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Mechanisms of estrogen-independent breast cancer growth driven by low estrogen concentrations are unique versus complete estrogen deprivation

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Abstract

Despite the success of the aromatase inhibitors (AIs) in treating estrogen receptor positive breast cancer, 15–20 % of patients receiving adjuvant AIs will relapse within 5–10 years of treatment initiation. Long-term estrogen deprivation (LTED) of breast cancer cells in culture mimics AI-induced estrogen depletion to dissect mechanisms of AI resistance. However, we hypothesized that a subset of patients receiving AI therapy may maintain low circulating concentrations of estrogens that influence the development of endocrine resistance. We expanded established LTED models to account for incomplete suppression of estrogen synthesis during AI therapy. MCF-7 cells were grown in medium with charcoal-stripped serum supplemented with defined concentrations of 17β -estradiol (E2) or the estrogenic androgen metabolite 5α -androstane- 3β , 17β -diol (3β Adiol), an endogenous selective estrogen receptor modulator. Cells were selected in concentrations of E2 or 3β Adiol that induce 10 or 90 percent of maximal proliferation (EC_{10} and EC_{90} , respectively), or estrogen deprived. Estrogen independence was evaluated during selection by assessing cell growth in the absence or presence of E2 or 3β Adiol. Following >7 months of selection, estrogen independence developed in estrogen-deprived cells and EC_{10} -selected cells.

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Functional analyses demonstrated that estrogen-deprived and EC₁₀-selected cells developed estrogen independence via unique mechanisms, ER α -independent and dependent, respectively. Estrogen-independent proliferation in EC₁₀-selected cells could be blocked by kinase inhibitors. However, these cells were resistant to kinase inhibition in the presence of low steroid concentrations. These data demonstrate that further understanding of the total estrogen environment in patients on AI therapy who experience recurrence is necessary to effectively treat endocrine-resistant disease.

Keywords

Breast cancer; Estrogen; Androgen; Aromatase inhibitor; Endocrine resistance

Introduction

Endocrine therapy using aromatase inhibitors has significantly improved disease-free survival and overall survival in post-menopausal women with ER-positive breast cancer. However, ~20 % of patients treated with adjuvant AI therapy will experience disease relapse within 10 years of treatment initiation [1, 2]. Mechanisms of resistance to AI therapy have been studied for over a decade, since the introduction of AIs as frontline endocrine therapy, and a number of interesting pathways and mechanisms have been implicated in both acquired and de novo resistance. Mechanisms of acquired resistance to AI therapy have been studied largely through the use of long term estrogen deprivation (LTED) models, both in cell culture [3–9] and xenograft [10, 11] settings. To mimic tumor conditions in patients on AI therapy, ER-positive breast cancer cells are maintained long term in the absence of steroid hormones, either in medium supplemented with charcoal-stripped calf serum (CCS) or in ovariectomized mice. Cells that adapt to LTED display an endocrine-independent phenotype, no longer requiring high concentrations of estrogen to maintain cell growth.

Though LTED models have implicated a number of mechanisms as drivers of estrogen-independent growth, these models assume that AI therapy depletes essentially all circulating estrogens. Cells that are maintained in medium supplemented with charcoal-stripped serum are almost totally deprived of any estrogenic stimulation. We have previously demonstrated that the androgen metabolite 5 α -Androstane-3 β ,17 β -diol (3 β Adiol) can provide an estrogenic stimulus to breast cancer cells by acting as an ER α ligand and endogenous selective estrogen receptor modulator (eSERM) [12]. Importantly, 3 β Adiol is synthesized in the body through a pathway that is independent of metabolism by aromatase, and indeed, 3 β Adiol concentrations may potentially be increased by AI therapy due to increased androgen production [13, 14]. In addition, low levels of residual aromatization are maintained in patients on AI therapy, sufficient to produce quantifiable, pM concentrations of circulating E2 in some patients [15, 16]. For MCF-7 cells, a well-established model for assessing the activity of estrogens in breast cancer cells, culture in E2 concentrations >100 fM is sufficient to induce proliferation, with half-maximal induction of proliferation at E2 concentrations of ~5 pM [12]. Based on these observations, we hypothesized that LTED may not accurately mimic the estrogenic environment for the subset of patients that maintain 3 β Adiol or E2 concentrations sufficient to activate ER α signaling. To model the conditions present in this subset of patients, MCF-7 cells were selected by long term culture in medium supplemented with CCS and defined concentrations of either E2 or 3 β Adiol (EC₁₀ and EC₉₀ concentrations of each steroid hormone, with respect to the stimulation of maximal MCF-7 cell proliferation). The development of acquired estrogen independence was assessed in these selected cell lines, and their phenotypes were compared to that of cells grown in CCS without steroid supplementation (LTED cells) and the parental MCF-7 cells maintained concurrently with the selection. The mechanisms driving estrogen-independent growth were

assessed in the selected cell lines following >7 months of selection in the defined steroid hormone conditions.

