Influence of Estrogen Treatment On *ESR1*⁺ and *ESR1*⁻ Cells In ER⁺ Breast Cancer: Insights From Single-Cell Analysis of Patient-Derived Xenograft Models

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Abstract

Background: Estrogen typically promotes the progression of hormone-dependent breast cancer through activation of estrogen receptor (ER)- α encoded by *ESR1*. While estrogen-induced tumor suppression in ER⁺ breast cancer has been clinically observed as an unexpected outcome of aromatase inhibitor (AI)-resistance, the molecular mechanisms have not yet been fully defined. Characterization of estrogen regulation in two ER⁺ breast cancer patient-derived xenograft (PDX) models with opposite responses to estrogen offered us an unprecedented opportunity to assess how 17 β -estradiol (E2) modulates ER⁺ cancer.

Methods: We established two PDX breast cancer models in mice using ER⁺ tumors from patients that responded (SC31) or were suppressed (GS3) by exogenous estrogen. *In vivo* tumor promotion or suppression by estrogen were confirmed through experiments by implanting E2 pellets in mice carrying SC31 or GS3, and then single-cell analysis was performed.

Results: E2 promoted SC31 tumor growth but suppressed growth of GS3 *in vivo*. The E2-mediated suppression of GS3 involves ER α , which was wild-type and not amplified. Single-cell RNA sequencing analysis showed that E2 treatment induced cell cycle promotion in SC31, while E2 induced cell cycle arrest in GS3. However, E2 treatment upregulated the expression of estrogen-regulated genes in both tumors. These gene-expression changes by E2 occurred in both *ESR1*⁺ cells and *ESR1*⁻ cells within the same tumor, demonstrating for the first time the influence of estrogen on *ESR1*⁻ cells in ER⁺ breast tumors. E2 also upregulated a tumor suppressor gene, *IL24*, only in GS3, and lower levels of *IL24* were linked to estrogen independence, after three rounds of intermittent E2 treatment. More *IL24*⁺ cells were *ESR1*⁺ and in G1 phase than *IL24*⁻ cells. Hallmark apoptosis gene sets were upregulated and the hallmark G2M checkpoint gene set was downregulated in *IL24*⁺ cells after E2 treatment.

Conclusions: Our study has revealed the effects of estrogen treatment on both *ESR1*⁺ and *ESR1*⁻ cells in ER⁺ tumors, but not all ER⁺ cancers respond the same manner to estrogen. SC31 is a tumor that is stimulated by E2, while GS3 is suppressed by E2 via cell cycle arrest. Our results indicate a potential role of *IL24* in estrogen-suppressive tumors.

Background

Estrogen plays a crucial role in the progression of hormone-dependent breast cancer through activation of estrogen receptor (ER). By suppressing estrogen production, aromatase inhibitor (AI) therapies are first-line therapy for ER⁺ postmenopausal breast cancers [1–3], and AI's improve the outcomes of these patients [4–6]. Unexpectedly, several clinical trials reported a therapeutic benefit of estrogen for AI-resistant, estrogen-deficient advanced breast cancer.

Patient-derived xenografts (PDXs) are superior to cell lines for cancer research because they typically maintain the biological features of original tumors and have proper multi-cellular architecture, e.g., ER⁺ tumors contain both ER⁺ and ER⁻ cells. In a previous study, we characterized a trastuzumab-resistant ER⁺/HER2⁺ breast cancer PDX named SC31 [7]. While trastuzumab resistance suggests downregulation of HER2 signaling, estrogen was found to act as a growth driver of SC31 tumors. Whole-genome RNA sequencing (RNAseq) transcriptome and reverse phase protein array (RPPA) proteome analyses revealed that ER α and mammalian target of rapamycin (mTOR) signaling predominantly regulate tumor growth of SC31. According to dissection of molecular features using omics approaches and prediction analysis of microarray 50 (PAM50) analyses, SC31, with high expression of *ESR1* (encoding ER α) and *BCL2*, presented as luminal-A subtype, and is thus an estrogen-stimulating tumor model [7]. While the expansion of ER⁺ tumors are typically stimulated by estrogen, we recently succeeded in establishing a unique ER⁺/HER2⁻ PDX model named GS3 from an AI-resistant brain metastatic breast cancer. Our GS3-PDX is a very important model because its characteristics of AI resistance and estrogen-mediated suppression was obtained clinically in the patient, rather than generated *in vitro*.

The functional characterization of these two ER⁺ PDXs suggests that estrogen can stimulate as well as suppress the progression of ER⁺ cancer. The differential responses to estrogen in SC31 and GS3 provided an opportunity to assess how estrogen modulates ER⁺ cancer. While PDX models are suggested to have “phenotypic drift”, estrogen responses of SC31 and GS3 are maintained after 14 passages and have been verified through *in vivo* estrogen treatment, indicating that these are biologically relevant models. Our investigation confirmed estrogen-stimulated growth of SC31 and revealed a new mechanism of estrogen-mediated suppression in GS3 that is different from those reported using other model systems. Considering the intrinsic heterogeneity in tumors, we performed single-cell RNA sequencing (scRNAseq) to evaluate gene expressions in both SC31 and GS3 at the individual cell level and to compare the signaling pathways between cells, especially between *ESR1*⁺ cells and *ESR1*⁻ cells.

Methods

PDX

Surgically resected human breast cancer pieces were implanted into the 4th mammary fat pad of 6–8 weeks old female NOD-scid/IL2Rγ^{-/-} (NSG) mice to establish the PDX lines. The details for PDX preparation were previously described [8]. We used two PDX models, SC31 and GS3, for this study. SC31, an estrogen-stimulating model, was established from a trastuzumab resistant chest wall metastatic postmenopausal breast cancer patient and whose subtype was ER⁺ (80%), progesterone receptor (PR)⁻ (0%), and HER2⁺. Consistent with the clinicopharmacological history of the corresponding patient, SC31 PDX derived from a trastuzumab-relapsed breast tumor was already found to be resistant to trastuzumab treatment with no considerable changes in either tumor volume or weight in the previous study [7]. Although this model is ER⁺/PR⁻/HER2⁺, our previous study demonstrated that the gene expression pattern of SC31 was Luminal-A like [7]. GS3, an estrogen-suppressing model, was established from an AI resistant brain metastatic postmenopausal breast cancer patient and whose subtype was ER⁺ (100%), PR⁺ (5%), and HER2⁻. To confirm that GS3 is indeed resistant to AI, NSG mice bearing serially transplanted GS3 were treated with placebo or letrozole (10 mg with 0.3% hydroxypropylcellulose in 0.9% NaCl solution, daily subcutaneous injection) [9] for 28 days. There was no difference in tumor growth rates and Ki-67 expression (determined by immunohistochemistry [IHC]) between placebo- and letrozole-treated GS3 (Fig. S1A, S1B), but letrozole treatment blocked the mouse mammary gland development. Cell viability assay also showed that organoids isolated from GS3 tumor were resistant to AI (Fig. S1C).

