Anti-estrogen Resistance in Human Breast Tumors Is Driven by JAG1-NOTCH4-Dependent Cancer Stem Cell Activity.

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Graphical Abstract

Highlights

- Anti-estrogen therapies selectively enrich for BCSCs and activate Notch signaling
- Notch pathway activation and ALDH1 predict for anti-estrogen treatment failure
- Targeting of Notch4 reduces the population of BCSCs
- Notch inhibitors might prevent relapse or overcome resistance in ER+ tumors

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In Brief
Breast cancers frequently develop resistance to anti-estrogen treatment, which makes it imperative to understanding how therapy resistance develops. Here, Simões et al. show that combining standard anti-estrogen therapies with anti-Notch4 drugs targeting breast cancer stem cells should improve treatment of ER+ breast cancer patients by preventing relapse due to therapy resistance.
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SUMMARY

Breast cancers (BCs) typically express estrogen receptors (ERs) but frequently exhibit de novo or acquired resistance to hormonal therapies. Here, we show that short-term treatment with the anti-estrogens tamoxifen or fulvestrant decrease cell proliferation but increase BC stem cell (BCSC) activity through JAG1-NOTCH4 receptor activation both in patient-derived samples and xenograft (PDX) tumors. In support of this mechanism, we demonstrate that high ALDH1 predicts resistance in women treated with tamoxifen and that a NOTCH4/HES/HEY gene signature predicts for a poor response/prognosis in 2 ER+ patient cohorts. Targeting of NOTCH4 reverses the increase in Notch and BCSC activity induced by anti-estrogens. Importantly, in PDX tumors with acquired tamoxifen resistance, NOTCH4 inhibition reduced BCSC activity. Thus, we establish that BCSC and NOTCH4 activities predict both de novo and acquired tamoxifen resistance and that combining endocrine therapy with targeting JAG1-NOTCH4 overcomes resistance in human breast cancers.

INTRODUCTION

Resistance to endocrine therapies such as selective estrogen receptor (ER) modulators (SERMs; e.g., tamoxifen), selective ER downregulators (SERDs; e.g., fulvestrant), and the aromatase inhibitors is seen in 50%–60% of early breast cancer (BC) cases and develops in almost all patients with advanced disease (Davies et al., 2011; Palmieri et al., 2014).

Evidence suggests that tumor-initiating or cancer stem-like cells (CSCs) are responsible for tumor recurrence after chemotherapeutic and endocrine therapy (Li et al., 2008; Creighton et al., 2009). Al-Hajj et al. (2003) were the first to show that tumor-initiating cells were capable of recapitulating the original tumor phenotype when transplanted into immunodeficient mice. In vitro functional assays for BC stem cell (BCSC) activity include aldehyde dehydrogenase 1 (ALDH1) enzyme activity and the capacity to form clonogenic mammospheres in suspension culture (Ginestier et al., 2007). It has been demonstrated that the BCSC population is ER negative/low and resistant to the direct effects of endocrine therapy (Simoões et al., 2011; Harrison et al., 2013; Piva et al., 2014).

We have shown that aberrant Notch activation transforms normal breast cells, is found in pre-invasive and invasive human BCs, and correlates with early recurrence (Stylianou et al., 2006; Farnie et al., 2007). Moreover, we reported that inhibition of Notch signaling, particularly NOTCH4 receptor, reduced BCSC activity (Simões et al., 2011; Harrison et al., 2013; Piva et al., 2014).

Here, using patient-derived ER+ BC samples and patient-derived xenografts (PDXs), we report that short-term treatment with endocrine therapies enriches for JAG1-NOTCH4-regulated BCSCs, suggesting that these effects are not through genetic selection. Furthermore, we show that ALDH1 expression and NOTCH4 activation in human primary tumors are predictive of resistance to endocrine treatments. Finally, we demonstrate that NOTCH inhibition in vivo reduces BCSC activity in long-term acquired resistant PDX tumors.
RESULTS

BCSC Activity Is Enriched by Tamoxifen and Fulvestrant

We tested the effect of the anti-estrogen tamoxifen on the mammosphere-forming efficiency (MFE) of patient-derived ER+ tumor cells and found that tamoxifen increases mammosphere self-renewal by about 2-fold (Figures 1A, S1A, and S1B). Next, we investigated ALDH activity, another functional assay for CSCs, in nine patient samples treated with tamoxifen or fulvestrant and showed significant increases in ALDH enzymatic activity in seven patients (Figures 1B and 1C). These data suggest that endocrine therapies, given for a period of a few days, enrich for stem cell activity.