Methods

Cell lines, culture conditions, and proliferation assays

MCF-7 cells were obtained from the Tissue Culture Shared Resource (TCSR) at the Lombardi Comprehensive Cancer Center (LCCC; Georgetown University, Washington, DC) and routinely maintained as previously described [12, 17]. For long term selections, MCF-7 cells were maintained in phenol red-free modified Improved Minimum Essential Medium (IMEM, Invitrogen, Carlsbad, CA; catalog # A10488)[18] supplemented with 10 % CCS (Valley Biomedical, Winchester, VA) and either vehicle (0.05 % ethanol) or defined E2 concentrations (1 or 50 pM) or 3 β Adiol concentrations (50 pM or 1nM). Steroid concentrations used during selection were chosen based on potential conditions during AI therapy, and matched to the dose– response curves generated using the parental MCF-7 cells (Supplementary Figure 1). Conditions and naming conventions for the selected cell lines are listed in Table 1. During selection, cells were assayed for growth in estrogen-free conditions (described below) until stable pheno-types developed as described in the Results section.

Before selection, and again following 7 months of selection, parental and selected cell lines were subjected to DNA microsatellite fingerprint analysis and mycoplasma testing at the TCSR at LCCC. Results from the DNA fingerprint analyses matched those of MCF-7 cells from the TCSR and ATCC (HTB-22), and all cell lines were negative for mycoplasma.

Cells were assayed for growth and protein/mRNA expression as indicated following 7+ months of selection. For assays under estrogen-free conditions (or defined steroid hormone concentrations), cells were washed 5 times daily for 3 days. With each wash, cells were rinsed twice with serum- and phenol red-free IMEM. Following washing, cells were plated in phenol red-free IMEM with 5 % CCS. After the final wash on the third day, cells are harvested and plated as necessary in IMEM + 5 % CCS, and were allowed to grow for 24 h before treatment. For assays in the presence of estrogens, cells were plated in their respective selection conditions (as listed in Table 1), and allowed to grow for 24 h before treatment. For growth assays, cells were allowed to grow for up to 5 days as indicated after treatment before assaying cell proliferation. Proliferation assays were performed using the FluoRe-porter Blue fluorometric dsDNA quantitation kit (F2692, Invitrogen) according to the manufacturer's instructions. Briefly, cells were lysed in hypotonic conditions via freeze/thaw cycles, and lysates were stained using buffer containing Hoechst 33258. Fluorescence was read at 360-nm excitation and 460-nm emission wavelengths in a PolarStar Omega plate reader (BMG Labtech, Cary, NC).

E2 was obtained from Sigma-Aldrich (St. Louis, MO). 3 β Adiol was obtained from Steraloids (Newport, RI). ICI 182,780 was obtained from Tocris Biosciences (Ellisville, MO). Lapatinib, U0126 and LY294002 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Western blotting

Western blot analysis was performed on whole cell lysates from breast cancer cells. Cells were lysed using RIPA buffer (Thermo Pierce, Rockford, IL) supplemented with Protease Inhibitor Cocktail Tablets and Phosphatase Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). Total protein from cell lysates was quantified by the Bradford assay (Bradford Reagent; Bio-Rad, Hercules, CA). Thirty micrograms of protein per lane was resolved on 4–20 % gradient polyacrylamide gels (Pierce, Rockford, IL), and

transferred to PVDF membrane. Primary antibodies are listed in Supplemental Table 1 (Cell Signaling; Boston, MA); antibody dilutions were prepared according to the manufacturer's instructions.

Real-time PCR

Total RNA was harvested using RNeasy mini columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. GREB1 mRNA expression was measured using a Taqman real-time PCR assay as described previously [17]. Reactions were performed using a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA). Gene expression was normalized against GAPDH (Hs99999905_m1, Applied Biosystems) and relative expression was determined using the ΔC_T method [19].

Gene expression microarray analysis

Whole genome expression analysis was performed using HumanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA). Selected cell lines were plated in 75-cm² flasks, washed as described above, and medium was replaced with IMEM + 5 % CCS. Cells were harvested, re-seeded in triplicate 75-cm² flasks, and allowed to grow for 3 days before sample harvest (IMEM + 5 % CCS). Proliferation assays in the absence of exogenous estrogens were performed in parallel to verify the estrogen-independent phenotypes. Total RNA was harvested for gene expression analyses as described above. Samples were prepared for the direct hybridization assay and scanned using a BeadArray reader (Illumina) by the University of Michigan DNA Sequencing Core Facility.

All microarray data has been deposited in the GEO database under accession # GSE33287; both raw and normalized data are available. Raw expression data were quantile normalized by GenomeStudio software (Illumina) and further analyzed by R software (<http://www.R-project.org>) The 47,313 probes on the array were filtered according to the median detection p value across triplicates for each sample; only probes with median detection $p < 0.05$ in at least one of the tested samples were used in further analyses. This filtering produced a set of 20,107 probes that we therefore define as expressed. For these probes, we computed average of the log₂-transformed expression levels across each of the three replicates, and used differentially expressed probes (with ratio of the mean > 1.1 or < 0.9) for the enrichment analysis using IPA (Ingenuity® Systems, www.ingenuity.com). Briefly, IPA is designed to transform gene lists into molecular pathways and interaction networks based on the curated IPA Knowledge Base. Of 20,107 probe sets that passed detection threshold across experiments, 17,723 were successfully mapped to entries in IPA Knowledge Base. The remaining 2,884 probes represented primarily EST's with no functional annotation available.