In vivo animal study

Tumor pieces from established SC31/GS3 lines were implanted into mammary fat pad of 8–10 weeks old female ovariectomized/intact NSG mice respectively (GS3 did not grow in ovariectomized NSG mice). Mice were randomized and implanted with SC31 tumors and pellets (17β-estradiol [E2] 1 mg or placebo) on their abdominal side. For GS3, mice were randomized after tumor volume reached approximately 200 mm³ and implanted with an E2 (1 mg) or placebo pellet on their backside. For intermittent E2 treatment, mice were implanted with the E2 (1 mg) pellet which remained in the mice 28 days, and then the pellet was removed on day 28. After 28 days interval with no treatment, the mice were again implanted with the E2 (1 mg) pellet for another 28 days. This intermittent E2 treatment was repeated three rounds. ICI (Fulvestrant, AstraZeneca, Cambridge, UK) was injected subcutaneously (5 mg in 100 μl sterile saline, once weekly) 4 times. Each treatment group of all animal experiments included at least three mice. All experiments were performed in replicate to confirm that the results we observed were statistically significant.

Histological analysis

Hematoxylin and eosin (H&E) staining and IHC of formalin-fixed tumor tissues were performed by the Pathology Core Facility at City of Hope. Antibodies used in IHC included ER α (ab16660, Abcam, Cambridge, UK), PR (PA0312, Leica Biosystems Inc., Wetzlar, Germany), HER2 (A0485, Dako, Glostrup, Denmark), and Ki-67 (M7240, Dako, Glostrup, Denmark). The pathologists evaluated five areas randomly, and ER, PR, HER2, and Ki-67 positivity was defined according to the guidelines [10].

Western blotting

Total protein from PDX tumors were extracted using the Precellys Lysing Kit (Bertin Technologies, Montigny-Le-Bretonneux, France) and lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). After centrifuging at $15,000 \times g$ for 30 min, the supernatants were collected for Western blotting analysis. Antibodies used in Western blotting included ER α (sc-8002, Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (#5174, Cell Signaling Technology, Danvers, MA).

RPPA analysis

Each of the two snap-frozen PDX tumor samples were subjected to RPPA analysis and probed for a total of 232/291 antibodies on SC31/GS3 samples. This was conducted by the MD Anderson Cancer Center Functional Proteomics RPPA Facility as described previously [11].

Real-time PCR analysis

Total RNA for real-time PCR was extracted from PDX tumors and organoids using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Reverse transcription reactions were performed with iScript RT reagent (BioRad, Hercules, CA) according to the manufacturer's instructions. Real-time PCR was performed with SYBER Green FastMix for Real-time PCR (Quantabio, Beverly, MA). The mRNA expression was normalized against both β -actin and GAPDH allowing comparison of mRNA levels. Primers used in this study are listed in Table S1.

Bulk RNAseq and analysis

Total RNA from PDX tumors were extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and then subjected to RNAseq conducted by the Integrative Genomics Core at the City of Hope. All RNA samples were extracted from two biological replicates. All sequencing data was submitted to the GEO database. Gene set enrichment analysis (GSEA) was performed using genes ranked by the fold changes between the different conditions to evaluate the significance activation of 50 HALLMARK gene sets in MSigDB22.

Dissociation into single-cell suspension

Following a treatment which lasted 6 weeks in SC31 and 7 days in GS3 using E2 (1 mg) or a placebo pellet, tumors were harvested and digested into a single-cell suspension. Although results of longer E2/placebo treatments of GS3 showed larger differences of gene expressions according to the bulk RNAseq analysis, the viability of single-cells isolated from GS3 tumors decreased after the tumors shrunk. Since the presence of a large number of dead cells would affect the quality of single-cell analysis, we decided to treat GS3-PDX for 7 days to keep single-cell viability over 80%. Single-cell samples were prepared for scRNAseq using a 10x Genomics platform. Single-cell preparations from two biological replicates from each treatment were combined and processed for scRNAseq (Fig. S2). SC31 and GS3 tumors were cut into small, 2 mm thick strips and digested with 1.5 mg/mL DNase I (#10104159001, Millipore Sigma, St. Louis, MO), 0.4 mg/mL Collagenase IV (CLS-4, Lot: 47E17528A, Worthington, Lakewood, NJ), 5% FBS, 10mM HEPES in HBSS. The mixture was strained through a 70 μ m cell strainer. 1 mL of ACK lysis buffer was used to remove residual red blood cells from the sample. Dead cells were removed using Dead Cells Removal Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

ScRNAseq and analysis

Cell number and viability were measured using a TC20 Automated Cell Counter (BioRad, Hercules, CA). We only processed samples that showed at least 80% viability. Cells were then loaded onto the Chromium Controller (10x Genomics, Pleasanton, CA) targeting 2,000–5,000 cells per lane. The Chromium v2 single-cell 3' RNA-seq reagent kit (10x Genomics, Pleasanton, CA) was used to process samples into scRNA-seq libraries according to the manufacturer's protocol. Libraries were sequenced with a HiSeq 2500 instrument (Illumina, San Diego, CA) with a depth of 50k–100k reads per cell. Raw sequencing data were processed using the 10x Genomics Cell Ranger pipeline (version 2.0) to generate FASTQ files and aligned to mm10 genome to gene expression count. All sequencing data was submitted to the GEO database. The subsequent data analysis was performed using the Seurat package and R software unless otherwise specified. Cells with mitochondrial read rate > 20% and < 200 detectable genes were considered as low-quality and filtered out. After normalization and scaling, Cell cycle scoring was performed using the Seurat package according to the developer's protocol [12]. Principal component analysis was then performed on 2,000 highly variable genes (HVGs). A uniform manifold approximation and projection (UMAP) was generated to summarize and visualize the data in a two-dimensional subspace. Cluster-specific markers were identified to generate heatmap and feature plots in the identified cell clusters. GSEA analysis was also performed on the single-cell level, using genes ranked by mean centered log2-normalized read counts and Hallmark gene sets in MSigDB.

Organoids and in vitro treatment study

Organoids with the E2-suppressing phenotype were established from PDX-derived surgical specimens of GS3 tumors using 3D culture conditions (Fig. S3). After tumor tissue was minced and digested, 10⁴ organoids were embedded in VitroGel 3D-RGD (TWG002, TheWell Bioscience, North Brunswick, NJ) on a 96 well plate and cultured in E2-free M87 medium at 37°C [13]. Organoids viability was evaluated using the CellTiter-Glo 3D Cell Viability Assay (G9682, Promega, Madison, WI) which measured ATP. We made at least five technical replicates and repeated each experiment twice.

Statistics

To assess statistical significance, values of treated groups were compared to those of control/placebo groups by either two-way ANOVA or Student's t-test, using GraphPad Prism 8 (GraphPad software, San Diego, CA). Error bars represent SEM. *P*-value of less than 0.05 was considered statistically significant.

Results

SC31 and GS3 behaved oppositely regarding tumor growth with estrogen

Measurements of tumor volume showed that E2 promoted growth of SC31 and suppressed growth of GS3 (Fig. 1A). A consistent promotion of SC31 and regression of GS3 were observed by the E2 treatments in which an E2 pellet (1 mg) was implanted in mice with SC31/GS3 tumor initially (Fig. 1A, *P* = 0.016, *P* = 0.008, respectively), or after tumors grew up to 150–200mm³ (Fig. 1A, *P* = 0.04, *P* = 0.0006, respectively). These growth phenotypes were maintained after serially transplanted SC31/GS3 tumors (up to 13/14 passages so far). H&E staining showed that cell density (the number of cells per microscopic field) increased in SC31 and decreased in GS3 after E2 treatment (Fig. 1B). IHC indicated that the proportion of ERα⁺ cells, ERα staining intensity, and the number of Ki-67⁺ cells were increased in SC31 and decreased in GS3 after E2 treatment (Fig. 1B). However, IHC showed that PR⁺ cells appeared in both SC31 and GS3 after E2 treatment (Fig. 1B).