Then, we tested the in vivo impact of endocrine therapies on stem cell activity in ER+ BC using PDXs grown subcutaneously in mice. We used both an early (treatment-naive; early BC) and a metastatic ER+ PDX tumor that both maintain biological characteristics (such as the expression of ER and estrogen receptor). In vivo for 14 days with tamoxifen (10 mg/kg/day, oral gavage; red bars) or fulvestrant (200 mg/kg/week, subcutaneous injection; blue bars). Gray bars correspond to vehicle control, FFPE, formalin-fixed paraffin-embedded. (E) Representative micrographs and quantification of Ki67 expression determined by immunohistochemistry (IHC), (F) Percentage of MFE, (G) ALDH-positive cells (%) determined using the ALDEFLUOR assay, (H) ALDH1 expression was assessed by immunohistochemistry in breast tumor epithelial cells, and the percentage of positive cells was scored. Representative micrographs of ALDH-high (ALDH+) and -low (ALDH-) epithelial expression are shown. Kaplan-Meier curves represent cumulative survival for the ALDH population and ALDH+ population of a cohort of 322 pre-menopausal ER+ BC patients who participated in a randomized trial of 2 years of adjuvant tamoxifen treatment versus no systemic treatment (control). Vertical bars on survival curves indicate censored cases. p values are based on a log-rank (Mantel-Cox) test of equality of survival distributions.

Scale bars, 100 μm. Data are represented as mean ± SEM, *p < 0.05; **p < 0.01. See also Figure S1.
Figure 2. Tamoxifen or Fulvestrant Treatment Upregulates Notch Target Genes in Patient-Derived Samples and PDXs

(A and B) Expression of Notch target genes HEY1 and HES1 was assessed by real-time qPCR analysis and compared to control to determine fold change. (A) Metastatic BC patient-derived cells were treated for 7–9 days with ethanol (control), tamoxifen (10⁻⁶ M), or fulvestrant (10⁻⁷ M) and a correlation between fold change of expression of HEY1 and HES1 and fold change of percentage of ALDH-positive cells is shown. (B) Early (HBCx34) and metastatic (BB3RC31) BC PDXs: the effect of in vivo treatment for 14 days with tamoxifen (10 mg/kg/day, oral gavage) or fulvestrant (200 mg/kg/week, subcutaneous injection) on HEY1 and HES1.

(legend continued on next page)
increased expression of tamoxifen or fulvestrant for 6 days preferentially suggest a mechanism for endocrine resistance driven by enrichment for a stem cell phenotype. The mechanism for this enrichment by anti-estrogens may be partly explained by more than 90% of sorted ALDH-positive cells being ER negative (Figure S1C). Thus, we hypothesized that frequency of ALDH-positive cells would predict for response to tamoxifen treatment, and we analyzed ALDH1 in 322 ER+ BC samples taken prior to a randomized trial of tamoxifen versus no systemic treatment. ALDH1 percentage dichotomized at the median value predicted benefit from tamoxifen so that improvement in survival (i.e., a response to treatment) was only seen in women with low epithelial ALDH1 expression (Figure 1H; Table S3). We saw no significant difference in recurrence between control treated patients with high versus low ALDH1 expression (p = 0.59). These data, from a prospective randomized trial, establish for the first time that ALDH-positive cell frequency predicts response to tamoxifen treatment, suggesting that stem cell numbers may be responsible for de novo endocrine resistance.