Statistical analyses and curve fitting

A two-tailed T test was used to compare treatments to respective controls (SigmaStat, Systat Software, Inc.). Curve fitting and effect concentration for half-maximal growth (EC₅₀) were determined using GraphPad Prism 4.03 (GraphPad Software, Inc.). Image analysis and densitometry were performed using NIH ImageJ 1.45 (National Institutes of Health).

Results

Estrogen independence develops in low-estrogen conditions

During selection, cells were tested for their ability to proliferate in estrogen-free conditions in the presence or absence of the anti-estrogen ICI 182,780. Stable pheno-types developed in selected cells within 7 months, and results described were obtained after 7–12 months of selection. Shown in Fig. 1, selected cells in estrogen-free conditions (following repeated

washing as described above) were treated with the indicated concentration of hormone or anti-estrogen. High estrogen-selected 50pE cells remained estrogen-dependent following long-term selection; in estrogen-free conditions, 50pE cell growth slowed and stopped (Fig. 1c). ICI 182,780 did not further decrease growth. 1 nM E2 maximally stimulated 50 pE cell proliferation, whereas 10 nM 3 β Adiol induced ~75 % maximal growth, similar to the behavior of the parental cells (Supplementary Figure 1) [12]. High 3 β Adiol selected 1n3 β cells also ceased proliferating in estrogen-free conditions after a short period of estrogen-independent proliferation during the first 3 days after seeding (Fig. 1e). ICI 182,780 did not further decrease proliferation. Treatment with either E2 (1 nM) or 3 β Adiol (10 nM) induced the growth of 1n3 β cells to a similar extent. LTED Veh cells demonstrated estrogen-independent proliferation (7.2-fold increase at day 5 versus pre-treatment); this growth was not inhibited by ICI 182,780 (Fig. 1a). Veh cells also remained responsive to both E2 and 3 β Adiol treatment. Growth above baseline and maximal steroid hormone-induced growth of Veh cells was less than that of 50pE or parental cells (Fig. 1 and Supplementary Figure 1). Both 1pE cells and 50p3 β cells were able to proliferate in estrogen-free conditions (>10-fold day 5 growth versus pre-treatment control). Unlike the Veh cells, this proliferation was inhibited by the addition of ICI 182,780. Both 1pE cells and 50p3 β cells remained responsive to the addition of E2 or 3 β Adiol; treatment with either steroid induced growth greater than vehicle control at 3- and 5-days post treatment. However, 50p3 β cells demonstrated minimal growth induction following steroid hormone treatment. Estrogen independence developed 9–12 weeks after initiation of selection in Veh cells (data not shown), consistent with previous reports [6, 20]. The emergence of estrogen independence in 1pE and 50p3 β cells was delayed, developing after 6–7mo of selection and 4–5mo selection, respectively (data not shown).

Dose–response curves were generated for E2- or 3 β Adiol-induced proliferation for each cell line to determine whether any of the selection lines had developed hypersensitivity to low concentrations of steroid hormone for growth induction, as previously reported [6, 20]. Minor increases in sensitivity (<1-log decrease in EC₅₀) were observed versus parental MCF-7 cells in all selections except 50pE cells, consistent with their parallel phenotype to parental MCF-7 cells (Supplementary Figure 2). Maximum E2-induced growth required 10⁻¹¹M E2 for all cell lines, ~2-logs greater than previously reported for hypersensitive MCF-7 cells. No hypersensitivity was observed in growth induction by 3 β Adiol in any selection cell line (data not shown).

Different mechanisms for estrogen independence develop under low estrogen conditions versus complete estrogen deprivation

To examine potential mechanisms responsible for estrogen-independent proliferation, the expression and activation of the MAPK and PI₃K/Akt signaling pathways was examined via western blot analysis. Selected cell lines were assessed in estrogen-free conditions and compared to the parental cells maintained in 10 % FBS (Fig. 2). High concentration steroid hormone-selected 50pE and 1n3 β cells in estrogen-free conditions exhibited decreased levels of phosphorylated MAPK (pMAPK) compared to the parental MCF-7 cells (0.5- and 0.3-fold versus parental, respectively), consistent with their lack of proliferation under these conditions. Low concentration steroid hormone-selected 1pE and 50p3 β cells maintained pMAPK levels similar to those in parental MCF-7 cells (each 1.1-fold versus parental), while Veh cells had slightly lower levels of pMAPK (0.8-fold versus parental). Activity of the PI₃K/Akt pathway was examined by assessing the phosphorylation of Akt on T308 and S473 as indications of Akt activation. pAkt-T308 was only observed in Veh, 1pE, and 50p3 β cells, suggesting that these cell lines had activated PI₃K signaling in the absence of estrogen. Similarly, pAkt-S473 was highest in 1pE and 50p3 β cells (2.0- and 2.6-fold versus parental, respectively), further suggesting the importance of Akt signaling in estrogen-independent