E2 downregulated the expression of ER α and cell cycle proliferation genes in GS3

Because the growth response to E2 in these two ER $^{+}$ breast cancers were unexpectedly different, we performed molecular analyses to determine the mechanism of the different response in GS3, the tumor suppressed by E2. These analyses included exome sequencing, bulk RNA sequencing and *in vitro* validation. Human whole exome sequencing of GS3 did not detect *ESR1* and *ESR2* variants (Table S2). ER α and ER β genes in GS3 were wild-type and not amplified. The only other reported estrogen-suppressing PDX model, WHIM16, has an *ESR1* amplification [14, 15]. To verify the effect of E2 on the proliferation of GS3 *in vitro*, organoids were generated from untreated GS3 tumors. E2 suppressed the proliferation of GS3 organoids in an E2 concentration-dependent manner (Fig. 2A). These findings demonstrated that estrogen/ER α signaling could modulate ER $^{+}$ tumor growth by unusual mechanisms. To determine which ER subtype is involved in E2-induced regression of GS3, we performed co-treatment of E2 and ER α -specific antagonist (MPP) or ER β -specific antagonist (PHTPP) *in vitro*. E2-mediated inhibition of GS3-organoids could be reversed by the co-treatment of MPP, but not by PHTPP (Fig. 2B), indicating the participation of ER α in this suppression process. Western blotting and RPPA analysis showed that the E2 treatment resulted in lower ER α protein level *in vivo* (Fig. 2C, 2D). E2 treatment decreased the *ESR1* expression of GS3-organoids at the mRNA level *in vitro* as well (Fig. 2E). E2 treatment increased the levels of PR protein and mRNA (Fig. 2D, 2E, respectively).

To investigate the overall effects of E2 treatment on GS3, bulk RNAseq was performed on tumors from mice treated with E2 for 5, 10 and 53 days. GSEA analysis showed that the hallmark estrogen response early and late gene sets were upregulated after E2 treatment (Table S3). Although the *ESR1* expression level decreased (possibly in part due to reduction of ER α^{+} cells, as indicated by IHC), expression levels of estrogen-regulated genes, such as *PGR*, *GREB1*, and *TFF1*, increased after E2 treatment (Fig. 2F). In addition, cell cycle progression genes, such as *CDK1*, *TOP2A*, *E2F2*, and *MKI67*, were down-regulated in E2-treated GS3 tumors (Fig. 2F). The organoids isolated from longer E2-treated tumors expressed estrogen-regulated genes at a high level (Fig. 2G). In addition, the expression of two tumor suppressor genes, *IL24* and *GADD45A*, were upregulated in E2-treated PDXs, as well as in organoids that were isolated from E2-treated tumors, depending on treatment time (Fig. 2F, 2G). Collectively, our examination of GS3 tumors after E2 treatment indicates that inhibition of GS3 growth is associated with a reduction of ER α^{+} cells, an increased expression of ER α target genes (indicating the presence of functional active ER α), and an induced cell cycle arrest and growth suppression.

Impact of E2 on gene expression at the single-cell level in SC31 and GS3

To better understand the mechanism of estrogen-regulatory effects and the potential contributions of ER α^{+} cells and ER α^{-} cells within the tumors, we performed scRNAseq on individual cells from SC31 and GS3. After removing mouse cells from the single cell suspension based on species-specific DNA markers (8% of total cells), 19,568 genes were detected from 5,581 cells in SC31 (3,110 cells from placebo-treated tumors and 2,471 cells from E2-treated tumors), and 20,467 genes were detected from 10,631 cells in GS3 (3,927 cells from placebo-treated tumors and 6,704 cells from E2-treated tumors). A UMAP plot led to the identification of 8 major cell clusters in SC31 (Fig. 3A) and GS3 (Fig. 3B), where each dot represented one cell. The number of cells in each cluster is specified in Table S4. In SC31, C3 and C6 included more placebo-treated and G1 phase cells while C2 and C5 included more E2-treated and G2/M phase cells (Fig. 3A). In GS3, C1 and C7 included more E2-treated and G1 phase cells while C3, C5 and C6 included more placebo-treated and G2/M phase cells (Fig. 3B).

Characteristics of single-cell clusters in SC31 and GS3

The relationships between 8 clusters of each single-cell analysis were visualized by a heat map using the top 10 differentially expressed genes (DEGs) and hierarchical clustering based on the average expression of HVGs. In SC31, top 10 DEGs in C2, C4, and C5 included genes belonging to the hallmark G2M checkpoint gene set (Fig. 3C, Table S5). A dendrogram showed that C2 and C5, where approximately 62 % of the cells were from E2-treated tumors, were most similar (Fig. 3D), and cells in those clusters were mainly in G2/M or S phase (Fig. S4A). GSEA analysis showed that the hallmark mTORC1 signaling gene set was upregulated ($P = 3.30E-41$) in C6 (Table S5). In GS3, top 10 DEGs in C0 and C2 included estrogen response genes (Fig. 3E), and cells in those clusters were mainly G1 phase cells (Fig. S4B). The hallmark G2M checkpoint gene set was downregulated in these clusters ($P = 2.02E-22$ and $P = 3.76E-25$, respectively) according to GSEA analysis (Table S6). In C1, which included approximately 90% of G1 phase cells (Fig. S4B), the hallmark estrogen response early and late gene sets were upregulated ($P = 5.13E-06$ and $P = 3.90E-07$, respectively), and the hallmark G2M checkpoint gene set was significantly downregulated ($P = 5.62E-25$) at the same time (Table S6). C1 and C0, where the hallmark G2M checkpoint gene set was downregulated, were relatively similar according to a dendrogram (Fig. 3F).

Comparison of E2-treated cells vs. placebo-treated cells in SC31 and GS3

In SC31, GSEA analysis showed that the hallmark G2M checkpoint gene set and the hallmark estrogen response early and late gene sets were significantly upregulated ($P = 8.99E-72$, $5.43E-52$, $5.43E-52$, respectively) after E2 treatment (Table 1). Cells expressing *ESR1* or estrogen-regulated gene *PGR* were distributed mainly in C1, C3, and C6 (Fig. S4C). The percentage of *ESR1*⁺ cells and *PGR*⁺ cells increased by 31% and 37% respectively in E2-treated cells compared to placebo-treated cells (Fig. 4A). A majority of *MKI67*⁺ cells were found in C3 and C6 (Fig. S4C), and the percentage of *MKI67*⁺ cells increased by 15% in E2-treated cells (Fig. 4A). In GS3, GSEA analysis showed that the hallmark estrogen response early and late gene sets were upregulated ($P = 1.63E-17$ and $4.55E-16$, respectively), while the hallmark G2M checkpoint gene set was downregulated ($P = 3.95E-13$) after E2 treatment (Table 1). Most *ESR1*⁺ cells were present in C1, C2 and C6, and the percentage of *ESR1*⁺ cells decreased by 10% in E2-treated cells compared to placebo-treated cells (Fig. 4B, S4D). Cells expressing *PGR*⁺ were found in C1, C2 and C6, in the same distribution as *ESR1*⁺ cells, and the percentage of cells expressing *PGR* increased by 11% in E2-treated cells (Fig. 4B, S4D). Furthermore, most of cells expressing *MKI67* were primarily in C6. The percentage of *MKI67*⁺ cells decreased by 4% in E2-treated cells (Fig. 4B, S4D). The percentage of cells expressing other estrogen-regulated genes, such as *AREG* and *PDZK1*, also increased in E2-treated cells in both SC31 and GS3 (Fig. S5A, S5B). The percentage of cells expressing other cell cycle progression genes, such as *CCNE2* and *CDK1*, increased in E2-treated cells in SC31, while decreased in GS3 (Fig. S5A, S5B).