Tamoxifen or Fulvestrant Treatment Upregulates Notch Target Genes

We analyzed the patient-derived BC cells that were treated with tamoxifen and fulvestrant in Figures 1B and 1C and found that increased numbers of ALDH-positive cells were strongly correlated to increased expression of Notch target genes (HEY1 and HES1) (Figure 2A). In addition, the BC PDX tumors treated in vivo with tamoxifen or fulvestrant (Figure 1D) for 2 weeks showed increased HEY1 and HES1 expression (Figure 2B), supporting an increased role for the Notch signaling pathway after endocrine therapies. In ER+ cell lines (MCF-7, T47D, and ZR-75-1) in vitro, treatment with tamoxifen or fulvestrant for 6 days preferentially increased expression of HEY1 and HES1 (Figure S2A). Similarly, in tamoxifen-resistant (TAMR) or fulvestrant-resistant (FULVR) MCF-7 models, which have acquired resistance after long-term tamoxifen or fulvestrant treatment, we found upregulation of Notch target genes and increased Notch transcriptional activity (Figure S4A).

JAG1 and NOTCH4 Receptor Signaling Drives Endocrine Resistance

Next, we assessed the expression of Notch receptors and ligands in parental, TAMR, and FULVR cell lines. NOTCH4 and its intracellular domain (ICD) were upregulated while NOTCH1, -2, and -3 were downregulated (Figure S4B) in the resistant versus parental cell lines. We found the Notch ligand JAG1 to be highly expressed in both resistant models (Figure S4B), while expression of the other four ligands was either unchanged (DLL1 and DLL4; Figure S4B) or absent (JAG2 and DLL3; data not shown). JAG1 and NOTCH4-ICD were also upregulated after a 14-day window treatment of PDXs in vivo, and after short-term treatment with tamoxifen or fulvestrant of MCF-7 cells in vitro, suggesting that activation of Notch signaling (demonstrated by increased HES1 expression) is an early event in the acquisition of endocrine resistance (Figures 2C and S2B). Importantly, JAG1, NOTCH4-ICD, and HES1 are expressed at higher levels in ALDH-positive cells, which suggests JAG1-NOTCH4 signaling between ALDH-positive cells (Figure 2D).

To further confirm the role of NOTCH4 activity in endocrine resistance and the stem cell phenotype, we analyzed loss-of- and gain-of-function phenotypes for NOTCH4-ICD in MCF-7 cells. Genomic disruption of exon 2 of NOTCH4 by using a CRISPR approach led to loss of protein expression (Figures S2C–S2E) and a significant inhibition of MFE and ALDH-positive cells, especially after tamoxifen and fulvestrant treatments (Figure 2E). In contrast, overexpression of NOTCH4-ICD or JAG1 conferred tamoxifen and fulvestrant resistance in parental MCF-7 cells (Figures 2F and 2G).

Overall, these results indicate that JAG1 ligand and cleavage of NOTCH4-ICD may be responsible for Notch signaling activation after endocrine treatment, which is in agreement with recent reports that NOTCH4 expression is increased in TAMR cell lines (Yun et al., 2013; Lombardo et al., 2014).

GSI RO4929097 Abrogates Tamoxifen- and Fulvestrant-Stimulated CSC Activity

In order to inhibit NOTCH4 signaling, we used the gamma-secretase inhibitor (GSI) RO4929097, which we found to be effective in reducing levels of the active NOTCH4 ICD in endocrine-resistant models (Figure S4C). RO4929097 inhibited HEY1 and HES1 expression, as well as CBF1-Notch transcriptional activity in TAMR and FULVR cell lines, but not in parental MCF-7 cells (Figure S4D). Therefore, we tested whether RO4929097 would abrogate increases in MFE and ALDH-positive cells induced in vivo by anti-estrogens administered in short-term window...
In Vivo Patient-Derived Xenografts

ER+ breast cancer cell lines

(3-day treatment in vitro)

MCF-7 (6-day treatment in vitro)

In Vivo Patient-Derived Xenografts

Secondary tumor formation at 90 days

ER+ breast cancer cell lines

(3-day treatment in vitro)

MCF-7 (6-day treatment in vitro)
treatments, using the same estrogen-dependent ER+ PDX tumors as in Figures 1D–1G. Tamoxifen and fulvestrant treatments reduced tumor growth and proliferation (Figures S3A and S3B) while increasing both MFE and ALDH activity (Figures 3A and 3B). RO4929097 had no impact on growth or proliferation (% Ki67; Figure S3B) but significantly inhibited endocrine-stimulated MFE and ALDH activity (Figures 3A and 3B). The gold standard for functionally determining tumor-initiating cells is xenograft formation in secondary mouse hosts, which we performed using dissociated cells from PDX tumors treated in vivo with anti-estrogens and/or RO4929097. Cells isolated from tumors treated in vivo with RO4929097 had significantly reduced tumor-initiating capacity 90 days post-implantation (Figure 3C).