growth in these cells. pAkt-S473 was also induced in Veh and 1n3 β cells (1.3- and 1.6-fold versus parental, respectively). Akt may play a role in Veh cell estrogen-independent growth, as well as the transient estrogen-independent growth in 1n3 β cells. Since both the Akt and MAPK pathways can activate ER α via phosphorylation [21–23], we examined ER α phosphorylation at S167. All five selected lines express ER α when grown in estrogen-free conditions. However, in estrogen-free conditions, pER α -S167 was only observed in the 1pE and 50p3 β cells. These data suggest that 1pE and 50p3 β cells maintain ER α in an active state in the absence of ligand. ErbB2 expression was also examined; all selected cell lines up-regulated ErbB2 equivalently in estrogen-free conditions compared to parental cells (Fig. 2).

To examine whether the observed phosphorylation of ER α was indicative of transcriptional activity of ER α in the absence of ligand, we examined GREB1 expression in estrogen-free conditions (Fig. 3). GREB1 is an ER α -specific target critically involved in the estrogen induced growth of breast cancer cells [17]. In the presence of estrogen, parental MCF-7 cells express very high levels of GREB1 (column 1). GREB1 expression is strongly decreased upon the addition of ICI 182,780 (column 2); GREB1 expression was similarly very low in MCF-7 cells in estrogen-free conditions (columns 3, 4). Veh, 50pE, and 1n3 β cells also expressed very low levels of GREB1 in estrogen-free conditions, consistent with ICI-insensitivity and lack of proliferation in estrogen-free conditions, respectively. However, both 1pE and 50p3 β cells maintained increased GREB1 expression in estrogen-free conditions (columns 7, 11). GREB1 expression in these cells was likely ER α -dependent, as expression is decreased by the addition of ICI 182,780 (columns 8, 12). Taken together with above observations, these data suggest that 1pE and 50p3 β cells maintain ER α activation in the absence of ligand to maintain estrogen-independent growth. Conversely, Veh cells do not appear to require ER α to maintain estrogen-independent growth.

Microarray analysis reveals potential drivers of estrogen-independent growth

We performed gene expression microarray analyses of selected cells in estrogen-free conditions to identify genes and pathways critical for estrogen-independent growth, as described in Materials and Methods. Importantly, the selection of cells in high estrogen concentration (50pE cells) provided an internal control for gene expression changes caused by long-term growth in charcoal-stripped serum, rather than specific to the removal of steroid hormones. Based on this, comparisons described below include only the selection cell lines, rather than parental MCF-7 cells.

Initial enrichment analysis identified common proliferation signatures (DNA synthesis, checkpoint progression, etc.) as enriched for Veh, 1pE, and 50p3 β cells in estrogen-free conditions (data not shown). However, the above observations strongly suggest that unique mechanisms are responsible for growth in Veh (LTED) cells, and the low steroid concentration-selected cells (1pE and 50p3 β). To assess this, gene expression data from Veh cells were compared to the other four selected lines and data from the 1pE and 50p3 β cells were compared to the other three selected lines. In each case, we defined differentially expressed genes as those with ratio of the target phenotype to the mean of expression in all other conditions of ≥ 1.1 or ≤ 0.9 compared to the mean of expression in all other conditions. In Veh cells, we identified 337 molecules that were differentially expressed according to our criteria. We used IPA Core Analysis to explore functions and networks/pathways represented in this gene set; 266 of the 337 genes were mapped by IPA Knowledge Base. At the top of the list of known biological functions/disease states, IPA enrichment showed 55 different functions/subcategories with 130 unique molecules in the “cancer” category. Enrichment p values for these functional groups range from 8.15×10^{-3} to 7.5×10^{-18} . Unsupervised connectivity analysis of molecules in the “cancer” category generated

numerous direct relationships and formed a large cohesive network shown in Supplemental Figure 3. This network has nodes consisting of molecules known to play key roles in tumor biology, including EGFR, TGF β 2, PRKCA, and others, suggesting that increased expression of genes in this network may be responsible for driving cell growth in Veh cells.

In 1pE and 50p3 β cell lines we identified 188 genes that were differentially expressed according to our criteria; 184 of these genes were mapped by IPA Knowledge Base. Similar to the comparison for Veh cells, the most enriched category mapped by IPA was “cancer” with 60 subcategories and 104 unique genes. However, unsupervised connectivity analysis of molecules in the “cancer” category enriched in 1pE/50p3 β cells yielded an entirely different network than observed with Veh cells. Network relationships between these genes are shown in Supplemental Figure 4. In contrast to the cancer signaling pathways observed in Veh cells, 1pE/50p3 β cells demonstrated activation of the ER α signaling pathway in estrogen-free conditions, consistent with our observations described above. These data strongly suggest that unique mechanisms are responsible for maintaining estrogen-independent growth in Veh versus 1pE/50p3 β cells.