Distribution of cells in the G1, S, or G2/M phases changed after E2 treatment. In SC31, the percentage of cells which progressed to the G2/M phase increased (+6.6%) and the percentage of cells arrested at the G1 phase decreased (-14.5%) in E2-treated cells (Fig. S6A). In GS3, the percentage of cells which progressed to the G2/M phase decreased (-4.7%) and the percentage of cells arrested at the G1 phase increased (+12.5%) in E2-treated cells (Fig. S6B). Collectively, the estrogen-regulated gene, *PGR*, was expressed after E2 treatment in both SC31 and GS3, while the percentage of cells expressing *ESR1* and *MKI67* or entering G2/M phase increased in SC31 but decreased in GS3.

Table 1. Hallmark gene sets analysis of SC31 and GS3 (E2 treated vs. Placebo)

	Upregulated in E2	P-value	Downregulated in E2	P-value
SC31	E2F_TARGETS	5.94E-84	INTERFERON_GAMMA_RESPONSE	5.43E-64
	MYC_TARGETS_V1	2.88E-76	INTERFERON_ALPHA_RESPONSE	6.33E-64
	G2M_CHECKPOINT	8.99E-72	TNFA_SIGNALING_VIA_NFKB	1.70E-33
	ESTROGEN_RESPONSE_EARLY	5.43E-52	APOPTOSIS	6.64E-25
	ESTROGEN_RESPONSE_LATE	5.43E-52	P53_PATHWAY	2.15E-18
GS3	ESTROGEN_RESPONSE_LATE	1.63E-17	TNFA_SIGNALING_VIA_NFKB	6.06E-21
	ESTROGEN_RESPONSE_EARLY	4.55E-16	G2M_CHECKPOINT	3.95E-13
			P53_PATHWAY	3.95E-13
			APOPTOSIS	5.90E-12
			E2F_TARGETS	6.23E-12

Footnote: This table included top five hallmark gene sets or those which *P* value was smaller than 1.0E-10.

Effects of E2 treatment on *ESR1*⁺ cells and *ESR1*[−] cells in SC31 and GS3

Since the percentage of *ESR1*⁺ cells changed oppositely in SC31 and GS3, we decided to compare *ESR1*⁺ cells and *ESR1*[−] cells by analyzing them separately. The percentage of *ESR1*⁺ and *ESR1*[−] cells in two tumors treated with E2 or placebo was shown in Table S7. In SC31, the percentage of cells progressing to the G2/M phase increased by 7.2% in *ESR1*⁺ cells and by 5.8% in *ESR1*[−] cells after E2 treatment. Furthermore, the percentage of cells in the G1 phase decreased by 15.9% and 10.9% in *ESR1*⁺ and *ESR1*[−] cells respectively (Fig. 4C). In GS3, the percentage of cells arrested at the G1 phase increased by 10.9% in *ESR1*⁺ cells and by 16.0% in *ESR1*[−] cells after E2 treatment. In addition, the percentage of cells progressing to the G2M phase decreased by 3.4% and 7.8% in *ESR1*⁺ and *ESR1*[−] cells, respectively. *ESR1*[−] cells included more G2M phase cells compared to *ESR1*⁺ cells in GS3 (Fig. 4D). However, the decreasing percentage of G2/M phase cells in GS3 after E2 treatment was greater in *ESR1*[−] cells compared to *ESR1*⁺ cells (Fig. 4D).

To clarify the difference of signaling pathways between *ESR1*⁺ cells and *ESR1*[−] cells, we performed GSEA analysis for *ESR1*⁺ cells and *ESR1*[−] cells separately. Firstly, we focused on placebo-treated cells to learn original characteristics of two tumors. In SC31, the hallmark G2M checkpoint gene set was upregulated in *ESR1*⁺ cells (*P* = 1.99E-08), and the hallmark mTORC1 signaling gene set was upregulated in *ESR1*[−] cells compared to *ESR1*⁺ cells (*P* = 1.56E-03, Table S8). In GS3, the hallmark TNFA/NFκB signaling gene set was upregulated in *ESR1*⁺ cells (*P* = 4.50E-34) and the hallmark G2M checkpoint gene set was upregulated in *ESR1*[−] cells (*P* = 7.91E-15, Table S8). Secondly, we focused on the comparison of placebo-treated vs. E2-treated cells. In SC31, the hallmark G2M checkpoint gene set and estrogen response early and late gene sets were upregulated not only in *ESR1*⁺ cells (*P* = 1.98E-60, 1.35E-06, 8.26E-08, respectively), but also in *ESR1*[−] cells (*P* = 1.64E-25, 1.13E-07, 4.72E-09, respectively) after E2 treatment (Table 2). In GS3, the hallmark estrogen response early and late gene sets were upregulated not only in *ESR1*⁺ cells (*P* = 2.69E-19, *P* = 7.36E-18, respectively), but also in *ESR1*[−] cells (*P* = 1.22E-16, *P* = 2.99E-15, respectively) after E2 treatment. Moreover, the hallmark G2M checkpoint gene set was significantly downregulated in *ESR1*[−] cells (*P* = 1.45E-30) after E2 treatment (Table 2). Therefore, our single-cell analysis revealed that E2 treatment upregulated the expression of estrogen response genes in all epithelial cells of both SC31 and

GS3. While estrogen upregulated the expression of cell cycle proliferation genes of SC31 in both *ESR1*⁺ and *ESR1*[−] cells, it downregulated the expression of cell cycle proliferation genes of GS3.

Table 2. Hallmark gene sets analysis of *ESR1*⁺ cells and *ESR1*[−] cells (E2 treated vs. Placebo)

		Upregulated in E2	<i>P</i> -value	Downregulated in E2	<i>P</i> -value
SC31	<i>ESR1</i> ⁺ cells	E2F_TARGETS	2.91E-83	INTERFERON_GAMMA_RESPONSE	6.11E-64
		G2M_CHECKPOINT	1.23E-72	INTERFERON_ALPHA_RESPONSE	6.81E-63
		MYC_TARGETS_V1	1.10E-69	TNFA_SIGNALING_VIA_NFKB	1.06E-34
		ESTROGEN_RESPONSE_EARLY	6.52E-57	APOPTOSIS	2.82E-23
		ESTROGEN_RESPONSE_LATE	1.67E-51	P53_PATHWAY	3.16E-21
	<i>ESR1</i> [−] cells	E2F_TARGETS	1.94E-62	INTERFERON_ALPHA_RESPONSE	4.80E-66
		MYC_TARGETS_V1	1.94E-62	INTERFERON_GAMMA_RESPONSE	7.63E-66
		G2M_CHECKPOINT	1.44E-48	TNFA_SIGNALING_VIA_NFKB	7.61E-34
		ESTROGEN_RESPONSE_EARLY	2.49E-43	APOPTOSIS	3.63E-25
		ESTROGEN_RESPONSE_LATE	4.75E-42	HYPOXIA	3.83E-16
GS3	<i>ESR1</i> ⁺ cells	ESTROGEN_RESPONSE_EARLY	1.11E-19	TNFA_SIGNALING_VIA_NFKB	2.20E-22
		ESTROGEN_RESPONSE_LATE	3.20E-18	P53_PATHWAY	3.69E-13
				APOPTOSIS	5.57E-12
	<i>ESR1</i> [−] cells	ESTROGEN_RESPONSE_LATE	5.61E-17	G2M_CHECKPOINT	3.05E-31
		ESTROGEN_RESPONSE_EARLY	1.44E-15	E2F_TARGETS	8.25E-23
				TNFA_SIGNALING_VIA_NFKB	5.48E-14

Footnote: This table included top five hallmark gene sets or those which *P* value was smaller than 1.0E-10.