Furthermore, the stimulation of tumorigenicity following in vivo tamoxifen and fulvestrant treatment was completely reversed by RO4929097 (Figure 3C). Overall, these data suggest that BCSCs, measured by tumor-initiating activity and enriched by short-term anti-estrogen treatments, are dependent on NOTCH4 signaling that can be blocked by combination treatment with a NOTCH4 inhibitor.

To further substantiate this finding, we analyzed MFE and ALDH activity of MCF-7, T47D, and ZR-75-1 cells treated for 3 days with tamoxifen or fulvestran in combination with RO4929097. In all cases, RO4929097 reduced MFE and ALDH-positive cells (Figures 3D and 3E). To confirm that RO4929097 reduced the tumor-initiating capacity, we conducted in vivo limiting dilution transplantation of MCF-7 cells. Extreme limiting dilution analysis (ELDA) revealed an 11-fold enrichment in tumor-initiating cell frequency following tamoxifen or fulvestrant pre-treatment, which was reversed by co-treatment with RO4929097 (Figure 3F). Inhibition of NOTCH4 cleavage/activation by RO4929097 was evidenced by decreased HEY1 and HES1 mRNA and protein levels (Figures 3G and 3H). Thus, we established, using PDX models and cell lines in tumor-initiating cell assays, that NOTCH4 inhibition reduces BCSC activity induced by anti-estrogen treatment.

**NOTCH4 Inhibition Targets CSCs in TAMR PDX Models**

The next question we asked was whether inhibiting NOTCH4 signaling to target BCSCs will overcome long-term acquired anti-estrogen resistance in ER+ BC patients. We investigated RO4929097 treatment in two established PDXs (HBCx22 and HBCx34) that have long-term acquired resistance to tamoxifen in vivo. Analysis of HES1 expression by immunohistochemistry revealed that these two TAMR PDXs displayed increased Notch signaling activation compared to the parental control (Figure 4A).

Notably, the TAMR HBCx34 PDX model has a higher percentage of MFE and ALDH activity than the endocrine-sensitive HBCx34 PDX model (compare Figures 1F and 1G with Figures 4B and 4C). These data suggest that acquired tamoxifen resistance in PDX models involves enrichment for BCSC activity through Notch signaling. Treatment with RO4929097 for 14 days demonstrates that MFE and ALDH activity can be significantly reduced in TAMR PDX tumors in vivo (Figures 4B–4D).

**NOTCH4/HES/HEY Gene Signature Predicts for Resistance to Tamoxifen Treatment and Prognosis in ER+ Tumors**

Based on the aforementioned observations, we hypothesized that NOTCH4 activity, comprising a NOTCH4/HES/HEY gene signature, would predict for response to tamoxifen treatment. In gene expression data from 669 pre-treatment tumors from four published Affymetrix microarray datasets of ER+ patients who subsequently received adjuvant tamoxifen therapy, we found NOTCH4, HES1, HEY1, and HEY2 to be co-expressed in some tumors, as demonstrated in the heatmap ordered from left to right by the sum of the four genes (Figure 5A). Importantly, elevated expression of these Notch genes before treatment was significantly associated with distant metastasis (Figure 5A) and with reduced overall survival in an independent cohort of 343 untreated ER+ patients (Figure 5B). Thus, NOTCH4 gene expression and activity in tumors before treatment with endocrine therapy predicts sensitivity to treatment, indicating that this signaling pathway predicts de novo as well as acquired endocrine resistance. These data strengthen the case for therapies against NOTCH4 to target the endocrine-resistant ALDH-positive cells responsible for relapse of ER+ tumors following hormonal therapy (Figure 5C).