Kinase inhibitors block estrogen-independent growth of low estrogen-selected cells

As demonstrated in Fig. 2, 1pE and 50p3 β cells maintain active MAPK and Akt in estrogen-free conditions. We hypothesized that activated MAPK and Akt may be responsible for phosphorylating ER α in the absence of ligand and that 1pE and 50p3 β cells would be growth-inhibited by the MEK inhibitor U0126 and the PI $_3$ K inhibitor LY294002. Shown in Fig. 4A-B, 1pE and 50p3 β cells in estrogen-free conditions were treated with kinase inhibitors as indicated alone or in combination with ICI 182,780. Veh cells were not growth inhibited by any combination of U0126, LY294002, or ICI 182,780 (data not shown). Under estrogen-free conditions, 50pE and 1n3 β cells were not growth inhibited by any combination of U0126, LY294002, or ICI 182,780 at the indicated concentrations (data not shown). U0126 and LY294002 partially inhibited the growth of 1pE and 50p3 β cells in estrogen-free conditions. Growth was inhibited by either inhibitor or the combination by 40–70 % versus control treatments. Neither drug alone or in combination could inhibit cell growth to the extent of ICI 182,780.

Based on the IPA results described above, implicating EGFR/ErbB2-driven gene expression in Veh cells, we hypothesized that inhibition of EGFR and/or ErbB2 would specifically block the growth of Veh cells, as observed in other LTED models [3, 4]. The small molecule inhibitor lapatinib is used clinically to inhibit the kinase activity of both EGFR and ErbB2 [24, 25]. Selected cells in estrogen-free conditions were treated with increasing concentrations of lapatinib. The transient estrogen-independent growth of 1n3 β cells was minimally inhibited by >100nM lapatinib (~20 % reduction in growth) (data not shown). In addition, lapatinib was not toxic to 50pE cells in estrogen-free conditions at the concentrations tested (data not shown). Surprisingly, Veh cells were relatively resistant to lapatinib, as growth could not be completely inhibited with the concentrations tested (estimated IC $_{50}$ > 1 μ M) (Fig. 4c). However, we observed that lapatinib is a potent inhibitor of estrogen-independent growth of 1pE and 50p3 β cells (Fig. 6c; IC $_{50}$ = 8.6nM and 1.2nM, respectively); maximal growth inhibition by lapatinib was equivalent to maximal inhibition by ICI 182,780 under these conditions (data not shown). These data are consistent with the hypothesis that an upstream receptor tyrosine kinase (i.e., a target of lapatinib, EGFR, or ErbB2) signals through MAPK and/or Akt to maintain ER α activation and growth of 1pE and 50p3 β cells in estrogen-free conditions.

Kinase inhibitors decrease estrogen-independent phosphorylation and activity of ER α in 1pE and 50p3 β cells

ER α , MAPK, and Akt phosphorylation were examined in 1pE and 50p3 β cells following treatment with kinase inhibitors to correlate the growth inhibition observed above with kinase and ER α activation. ER α phosphorylation at S167, Akt phosphorylation at T308 and S473, and MAPK phosphorylation were examined by western blotting as described above. 1pE and 50p3 β cells were seeded in estrogen-free conditions and treated with ICI 182,780 or kinase inhibitors as indicated (Fig. 5). Levels of pMAPK or pAkt were specifically decreased using U0126 or LY294002, respectively, in both cell lines. However, lapatinib completely eliminated all pMAPK and strongly decreased pAkt in both cell lines, suggesting that EGFR or ErbB2 is upstream of these signaling pathways in 1pE and 50p3 β cells.

pER-S167 levels were strongly decreased by ICI 182,780 treatment, consistent with the concurrent decrease in total ER observed following ICI 182,780 treatment. U0126 minimally decreased pER-S167 levels in either cell line (1.0- and 0.9-fold versus control in 1pE and 50p3 β cells, respectively), whereas LY294002 treatment decreased pER-S167 more (0.7-fold versus control in both cell lines). This is consistent with previous reports that the PI₃K/Akt pathway is responsible for phosphorylating ER α at S167. Lapatinib strongly decreased pER-S167 levels in both 1pE and 50p3 β cells, in particular in 50p3 β cells (0.7- and 0.5-fold versus control, respectively). This differential ability to decrease pER-S167 parallels differential growth inhibition by each drug (as described above).

1pE and 50p3 β cells are kinase inhibitor resistant in the presence of estrogens

As described above, 1pE and 50p3 β cells were growth inhibited by kinase inhibitors under estrogen-free conditions. We hypothesized that the selection conditions of these cells would mimic conditions in patients receiving AI therapy. It is likely that in these patients, estrogen-free conditions would not be clinically achievable, and therefore recurrent tumors would not be treated in an estrogen-free setting. Based on this, we examined whether 1pE and 50p3 β cells were also sensitive to kinase inhibition in their normal selection conditions, in the presence of low concentrations of estrogens. Selected cell lines were treated with kinase inhibitors alone or in combination with ICI 182,780 as in Fig. 6. Ligand-induced growth was inhibited by 100nM ICI 182,780. However, in the presence of low E2 or 3 β Adiol concentrations, both 1pE and 50p3 β cells were resistant to U0126 or LY294002 treatment (Fig. 6a, b). Neither agent alone blocked cell growth >10 % versus control treatment, and the combination only reduced growth by ~25 % versus control. Similarly, though both 1pE and 50p3 β cells were very sensitive to lapatinib in estrogen-free conditions, both cell lines were lapatinib-resistant in the presence of low estrogen concentrations (Fig. 6c). Growth was not decreased versus control treatment for any concentration of lapatinib tested for either cell line.