E2-induced *IL24*⁺ cells through ERα only in GS3

Interleukin-24 (IL24) has been shown to be a tumor suppressor [16–20]. In SC31 cells, no *IL24* expression was observed in either E2-treated or placebo conditions. (Fig. 5A). In GS3 cells, the percentage of *IL24*⁺ cells in the E2-treated category was 2.75-fold of that in placebo-treated cells (Fig. 5A). More *IL24*⁺ cells expressed *ESR1*⁺ compared to *IL24*[−] cells (Fig. 5B). In *IL24*⁺ cells, the percentage of cells that progressed to the G2/M phase decreased (−12.4%) and those that arrested at the G1 phase increased (+9.7%) when compared to those in the *IL24*[−] cells (Fig. 5C). When comparing *IL24* expression levels in total mRNA extracted from GS3 tumors, *IL24* expression level was upregulated after E2 treatment in a time dependent manner, and its expression was inhibited by ICI (Fig. 5D). To clarify the difference of signaling pathways between *IL24*[−] cells and *IL24*⁺ cells, we performed GSEA analysis for *IL24*[−] cells and *IL24*⁺ cells separately. The hallmark TNFA signaling via NFkB, hallmark estrogen response early and late, and hallmark apoptosis gene sets were upregulated in *IL24*⁺ cells after E2 treatment. On the other hand, the hallmark G2M checkpoint gene set was downregulated in *IL24*⁺ cells after E2 treatment (Table 3).

Table 3. GSEA hallmark gene sets analysis of genes expressed on *IL24*⁺ cells vs. *IL24*[−] cells

Upregulated in <i>IL24</i> ⁺ cells	<i>P</i> -value	Downregulated in <i>IL24</i> ⁺ cells	<i>P</i> -value
TNFA_SIGNALING_VIA_NFKB	1.33E-17	G2M_CHECKPOINT	8.25E-09
ESTROGEN_RESPONSE_EARLY	1.73E-09	E2F_TARGETS	8.25E-09
ESTROGEN_RESPONSE_LATE	1.73E-09	MYC_TARGETS_V1	5.24E-05
APOPTOSIS	2.45E-06	P53_PATHWAY	2.46E-03

Footnote: This table included all hallmark gene sets from the results of GSEA.

Intermittent E2 treatment in GS3

To investigate possible changes in response to E2 in GS3 tumors, we exposed PDX mice to cycles of intermittent E2 treatment (on and off every 28 days). A repeating pattern of tumor growth inhibition (E2 exposed) and growth (E2 absent) was initially observed. However, after three rounds of intermittent E2 treatment on GS3, an E2 independent growth developed (Fig. 6A). The decrease of ERα⁺ cells and Ki-67⁺ cells evaluated by after E2 treatment was reversed after intermittent E2 treatment (Fig. 6B), ERα protein level by Western blotting, as well (Fig. 2C). The increase of PR⁺ cells by IHC after E2 treatment was not reversed after intermittent E2 treatment (Fig. 6B). To identify the key pathway driving this emerging E2 independence, we performed bulk RNAseq on GS3 tumors treated with placebo, E2 (for 28 days), or intermittent E2. The hierarchical clustering using 1,312 DEGs showed that approximately 60% of genes in the intermittent E2-treated sample had the same trend as E2-treated samples (mainly estrogen-regulated genes), in which 40% of the genes behaved similarly to the placebo-treated sample (mainly cell cycle progression genes) (Fig. 6C). After E2 treatment for 28 days, the hallmark estrogen response late gene set was upregulated and the hallmark G2M checkpoint gene set was downregulated: the same results as scRNAseq analysis (Fig. S7A, S7B). Interestingly, the hallmark Reactive Oxygen Species (ROS) pathway gene set was upregulated after the long-term E2 treatment, despite the effective pathway size being relatively small (Fig. S7A). Upregulation of *IL24* expression level and the hallmark ROS pathway in 28-day E2 was reversed in intermittent E2 samples (Fig. 5D, S7B). Downregulation of the hallmark G2M checkpoint gene set in 28-day E2 was also reversed in intermittent E2 samples (Fig. S7B).

Discussion

A better understanding of breast cancer molecular and cellular biology has benefited from entirely new methods and technologies. For example, scRNAseq can identify the differences in expression levels among thousands of genes in individual cells in a nonbiased manner. It is also capable of assigning cell types based on gene expression profiles and can easily check the expression a selected gene among all single-cells in the study [21]. Furthermore, scRNAseq enables the identification of each cell with respect to cell cycle phase and tumor-associated gene expression.

Through scRNAseq on tumors with definitive responses to estrogen treatment, we could analyze gene expression profiles of individual *ESR1*⁺ and *ESR1*⁻ cells from ER⁺ breast cancers with vs. without estrogen treatment. ER⁺ disease is typically treated with AIs to reduce estrogen, since the latter is thought to promote growth of these tumors. ER⁺ cancer is identified by IHC analysis (1 – 100%) [22]. Although clinical data on the subgroup of cancers with ER-low-positive (1 – 10%) are less definitive, 100% ER positivity is not required for an endocrine therapy response. There has been little information about ER⁻ cells in endocrine-responsive tumors.

Our scRNAseq analysis of SC31 and GS3 offered an unprecedented opportunity to address the molecular and functional differences between *ESR1*⁺ and *ESR1*⁻ cells in ER⁺ tumors. While *ESR1*⁺ and *ESR1*⁻ cells within the tumors have different gene expression profiles, after analyzing DEGs on *ESR1*⁺ and *ESR1*⁻ cells separately, the G2M checkpoint gene set and the estrogen response early and late gene sets in SC31 were upregulated in not only *ESR1*⁺ cells, but also *ESR1*⁻ cells. In GS3, the G2M checkpoint gene set was downregulated mainly in *ESR1*⁻ cells, and the E2 response gene sets were upregulated in both *ESR1*⁺ cells and *ESR1*⁻ cells. This means that *ESR1*⁻ cells were also affected by E2 and behaved similarly to *ESR1*⁺ cells. While E2 should activate ER α and regulate *ESR1*⁺ cells, our findings regarding *ESR1*⁻ cells were new and suggested that the proliferation of *ESR1*⁻ cells in ER⁺ cancer is also affected by estrogen. We compared the percentage of *ESR1*⁺ and *ESR1*⁻ cells isolated from placebo and E2-treated PDXs (Table S7). The results further support that E2 affects both *ESR1*⁺ and *ESR1*⁻ cells. Otherwise, we would expect to find mainly *ESR1*⁺ cells in SC31 and *ESR1*⁻ cells in GS3 after E2 treatment. The activation of mTOR signaling pathway in SC31 was observed in *ESR1*⁻ cells. The findings in the previous study [7] on the combined effect of ICI and mTOR inhibitors for SC31 further support the case for effects of estrogen on *ESR1*⁻ cells in this tumor model. This was the first time to demonstrate the estrogen-induced responses in *ESR1*⁺ cells and *ESR1*⁻ cells in ER⁺ tumors using the scRNASeq. While the detailed mechanisms of the potential interactions between *ESR1*⁺ cells and *ESR1*⁻ cells are yet to be better defined, this finding offers an important explanation as to why most of the estrogen-responsive breast cancers are not homogeneously 100% ER⁺. Furthermore, it is clinically unusual that endocrine therapy of ER⁺ cancer results in a total loss of ER positivity in the residual tumors.