**DISCUSSION**

Here, we report that BCSC activity and frequency are increased in response to the common endocrine therapies tamoxifen and fulvestran in ER+ patient samples and in early and metastatic PDXs. Our findings suggest that endocrine therapies do not target BCSCs, and this may explain how residual drug-resistant cells are responsible for the relapse of ER+ tumors following hormonal therapy. Although we observed increased BCSC frequency after endocrine treatments, we do not know whether absolute BCSC numbers remain the same and are selected for or whether

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**Figure 3. NOTCH4 Inhibition using RO4929097 Abrogates Tamoxifen and Fulvestran Enrichment of CSC Activities**

(A–C) Early (HBCx34) and metastatic (Met) (BDBCRT31) PDX tumors treated in vivo for 14 days with tamoxifen (10 mg/kg/day, oral gavage) or fulvestran (200 mg/kg/week, subcutaneous injection) in the presence or absence of the NOTCH4 inhibitor RO4929097 (3 mg/kg/day, oral gavage). (A) MFE (%). (B) Percentage of ALDH-positive cells. (C) Secondary tumor formation. 100,000 cells of metastatic (BDBCRT31) PDX were re-implanted subcutaneously in NSG mice with 90-day slow-release estrogen pellets. Tumor growth (>100 mm³) was determined at day 90 after cell injection. (D and E) MCF-7, T47D, and ZR-75-1 cells were pre-treated in adherence with 10⁻⁵ M tamoxifen (red bars) and 10⁻⁷ M fulvestrant (blue bars) with RO4929097 (10 μM; hatched bars) or DMSO (filled bars) for 72 hr. (D) MFE and (E) percentage of ALDH-positive cells were assessed after pre-treatments.

(F–H) MCF-7 cells were pre-treated in adherence for 6 days in the presence of RO4929097 (10 μM; hatched bars) or DMSO (filled bars). (F) In vivo experiments were carried out in NSG mice with 90-day slow-release estrogen pellets. Tumor growth (>100 mm³) was assessed at day 60 and is represented as mice positive for growth/mice tested for each cell number tested. ELDA of tumor-initiating cell frequency is shown. (G) Expression of HEY1 and HES1 by real-time PCR was compared to the control. (H) HES1 protein expression levels determined by western blot.

Data are represented as mean ± SEM. p values refer to hatched bars compared to filled control bars. *p < 0.05; **p < 0.01.

See also Figures S3 and S4.
they can be induced by anti-estrogen treatment. Tamoxifen and fulvestrant are clearly successful in reducing BC recurrence in some patients. In other patients with poorer outcome after endocrine therapies, we demonstrate that tumors have high pre-treatment levels of ALDH1 expression and NOTCH4 activation. Moreover, we found that treating ER+ BC cells with endocrine therapies specifically increases JAG1-NOTCH4 signaling and that combining endocrine therapies with a Notch pathway inhibitor can prevent BCSC enrichment induced by endocrine therapies. Thus, our findings in patient-derived BCSCs establish that JAG1 ligand signaling through the NOTCH4 receptor in ALDH-positive cell populations is a determining factor in the acquisition of endocrine resistance.

The best described strategy for inhibition of Notch signaling is the use of small-molecule GSIs, which prevent the release of Notch ICD (NICD). In our study, the GSI RO4929097 specifically targets NOTCH4 cleavage in anti-estrogen-treated cells, and, thus, decreases BCSC activity in vitro (MFE and ALDH activity).
and tumor initiation in vivo. Our investigations in ER+ PDX tumors provide the rationale for the use of NOTCH4 inhibitors together with endocrine therapies in the adjuvant or advanced settings (Figure 5C). Significantly, we demonstrated the utility of RO4929097 to target BCSCs in pre-clinical models of TAMR patient tumors.

