradation with response to SERDs, we compared the effects of ICI 182,780 (fulvestrant) to the novel potent and orally bioavailable SERD AZD9496. ICI 182,780 was equally efficacious in IDC and ILC cell lines including in MDA-MB-134-VI cells that lacked E2-mediated degradation of ERα, rendering these cells as a great model for probing differences between E2 and SERD-mediated degradation of ERα. However, we made the unexpected discovery that AZD9496 acts as a partial agonist in the ILC cell lines tested. Given that SERDs are generally considered to lack agonist activity in breast cancer, this is the first report of AZD9496 displaying agonist activity in breast cancer cells. Of note, Weir et al have recently described an agonist activity of AZD9496 in uterine cells, an effect that was not seen with ICI 182,780 treatment \(^34\). The agonist activity in ILC cells observed herein was accompanied by decreased effects on ERα protein degradation, but further studies are required to test if and how these observations are causatively linked. It is also possible that AZD9496 causes a unique recruitment of classical ERα co-factors in ILC cells, similar to what has been described as a mechanism for tamoxifen's agonist activity \(^84,85\). A prime candidate is FoxA1 which has been shown to be more frequently altered in ILC compared to IDC, and has been suggested to play a role on endocrine resistant ILC \(^16,86\). Finally, given the recent studies showing that partial ER agonists cause increases in chromatin accessibility, ER dynamics in ILC cells should be further explored as it is not
currently known how intra-nuclear mobility of ER differs between ILC and IDC, and the relationship of this mobility to their response to novel SERDs.

In summary, our study adds to the existing evidence for unique ERα biology in ILC, which could contribute to endocrine resistance in some tumors. Further pre-clinical studies are warranted to understand the mechanisms underlying this unique biology and pharmacology. Importantly, our data calls for consideration of histological breast cancer subtypes in pre-clinical studies, as well as in clinical trials testing SERDs.
Acknowledgements

We thank Dr. Elaine Alarid, (University of Wisconsin-Madison), for her suggestions and help with the ubiquitination assay, Dr. Britta Jacobson (University of Colorado, Denver) for the BCK4 cell line, and AstraZeneca for AZD9496. This project used the UPMC Hillman Cancer Center and Tissue and Research Pathology/Pitt Biospecimen Core shared resource which is supported in part by award P30CA047904.
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64. RRID:AB_621842, https://scicrunch.org/resolver/RRID:AB_621842


68. RRID:AB_628423, https://scicrunch.org/resolver/RRID:AB_628423


Figure Legends

Fig. 1 Correlation of ESR1 mRNA and ERα protein levels in ER+ ILC and IDC tumors
(A) ESR1 mRNA and (B) RPPA ERα protein levels in ER+ invasive lobular carcinoma (ILC) and ER+ invasive ductal carcinoma (IDC) samples analyzed from the TCGA data set. (C) PGR mRNA and (D) PGR protein levels from the TCGA data set. (E) Correlation between RPPA ERα protein and ESR1 mRNA levels between ILC and IDC samples analyzed from TCGA data set using Pearson’s (r) correlation. \( P = 5.9 \times 10^{-6} \) for Wilcoxon rank-sum test comparison of Pearson correlations. (F) ESR1 mRNA levels in IDC and ILC tumor samples as analyzed by qRT-PCR in the UPMC cohort. (G) Immuno-histochemical semi quantitation of ER in IDC and ILC tumor samples determined using a modified H-score. (H) PGR mRNA by qRT-PCR and (I) immuno-histochemical semi quantitation of PGR in IDC and ILC tumor samples determined using a modified H-score in the UPMC cohort. (J) Correlation between ER IHC H-score and ESR1 mRNA levels between ILC and IDC samples as analyzed using Pearson’s coefficient (r) correlation. \( P = 0.0017 \) for Wilcoxon rank-sum test comparison of Pearson correlations.

Fig. 2 ERα protein levels and estrogen response in ILC and IDC cell line models
(A) Expression of ESR1 mRNA in IDC and ILC cell lines. Cell lines were grown in standard culture conditions and ESR1 mRNA levels were quantified by qRT-PCR. Data are shown as mean ± SEM from three biological replicates. *\( P < 0.05 \); ****\( P < 0.0001 \); calculated by two-way ANOVA followed by Dunnett’s multiple comparison test; comparing the ESR1 mRNA levels to that of MCF-7. (B) Expression of ERα protein in IDC and ILC cell lines. Western blot analysis of ERα in total protein extracts from IDC and ILC cell lines. The protein quantity is expressed as average fold change versus MCF-7 cells. Results represent the mean ± SEM of three independent experiments. (C) Pelleted, fixed and paraffin embedded cells were immuno-stained for ERα. Representative images taken at 40x objective are shown (scale bar = 100μM). (D) Effect of 17β-estradiol (E2) on growth of IDC and ILC cell lines. Hormone deprived cell lines were treated with Vehicle (Veh, 0.01% DMSO) or increasing doses of E2 (10^{-14} to 10^{-7} M) for 6 (MCF-7, T47D) or 7 (ZR-75-1, BCK-4, MDA-MB-134-VI, SUM44) days and proliferation assessed by FluoReporter® Blue Fluorometric dsDNA Quantitation Kit. Data are shown as fold growth vs. Veh. control. Points represent mean of 6 biological replicates; error bars denote SD. (E) Heat maps depicting gene expression changes (log₂ fold change (FC) vs. Veh) after treatment with E2 ± ICI 182,780 in IDC and ILC cell lines. Cell lines were hormone deprived and treated with Veh or 1nM E2 ± 1μM ICI 182,780 for 6h.
GREB1 and PGR mRNA levels were quantified by qRT-PCR. Data expressed as log2FC vs. Veh from 3 biological replicates.