Our results also showed that estrogen-regulated genes (as exemplified by *PGR*) in ER⁺ breast cancer were induced by E2 regardless of the tumors which growth were promoted or suppressed by E2. SC31 is a trastuzumab-resistant, ER⁺/HER2⁺ PDX model with gene expression characteristic of luminal-A breast cancer [7]. As shown previously [23, 24], E2 signaling increased expression of estrogen-regulated genes and genes involved in cell proliferation (Fig. 4A, S5A). Ingenuity Pathway Analysis revealed that Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/mTOR pathway was the mostly significant network, and GSEA supportively identified that mTOR signaling and the ER α target, Cyclin D1, were remarkably activated in SC31 [8]. RPPA results of 232 pan-/phosphor-proteins independently revealed that expression levels of the active forms of signaling components in the PI3K/AKT/mTOR pathway, as well as those of ER signaling were relatively higher at the posttranslational level. These results have indicated that PI3K/AKT/mTOR signaling is a critical oncogenic pathway, and ER α signaling plays a dominated role in SC31 [8]. This is an important example for the activation of ER-mediated pathway in ER⁺/HER2⁺ cancers [25], and ER regulation becomes dominant after HER2 signaling is no longer the driver. SC31 responds to E2 in the same manner as typical luminal-A ER⁺ breast cancer.

Als are first line adjuvant therapy for Luminal-A type postmenopausal breast cancer. Clinically, AI resistance is not uncommon [5], and estrogen-induced tumor regression has been reported in some AI resistant ER⁺ breast cancer cases [26–31]. Considering that AI resistant cancers develop an ability to grow without estrogen, important AI resistant cell lines have been established from hormone-dependent breast cancer cell lines, such as MCF-7 and T-47D cells, through culture under long-term estrogen deprivation (LTED) conditions [32–36]. Some of these LTED cell lines showed higher ER expression and estrogen hypersensitivity [35]. In some LTED cell lines, growth factor receptors and downstream signaling pathways, including the MAPK, PI3K/AKT/mTOR, and JNK pathways, could function as alternative growth signaling pathways, independent of ER [37].

Cell lines and a PDX model have been used to investigate the mechanism of the varying response to E2 in breast cancer [14, 38–42]. In our investigations of PDX models SC31 and GS3 by scRNAseq, we obtained new and valuable information on how E2 suppresses growth of patient derived tumors in the PDX murine model. While as expected, E2 exposure increased the expression of ER α -regulated genes in the GS3 model, our single-cells analyses revealed that E2 reduced the proportion of cells expressing genes involved in cell proliferation (Fig. 4B and S6B). While others have suggested that AI resistance is caused by mutation [43, 44] or amplification [14, 15] of *ESR1*, our results of GS3 indicated that this is not so. AI-resistant tumors often retain ER expression and ER signaling [45]. ER is constitutively active in GS3, as reported for AI-resistant tumors [45]. To verify the important role of ER in GS3, *in vivo* treatment of placebo/E2/ICI/E2 + ICI for GS3 indicated that E2, ICI, and E2 + ICI treatments were significantly suppressed GS3 tumor growth compared to placebo treatment. (Fig. S8).

Importantly, the results of bulk RNAseq of GS3 tumors revealed that the *IL24* expression level increased markedly after E2 treatment in a time dependent manner (Fig. 2F); we confirmed that *IL24* was upregulated by ER α in GS3 (Fig. 5D). *IL24* was not included in the hallmark estrogen response early and late gene sets, but it has been documented that *IL24* was upregulated by E2 preferentially through ER α [46]. IL-24 is a tumor suppressor cytokine that selectively induces cell cycle arrest and apoptosis in a wide variety of human cancer cells, including breast cancer [16–20]. *IL24*⁺ cells were arrested in the G1 phase of the cell cycle (Fig. 5C), downregulating the hallmark G2M checkpoint gene set, and upregulating the hallmark apoptosis gene set (Table 3). These results were consistent for IL-24 as a tumor suppressor. Publicly available data showing that high expression of IL-24 at the protein level is related to longer overall survival in breast cancer patients also further supported our results ($P = 0.00051$, Fig. S9). Therefore, our results suggest that estrogen-mediated suppression of GS3 is through its induction of IL24 expression vs. ER. Our analysis has revealed that TNFA Signaling via NFKB gene set has a strong association with *IL24*⁺ cells. The NFKB pathway has been linked to endocrine therapy resistance [47].

Intermittent E2 treatment tumor serves as a good reference control because when mice bearing GS3 tumors were treated intermittently with E2, the tumor was initially E2 dependent but after several cycles became E2-independent. Upregulation of the hallmark ROS pathway gene set and downregulation of the hallmark G2M checkpoint gene set after E2 treatment for 28 days were reversed in intermittent E2 samples (Fig. S7A, S7B). The increase of *IL24* expression level after E2 treatment for 28 days was also reversed in intermittent E2 samples (Fig. 5D). These results confirm our data on the PDX models that the ROS pathway and IL24 expression play important roles in the suppression of tumor growth induced in these tumors by E2.

Based in our data on single tumor cells in a valid breast cancer model, we propose a mechanism for E2 induced promotion and repression (Fig. 7). The expression of estrogen-regulated gene expression in both *ESR1*⁺ cells and *ESR1*[−] cells in ER⁺ tumors was shown for the first time through single-cell analysis of estrogen-responsive PDXs containing both types of cells. The expression of estrogen-regulated genes, such as *PGR* and *AREG*, is upregulated in ER⁺ breast cancer regardless of the tumors in which growth are promoted or suppressed by E2. On the contrary to the common ER⁺ breast cancer like SC31, decreasing the number of *ESR1*⁺ cells and G2/M phase cells following the E2 treatment were observed

in a part of ER⁺ AI resistance breast cancer like GS3. Elimination of ER⁺ cells played a crucial role for E2-suppressed tumor growth. More importantly, E2 increased the percentage of cells expressing *IL24*, which has been identified as a tumor suppressor gene.

Estrogen-induced cell cycle arrest can be an unexpected outcome of AI resistance, suggesting that estrogen therapy may be viable for such patients. In ER⁺ breast cancer, estrogen is typically assumed to promote tumor growth. Therefore, clinicians hesitate to use E2 as a general treatment option for recurrent ER⁺ tumors even when tumors are completely resistant to AI. Our mechanistic studies of GS3 offer leads into identifying such tumors that can respond to E2 therapies. The clinical benefit rate of estrogen therapies for AI resistant cases is 26–56% in 7 trials (Table S9). Our findings point out that measurements of ER and PR expression alone are not sufficient to propose an E2 therapy. We need to identify appropriate biomarkers to select patients who will benefit from undergoing E2 therapy. Our study has shown that *IL24* is a biomarker candidate for selecting patients and predicting effect of E2 treatment in AI-resistant ER⁺ postmenopausal breast cancer.