In conclusion, our data establish that tamoxifen and fulvestrant select for stem cell activity in short- and long-term-treated BC cells, as well as in early endocrine therapy naive and metastatic-endocrine-treated patient-derived samples and PDXs. Importantly, we report that low numbers of stem cells and low Notch signaling activation in patient tumors predict response to tamoxifen therapy and better survival. Overall, these results suggest that ER+ BC recurrence after endocrine therapies, which target the majority of cells (ER+ cells), will be reduced by targeting the JAG1+/NOTCH4+/ALDH1+/ER/C0 BCSC population.

**EXPERIMENTAL PROCEDURES**

**Patient-Derived Samples**

Early BC samples were collected in RPMI (GIBCO), dissected into 1- to 2-mm³ cubes and digested with the Human Tumor Dissociation Kit (Miltenyi Biotec) for 2 hr at 37°C. Digested tissue was filtered sequentially through 100- and 40-μm cell strainers, then centrifuged at 300 x g for 5 min and washed in PBS. Metastatic samples (ascites or pleural effusions) were centrifuged at 1,000 x g for 10 min at 4°C. The cell pellets were diluted in PBS. Erythrocytes and leucocytes were removed using Lymphoprep (Axis-Shield) and CD45-negative magnetic sorting (Miltenyi Biotec), respectively. Cells were cultured in adherence for 7–9 days in DMEM/F-12 medium, GlutaMAX (GIBCO) with 10% fetal bovine serum (FBS; GIBCO), 10 μg/ml insulin (Sigma-Aldrich), 10 μg/ml hydrocortisone (Sigma-Aldrich), and 5 ng/ml epidermal growth factor (EGF; Sigma-Aldrich), in 10⁻⁶ M 4-OH tamoxifen (Sigma-Aldrich, H7904), 10⁻⁷ M fulvestrant (ICI 182,780, Tocris, 1047), or ethanol (control). Clinico-pathological details of the samples are summarized in Tables S1 (primary BC) and S2 (metastatic BC).

Please refer to the Supplemental Experimental Procedures for further details.

**PDXs and In Vivo Experiments**

Mouse studies commenced in 8- to 12-week-old female mice and were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, using NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice. All in vivo work was performed with a minimum of n = 4 mice per condition. Serial passaging of the PDX was carried out by implanting small fragments of the tumor subcutaneously into dorsal flanks of NSG mice. Early (HBCx34) and metastatic (BB3RC31) BC estrogen-dependent PDXs were administered with 8 μg/ml of 17-beta estradiol in drinking water at all times and were treated with drugs when tumors reached 200–300 mm³. Experiments were performed using PDX tumors between passages 5 and 8. Animal weight and tumor size was measured bi-dimensionally using callipers twice a week.

Tamoxifen citrate (Sigma, T9262, 10 mg/kg/day) and RO4929097 (Cellagen Technology, 3 mg/kg/day) were administered by oral gavage on a basis of 5 days out of 7 (weekends excluded) for 14 days. Tamoxifen citrate and RO4929097 were prepared in 1% carboxymethylcellulose (Sigma, C9481) dissolved in distilled water. Fulvestrant (kindly provided by AstraZeneca, 200 mg/kg/week) was administered by subcutaneous injection
Mammosphere Colony Assay
MFE was calculated by dividing the number of mammospheres formed (≥ 50 μm) by the original number of single cells seeded (500 cells per square centimeter for primary cells) and is expressed as fold change normalized to control or as the mean percentage of MFE (Shaw et al., 2012). Please refer to the Supplemental Experimental Procedures for further details.

Tamoxifen Trial Study
Premenopausal BC patients with invasive stage II disease were enrolled in SBIll2a, a Swedish clinical trial in which patients were randomly assigned to receive 2 years of adjuvant tamoxifen or no treatment (control) and followed up for recurrence-free and overall survival (Prydein et al., 2005). Our data represent cumulative survival for a cohort of 322 premenopausal ER+ BC patients stratified by ALDH-low (below median) and ALDH-high (above median) expression over time.

Statistical Analysis
If not stated otherwise, a two-tailed Student’s t test was performed for statistical analysis. A value of p < 0.05 was considered to be statistically significant. If not stated otherwise, a two-tailed Student’s t test was performed for statistical analysis. A value of p < 0.05 was considered to be statistically significant.

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