Discussion

Long-term estrogen deprivation (LTED) cell culture models and aromatase over-expression models have been used to examine changes in breast cancer cells following long term estrogen withdrawal [3–11]. Though successful in identifying potential mechanisms of AI resistance, these models do not account for estrogenic stimuli provided by alternative estrogens and eSERMs (e.g., 3 β Adiol [12]), estrogens produced from residual aromatization [15, 16] or sub-optimal patient adherence to AI therapy [26]. In this report, we expanded the LTED culture paradigm to include defined concentrations of either E2 or 3 β Adiol, and selected cells in these conditions for estrogen-independence, in parallel with cells maintained in LTED. We hypothesized that these selection conditions may more accurately

mimic conditions in a subset of patients on AI therapy, and would lead to the identification of novel, clinically relevant mechanisms of AI resistance.

Clear differences in the adaptation to the changes in levels of estrogens emerged across selected cell lines. In 50pE cells, the near-saturating concentration of E2 present in the selection conditions effectively maintained the parental phenotype; 50pE cells remained dependent on estrogen for proliferation (Fig. 1, Supplemental Figure 2). However, while ER α was also near-saturated in 1n3 β cells while under selection, the partial agonist 3 β Adiol [12] was not equivalent to E2. 1n3 β cells recognized 3 β Adiol as a full agonist (Fig. 1), suggesting possible changes in the cofactor cohort of the cells in recognizing activated ER α . 1n3 β cells did however remain dependent on estrogens and cease growth after 3–5 days in estrogen-free conditions. In the absence of E2, high concentrations of 3 β Adiol are sufficient to maintain tumor cell growth. Changes in the cofactor cohort allowing the recognition of 3 β Adiol as a full ER agonist may be consistent with proposed mechanisms for resistance to anti-estrogens with partial agonist activity, such as tamoxifen.

Though high concentrations of estrogens maintained estrogen dependence in 50pE and 1n3 β cells, low estrogen concentrations or LTED promoted the development of an estrogen-independent phenotype. Following selection, Veh, 1pE, and 50p3 β cells remained endocrine responsive (Fig. 1, Supplemental Figure 2) and continued to express ER α (Fig. 2). Unlike 50pE and 1n3 β cells, however, Veh, 1pE, and 50p3 β cells demonstrated cell proliferation in estrogen-free conditions (Fig. 1). Different mechanisms appear to drive estrogen-independent growth in the LTED Veh cells versus those maintained in low estrogen concentrations (1pE and 50p3 β). IPA of gene expression microarray data suggested that whereas 1pE and 50p3 β cells are driven by an ER-dependent pathway, Veh cells may instead be dependent on signaling through EGFR or PRKCA (Supplemental Figures 3–4). Consistent with these data, estrogen-independent growth in both 1pE and 50p3 β cells can be blocked by the addition of ICI 182,780, whereas Veh cells are ICI 182,780-resistant (Fig. 1). This suggests that estrogen-independent growth in 1pE and 50p3 β is ER-dependent, but ER-independent in Veh cells. Consistent with this, GREB1 expression was maintained in 1pE and 50p3 β cells in estrogen-free conditions and was decreased by ICI 182,780; Veh cells expressed very low levels of GREB1 in the absence of estrogen (Fig. 3). Also, phospho-ER α was detected only in 1pE and 50p3 β cells in estrogen-free conditions (Fig. 2). These data suggest that following selection in low estrogen concentrations, breast cancer cells can maintain ER α activation in the absence of ligand, whereas LTED Veh cells no longer require ER α activity. Our data with inhibitors of kinase signaling pathways suggest that ER α activation in 1pE and 50p3 β cells is maintained by ErbB receptor tyrosine kinases that signal via the MAPK or PI $_3$ K pathways. Further elucidation of the mechanisms driving estrogen-independent growth of the Veh cells is currently under investigation. Though IPA suggested roles for EGFR and PRKCA, Veh cell growth could not be inhibited using single agent treatment with a panel of kinase inhibitors. In addition, though not evident in our microarray analysis, IGF1R signaling has been demonstrated to play a critical role in MCF-7 cell survival [27] and recently in endocrine resistance [28]. IGF1R/InsR signaling and other pathways will be examined further in Veh cells. The mTOR signaling pathway has also been demonstrated to play a role in promoting endocrine resistance in pre-clinical models [29, 30] and in clinical trials [31]. We did not observe any direct evidence of active mTOR signaling in our microarray studies, but this pathway will also be further examined in these models.