We recognize several limitations in this study. There is a technical limitation to isolate 100% living cells after tumor digestion for single-cell analysis. Furthermore, we observed differences between the ER α positivity rate of placebo-treated SC31/GS3 at the protein level by IHC and the *ESR1* positivity rate at mRNA level. The single-cell isolation from solid tissue remains empirical. It is likely that different types of cells are fractionated with different efficiency. Another limitation is that our study included a single-estrogen-suppressing PDX model. There are only two estrogen-suppressing PDXs so far, GS3 and WHIM16 [14, 15]. We consider GS3 as a valuable tumor model derived from a patient that reveals a new mechanism of estrogen-induced cell-cycle arrest, indicating that there may be more than one way for estrogen-mediated suppression of breast cancer. With this in mind, additional estrogen-suppressing tumor specimens will be needed to verify these mechanisms.

Conclusions

Estrogen/ER α signaling increases the expression of estrogen-regulated genes (e.g., *PGR* and *AREG*), but it can modulate ER⁺ tumor growth in different manners. E2 promotes ER⁺ breast cancer growth as seen in the case of SC31, while E2-induced suppression is an unexpected outcome of AI resistance (such as GS3). In the latter case, elimination of estrogen by AI results in maintaining tumor growth. Analysis of GS3-PDX has revealed that estrogen can induce cell cycle arrest. As a conceptual advancement, our results reveal potential interactions between *ESR1*⁺ and *ESR1*[−] cells in both estrogen-stimulating and -suppressing ER⁺ tumors as well as potential roles of *IL24* that expresses in some breast cancers. Our findings point to the need to identify biomarkers for patients with estrogen-suppressing tumors who can benefit from E2 treatment after AI resistance; measurements of ER and PR expression are insufficient. Expression of *IL24* in AI-resistant tumors may be one such indicator for favorable response to E2 therapy.

List Of Abbreviations

AIs, aromatase inhibitors; AKT, Protein Kinase B; DEGs, differentially expressed genes; E2, 17 β -estradiol; ER, estrogen receptor; GSEA, Gene set enrichment analysis; H&E, Hematoxylin and eosin; HVGs, highly variable genes; IHC, immunohistochemistry; IL-24, Interleukin-24; LTED, long-term estrogen deprivation; mTOR, mammalian target of rapamycin; NSG, NOD-scid/IL2R γ ^{−/−}; PAM50, prediction analysis of microarray 50; PDX, patient-derived xenograft; PI3K, Phosphoinositide 3-kinase; PR, progesterone receptor; RNAseq, RNA sequencing; ROS, Reactive Oxygen Species; RPPA, Reverse phase protein array; scRNAseq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection.

Declarations

Ethics approval and consent to participate

This study was approved by City of Hope Institutional Review Board, and the patients provided written informed consent prior to tissue collection (IRB # 17030). All animal research procedures used in this study were approved by the Institutional Animal Care and Used Committee at City of Hope (IACUC #91051). Facilities are created by Association for Assessment and Accreditation of Laboratory Animal Core and operated according to National Institutes of Health guideline.

Consent for publication

Not applicable

Availability of data and materials

The raw bulk RNAseq and scRNAseq data generated in this study has been deposited in GEO.

SC31; scRNAseq, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE176532>

GS3; bulk RNAseq, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156922>

GS3; scRNAseq, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156752>

Competing interests

All authors declare no conflicts of interest.

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Authors' contributions

S.C. and H.M. designed the study; H.M., G.C., P.Y.H, N.K. and X.W. (Xiaoqiang Wang) performed the experiments; J.W. performed the 10X library production and sequencing; H.M., K.S., G.C., A. B., and X.W. (Xiwei Wu) analyzed the data; G.S. provided the clinical sample; H.M. and S.C. wrote the paper; M.N. is responsible person of JSPS funding acquisition; S.C. supervised the research.

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References

1. Chlebowski R, Cuzick J, Amakye D, Bauerfeind I, Buzdar A, Chiaf S, Cutulig B, Linforthh R, Maassi N, Noguchij S, et al. Clinical perspectives on the utility of aromatase inhibitors for the adjuvant treatment of breast cancer. *The Breast*. 2009;18(Suppl 2):1–11. doi:10.1016/S0960-9776(09)70002-5.
2. Chumsri S, Howes T, Bao T, Sabnis G, Brodie A. Aromatase, aromatase inhibitors, and breast cancer. *The Journal of Steroid Biochemistry Molecular Biology*. 2011;125(1–2):13–22. doi:10.1016/j.jsbmb.2011.02.001. PubMed PMID: 21335088; PubMed Central PMCID: PMC3104073.
3. Lao Romera J, Puertolas Hernandez TJ, Pelaez Fernandez I, Sampedro Gimeno T, Fernandez Martinez R, Fernandez Perez I, Iranzo Gonzalez Cruz V, Illarramendi Manas JJ, Garcera Juan S, Ciruelos Gil EM. Update on adjuvant hormonal treatment of early breast cancer. *Advances in Therapy*. 2011;28(Suppl 6):1–18. doi:10.1007/s12325-011-0017-1. PubMed PMID: 21922392.
4. The BIG 1–98 Collaborative Group. Letrozole Therapy Alone or in Sequence with Tamoxifen in Women with Breast Cancer. *N Engl J Med*. 2009;361(8):766–76. doi:10.1056/NEJMoa0810818.
5. Cuzick J, Sestak I, Baum M, Buzdar A, Howell A, Dowsett M, Forbes JF, investigators AL: Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncology* 2010, 11(12):1135–1141. doi: 10.1016/S1470-2045(10)70257-6.
6. van de Velde CJ, Rea D, Seynaeve C, Putter H, Hasenburg A, Vannetzel JM, Paridaens R, Markopoulos C, Hozumi Y, Hille ETM, et al. Adjuvant tamoxifen and exemestane in early breast cancer (TEAM): a randomised phase 3 trial. *Lancet*. 2011;377(9762):321–31. doi:10.1016/S0140-6736(10)62312-4.
7. Hsu PY, Wu VS, Kanaya N, Petrossian K, Hsu HK, Nguyen D, Schmolze D, Kai M, Liu CY, Lu H, et al. Dual mTOR Kinase Inhibitor MLN0128 Sensitizes HR(+)/HER2(+) Breast Cancer Patient-Derived Xenografts to Trastuzumab or Fulvestrant. *Clin Cancer Res*. 2018;24(2):395–406. doi:10.1158/1078-0432.CCR-17-1983. PubMed PMID: 29079660.
8. Kanaya N, Somlo G, Wu J, Frankel P, Kai M, Liu X, Wu SV, Nguyen D, Chan N, Hsieh MY, et al. Characterization of patient-derived tumor xenografts (PDXs) as models for estrogen receptor positive (ER + HER2- and ER + HER2+) breast cancers. *Journal of Steroid Biochemistry Molecular Biology*. 2017;170:65–74. doi: 10.1016/j.jsbmb.2016.05.001. PubMed PMID: 27154416; PubMed Central PMCID: PMC5094906.
9. Sabnis GJ, Goloubeva OG, Kazi AA, Shah P, Brodie AH. HDAC inhibitor entinostat restores responsiveness of letrozole-resistant MCF-7Ca xenografts to aromatase inhibitors through modulation of Her-2. *Mol Cancer Ther*. 2013;12(12):2804–16. doi:10.1158/1535-7163.MCT-13-0345. PubMed PMID: 24092810; PubMed Central PMCID: PMC3858401.
10. Fitzgibbons PL, Bartley AN, Connolly JL. Template for Reporting Results of Biomarker Testing of Specimens From Patients With Carcinoma of the Breast. In 2018 CAP Accreditation Program Checklist and the CAP/ASCO HER2 Guidelines 2018; Version: Breast Biomarkers 1.2.0.1. College of American Pathologist (CAP): 1–15.
11. Hennessy BT, Lu Y, Gonzalez-Angulo AM, Carey MS, Myhre S, Ju Z, Davies MA, Liu W, Coombes K, Meric-Bernstam F, et al. A Technical Assessment of the Utility of Reverse Phase Protein Arrays for the Study of the Functional Proteome