**Fig. 3** 17β-estradiol (E2) induced changes in ERα protein levels, ubiquitination and turnover in IDC and ILC cell lines. Cells were hormone deprived and treated with (A) Vehicle (V, 0.01% DMSO) or 1nM E2 for varying time points (0, 3, 6, 24, 48 h) or (B) increasing doses of E2 (0.01, 0.1, 1, 10, 100 nM) for 24h. ERα protein levels were assessed by immunoblotting. Protein levels are expressed as percentage of ERα remaining as compared to the corresponding Veh treated controls. Results represent the mean +/- SEM of three experiments. *P<0.05; **P<0.01; ****P<0.0001; calculated by one-way ANOVA followed by Dunnett’s multiple comparison test. (C) Half-life of ERα protein calculated by cycloheximide (CHX) chase assay. Cells were treated with CHX (50 µg/ml) in complete growth media or in combination with Veh or E2 (1nM) after hormone deprivation. ERα protein bands were normalized to β-actin and then to the time 0 h control. Half-life of ERα protein was calculated based on one-phase decay. *P<0.05; **P<0.01 calculated by two-way ANOVA followed by Bonferroni multiple comparison test. (D) Effect of E2 on ERα ubiquitination. MCF-7 and MDA-MB-134-VI cells were pretreated with 10 µM MG132 for 30 min followed by 5 h treatment with Veh, or E2 (1 nM). ERα was immunoprecipitated (IP) from the total protein lysates, and ubiquitination was evaluated by IB using a Ubiquitin (Ub) specific antibody. Input lanes represent 5% of the amount of protein lysates used for IP. The bottom panel shows the blots re-probed with ERα specific antibody.
Fig. 4 Oral SERD AZD9496 activity in blocking ILC and IDC growth, and ability to degrade ERα (A) Cells were hormone deprived and treated with Vehicle (Veh, 0.01% DMSO) or increasing doses (10^-12 to 10^-6 M) of SERDs ICI 182,780 (ICI) or AZD9496 (AZD) for 24h. ERα protein levels were assessed by immunoblotting. Protein levels are expressed as percentage of ERα remaining as compared to the corresponding Veh treated controls. Results represent the mean +/- SEM of two to three experiments. (B) Half-life of ERα protein calculated by cycloheximide (CHX) chase assay. Cells were treated with CHX (50 µg/ml) in complete growth media or in combination with Veh, or ICI 182,780 (100 nM) or AZD (100 nM) after hormone deprivation. Cells were harvested after 0, 3, 6, 12, 24 and 48h. Protein lysates were prepared and ERα protein levels were assessed by Western blotting. ERα protein bands were normalized to β-actin and then to the time 0 h control. Half-life of ERα protein was calculated using GraphPad Prism software based on one-phase decay. (C, D) Hormone deprived cells were treated with Veh, 100 nM ICI 182,780 or AZD with (C) or without (D) 100 pM E2 for 0, 3, 5, 7 days and proliferation was assessed by FluoReporter ® Blue Fluorometric dsDNA Quantitation Kit. Data are shown as fold growth vs. Day 0. Points represent mean of 6 biological replicates; error bars denote SD. **P<0.01; ***P<0.001; ****P<0.0001; comparing the growth rate between treatment groups.
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**ESR1 mRNA levels** (Fold change vs. MCF-7)

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**ERα protein levels** (Fold change vs. MCF-7)

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**Image:**

- **ERα** protein levels
- **β-Actin**

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**Images:**

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  - MCF-7
  - T47D
  - BCK4
  - MM134
  - SUM44

- ILC
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  - T47D
  - ZR-75-1
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  - MM134
  - SUM44

D

**Graphs:**

- **Fold growth vs. Veh**
  - Log [E2], M

- **Graphs:**
  - BCK-4
  - MM134
  - SUM44PE

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**Log2 FC Matrix:**

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**Gene Expression:**

- **GREB1**
- **PGR**