Observations of ligand-independent ER α activation are consistent with those reported for LTED cells developed by the Dowsett and Santen laboratories [3–6] and recently by the Arteaga laboratory [7]. These LTED models also appear to require kinase signaling pathways to maintain estrogen-independent growth, and respond to inhibitors of these pathways. However, while 1pE and 50p3 β cells are growth inhibited by lapatinib (in

addition to MEK and PI₃K inhibitors), they are only sensitive to kinase inhibitors in the absence of estrogen. In their normal selection conditions (low estrogen concentrations), 1pE and 50p3 β cells were resistant to all kinase inhibitors tested, and were only growth inhibited by ICI 182,780. These observations may suggest that in patients with low serum estrogen concentrations on AI therapy, recurrent tumors will not respond to kinase inhibitors, since sufficient ligand will still be present to maintain ER α signaling. The removal of AIs from a treatment regimen in this scenario would allow estrogen concentrations to increase, which may also prevent sensitivity to other targeted therapies. The specific sensitivity of 1pE and 50p3 β cells to ICI 182,780 suggests that in similar tumors, increased antagonism of estrogen signaling may be critical to blocking tumor growth. Increased estrogen depletion may be insufficient in these tumors, as EGRF/ErbB2 may then be sufficient to maintain growth.

Our cell culture studies and the majority of those described above have focused on the use of the MCF-7 cell line as a representative model of endocrine response and resistance. However, the broad applicability of our findings should be applied with caution until they can be expanded to additional cell lines as well as in vivo models. Importantly, a number of estrogen-independent breast cancer cell lines were reported by the Arteaga laboratory, with consistent phenotypes among different cell lines [7]. Adaptation to low estrogen concentrations, however, has not been investigated previously to date to our knowledge. Our study represents an important proof-of-principle that demonstrates that low estrogen concentrations can in fact profoundly influence the development of estrogen-independence.

Exogenous sources of estrogens remain a confounding factor in modeling endocrine response and resistance in pre-clinical systems. In addition to potentially masking low potency alternative estrogens (e.g., 3 β Adiol), low concentrations of estrogens and estrogenic chemicals present in CCS, plasticware, etc., may impact how cells respond to long term selection in the presumed absence of estrogens. Ishikawa and colleagues reported that estrogenic compounds could be extracted from plasticware from different manufacturers [32], consistent with observations in our laboratory and others (Richard Santen, personal communications). These phytoestrogens may play a role in what we and others have observed to be ligand-independent activation of estrogen receptor. The lack of ER α -mediated transcription observed in parental MCF-7, Veh, 50pE, and 1n3 β cells in estrogen-free conditions and the unique phenotype between Veh and 1pE/50p3 β suggest that this may not be a major confounder in our study. However, the effect of exogenous estrogens/phytoestrogens can not be ignored when modeling endocrine resistance.

Non-traditional ER α ligands that act as eSERMs may modulate the development of AI resistance. Of particular interest in addition to 3 β Adiol may be the cholesterol metabolite 27-hydroxycholesterol. As reported by Donald McDonnell and colleagues, 27-hydroxycholesterol can function as a SERM but specifically induces ER α -mediated transcription and proliferation in breast cancer cells [33]. The steroid metabolite androstenediol has also been demonstrated to induce breast cancer cell growth as an estrogen, independent of metabolism by aromatase [34, 35]. 3 β Adiol, 27-hydroxycholesterol and androstenediol are low potency estrogens; however, in patients on AIs these ligands are likely free to bind and activate the receptor. Clearly, quantifying serum concentrations of eSERMs will be critical in understanding their role in promoting breast cancer growth. Circulating 27-hydroxycholesterol concentrations are likely sufficient to maintain estrogen signaling [33]. In one study, plasma concentrations of 3 β Adiol in men measured by radioimmunoassay were reported to be approximately 1.5 nM [36]. Based on our data, this would be sufficient to maintain breast cancer cell growth in the absence of estrogen; however, no data exist on 3 β Adiol concentrations in women. Measurement of 3 β Adiol in women before and after AI therapy is an important area of future research.

In addition, though recent mass spectrometry-based methods report serum E2 concentrations of AI-treated patients below a limit of detection of 2.3 pM [37, 38], our data demonstrate that extremely low estrogen concentrations below current limits of quantification (1 pM estradiol in 1pE cells) can alter the LTED phenotype. Importantly, a significant proportion of patients maintain circulating estradiol levels above this limit of quantification (17–20 % [15, 16, 37, 38]). These concentrations of estrogen are sufficient to drive breast cancer cell growth in breast cancer cell models, and quantification of alternative estrogens and E2 concentrations as low as 100 fM (Supplemental Figure 1) may be necessary to accurately identify patients with potentially active ER α signaling on AI therapy.

A number of pre-clinical and clinical studies support a role for maximizing ER α blockade in recurrent tumors (reviewed in [39]). However, it is clear that mechanisms of resistance to endocrine therapies are heterogeneous. Using MCF-7 cells, we selected populations that were either sensitive or resistant to ICI 182,780 (1pE/50p3 β cells and Veh cells respectively). The estrogen environment in which AI resistance develops is likely critical to the recurrent tumor phenotype. Biomarkers of ER α activation (such as phosphorylation at S167) may be necessary to identify patients that will benefit from ICI 182,780 or further AI treatment. Importantly, recent results of the CONFIRM trial (Comparison of Faslodex in Recurrent or Metastatic breast cancer, NCT00099437) demonstrated that increasing the dose of ICI 182,780 from 250 to 500 mg significantly reduced the risk of disease progression in patients who had recurred on prior endocrine therapy [40]. Thus, not only is it critical to identify recurrent tumors with ER α activity, but also the relative concentrations of ICI 182,780 and competing estrogen concentrations at the site of action in recurrent tumors are likely determinants of whether ICI 182,780 can effectively antagonize ER α and block tumor growth.

Our cell culture models suggest that the estrogen environment in which resistance to endocrine therapy develops promotes unique mechanisms of resistance that will display diverging sensitivity to targeted or endocrine therapies. The presence of residual E2 or alternative estrogens such as 3 β Adiol may drive tumor growth in the presence of AIs and subsequently maintain the growth of recurrent tumors during additional therapies. These observations highlight the heterogeneity in mechanisms of endocrine resistance, and the need for specific biomarkers that will indicate which therapies may benefit individual patients. Further understanding of the total estrogen environment in patients on AI therapy who experience recurrence is necessary to effectively treat these patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3βAdiol	5 α -Androstane-3 β ,17 β -diol
LTED	Long-term estrogen deprivation
AI	Aromatase inhibitor

CCS	Charcoal-stripped calf serum
SERM	Selective estrogen receptor modulator
eSERM	Endogenous selective estrogen receptor modulator
IPA	Ingenuity pathway analysis

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Fig. 1. MCF-7 selected cell line growth in estrogen-free conditions. Cells were washed as described in Materials and Methods, and treated as indicated. **a** Veh cells, **b** 1pE cells, **c** 50pE cells, **d** 50p3β cells, and **e** 1n3β cells. Growth was assessed at baseline, 3 days or 5 days after treatment. Points represent the average of 6 replicates $\pm$ SEM. Student's *t* tests were used to compare growth after 5 days of treatment with ICI 182,780 versus control. † *p* > 0.05. * *p* 0.0005

Fig. 2.

Kinase signaling pathways in selected cell lines in estrogen-free conditions. Parental cells were maintained in normal conditions with FBS; selected cells were washed and seeded in estrogen-free conditions before sample harvesting. *N.S.* non-specific band

Fig. 3. GREB1 expression in MCF-7 parental and selected cell lines, presented in arbitrary units. MCF-7 refers to the parental cell line; CCS indicates estrogen-free conditions. Veh, 1pE, 50pE, 50p3β, and 1n3β cells were assayed in estrogen-free conditions. Cells were treated with either vehicle (0.1 % EtOH) or 1 μM ICI for 72 h. Student's T-tests were used to compare expression in ICI 182,780-treated cells versus controls. * $p < 0.001$. ** $p = 0.032$. # $p > 0.05$

Fig. 4.

Kinase inhibition blocks estrogen-independent growth. **a** 1pE and **b** 50p3 β cells in estrogen-free conditions were treated with 100 nM ICI, 100nM U0126 (U), 250nM LY294002 (LY) or combinations as indicated. Growth was assessed 4 days after treatment. Bars represent average of 6 replicates $\pm$ SEM. * p value versus control < 0.05 . **c** Selected cell lines were treated with increasing concentrations of lapatinib as indicated. Growth was assessed 5 days after treatment. Points represent the average of 6 replicates $\pm$ SEM

Fig. 5.

Kinase inhibitors decrease kinase and ERα activation in estrogen-free conditions. 1pE or 50p3β cells in estrogen-free conditions were treated with 100 nM ICI, 100nM U0126 (U), 250 nM LY294002 (LY), 1 μM lapatinib (Lap) or combinations as indicated. Lysates were harvested 72 h after treatment. *N.S.* non-specific band

Fig. 6. Kinase inhibitor resistance in the presence of low estrogen concentrations. **a** 1pE and **b** 50p3 β cells in normal selection conditions were treated with 100 nM ICI, 100 nM U0126 (U), 250 nM LY294002 (LY) or combinations as indicated. Growth was assessed 4 days after treatment. Bars represent average of 6 replicates + SEM. *, p value versus control < 0.05. ** p value versus control > 0.05. **c** Selected cell lines were treated with increasing concentrations of lapatinib as indicated. Growth was assessed 5 days after treatment. Points represent the average of 6 replicates $\pm$ SEM. Veh and estrogen-free data are replicated from Fig. 4 for clarity, and are representative of repeat experiments

Table 1

MCF-7 selection conditions and nomenclature

Estrogen environment 10 % FBS (Standard cond.)	Cell line name Parental
10 % CCS + Vehicle (0.05 % EtOH)	Veh
1 pM E2	1pE
50 pM E2	50pE
50 pM 3 β Adiol	50p3 β
1 nM 3 β Adiol	1n3 β