- in Non-microdissected Human Breast Cancers. *Clin Proteomics*. 2010;6(4):129–51. doi: 10.1007/s12014-010-9055-y. PubMed PMID: 21691416; PubMed Central PMCID: PMC3116520.
12. Tirosh I, Izar B, Prakadan SM, Wadsworth MH, Treacy D, Trombetta JJ, Rotem A, Rodman C, Lian C, Murphy G, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science*. 2016;352(6282):189–96. doi:10.1126/science.aad0501.
 13. DeRose YS, Gligorich KM, Wang G, Georgelas A, Bowman P, Courdy SJ, Welm AL, Welm BE. Patient-derived models of human breast cancer: protocols for in vitro and in vivo applications in tumor biology and translational medicine. *Current Protocols in Pharmacology* 2013, Chap. 14:Unit14 23. doi: 10.1002/0471141755.ph1423s60. PubMed PMID: 23456611; PubMed Central PMCID: PMC3630511.
 14. Hosford SR, Shee K, Wells JD, Traphagen NA, Fields JL, Hampsch RA, Kettenbach AN, Demidenko E, Miller TW. Estrogen therapy induces an unfolded protein response to drive cell death in ER + breast cancer. *Mol Oncol*. 2019;13(8):1778–94. doi: 10.1002/1878-0261.12528. PubMed PMID: 31180176; PubMed Central PMCID: PMC6670014.
 15. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, He X, Liu S, Hoog J, Lu C, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Reports*. 2013;4(6):1116–30. doi: 10.1016/j.celrep.2013.08.022. PubMed PMID: 24055055; PubMed Central PMCID: PMC3881975.
 16. Lebedeva IV, Su ZZ, Chang Y, Kitada S, Reed JC, Fisher PB. The cancer growth suppressing gene mda-7 induces apoptosis selectively in human melanoma cells. *Oncogene*. 2002;21(5):708–18. doi:10.1038/sj.onc.1205116.
 17. Sauane M, Gopalkrishnan RV, Sarkar D, Su ZZ, Lebedeva IV, Dent P, Pestka S, Fisher PB. MDA-7/IL-24: novel cancer growth suppressing and apoptosis inducing cytokine. *Cytokine Growth Factor Rev*. 2003;14(1):35–51. doi:10.1016/s1359-6101(02)00074-6.
 18. Saito Y, Miyahara R, Gopalan B, Litvak A, Inoue S, Shanker M, Branch CD, Mhashilkar AM, Roth JA, Chada S, et al. Selective induction of cell cycle arrest and apoptosis in human prostate cancer cells through adenoviral transfer of the melanoma differentiation-associated – 7 (mda-7)/interleukin-24 (IL-24) gene. *Cancer Gene Ther*. 2005;12(3):238–47. doi:10.1038/sj.cgt.7700780. PubMed PMID: 15578066.
 19. Zheng M, Bocangel D, Doneske B, Mhashilkar A, Ramesh R, Hunt KK, Ekmekcioglu S, Sutton RB, Poindexter N, Grimm EA, et al. Human interleukin 24 (MDA-7/IL-24) protein kills breast cancer cells via the IL-20 receptor and is antagonized by IL-10. *Cancer Immunol Immunother*. 2007;56(2):205–15. doi:10.1007/s00262-006-0175-1. PubMed PMID: 16710719.
 20. Ma C, Zhao LL, Zhao HJ, Cui JW, Li W, Wang NY. Lentivirus-mediated MDA7/IL24 expression inhibits the proliferation of hepatocellular carcinoma cells. *Mol Med Rep*. 2018;17(4):5764–73. doi:10.3892/mmr.2018.8616. PubMed PMID: 29484443; PubMed Central PMCID: PMC5866019.
 21. Yuan W, Goldstein LD, Durinck S, Chen YJ, Nguyen TT, Kljavin NM, Sokol ES, Stawiski EW, Haley B, Ziai J, et al. S100a4 upregulation in Pik3caH1047R;Trp53R270H;MMTV-Cre-driven mammary tumors promotes metastasis. *Breast Cancer Res*. 2019;21(1):152. doi:10.1186/s13058-019-1238-5.
 22. Allison KH, Hammond MEH, Dowsett M, McKernin SE, Carey LA, Fitzgibbons PL, Hayes DF, Lakhani SR, Chavez-MacGregor M, Perlmutter J, et al. Estrogen and Progesterone Receptor Testing in Breast Cancer: ASCO/CAP Guideline Update. *J Clin Oncol*. 2020;38(12):1346–66. doi: 10.1200/JCO.19.02309. PubMed PMID: 31928404.
 23. Clarke R, Leonessa F, Welch JN, Skaar TC. Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev*. 2001;53(1):25–71.
 24. Hayashi S, Niwa T, Yamaguchi Y. Estrogen signaling pathway and its imaging in human breast cancer. *Cancer Sci*. 2009;100(10):1773–8. PubMed Central PMCID: PMC1171938.
 25. Wu VS, Kanaya N, Lo C, Mortimer J, Chen S. From bench to bedside: What do we know about hormone receptor-positive and human epidermal growth factor receptor 2-positive breast cancer? *J Steroid Biochem Mol Biol*.

2015;153:45–53. doi: 10.1016/j.jsbmb.2015.05.005. PubMed PMID: 25998416; PubMed Central PMCID: PMC4568143.

26. Lønning P, Taylor P, Anker G, Iddon J, Wie L, Jørgensen L-M, Mella O, Howell A. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat*. 2001;67(2):111–6. doi:10.1023/a:1010619225209.
27. Agrawal A, Robertson JF, Cheung KL. Efficacy and tolerability of high dose "ethinylestradiol" in post-menopausal advanced breast cancer patients heavily pre-treated with endocrine agents. *World Journal of Surgical Oncology*. 2006;4:44. doi:10.1186/1477-7819-4-44. PubMed PMID: 16834778; PubMed Central PMCID: PMC1533829.
28. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA*. 2009;302(7):774–80. doi:10.1001/jama.2009.1204.
29. Iwase H, Yamamoto Y, Yamamoto-Ibusuki M, Murakami KI, Okumura Y, Tomita S, Inao T, Honda Y, Omoto Y, Iyama KI. Ethinylestradiol is beneficial for postmenopausal patients with heavily pre-treated metastatic breast cancer after prior aromatase inhibitor treatment: a prospective study. *Br J Cancer*. 2013;109(6):1537–42. doi: 10.1038/bjc.2013.520. PubMed PMID: 24002591; PubMed Central PMCID: PMC3777011.
30. Chalasani P, Stopeck A, Clarke K, Livingston R. A pilot study of estradiol followed by exemestane for reversing endocrine resistance in postmenopausal women with hormone receptor-positive metastatic breast cancer. *Oncologist*. 2014;19(11):1127–8. doi:10.1634/theoncologist.2014-0306. PubMed PMID: 25260365; PubMed Central PMCID: PMC4221379.
31. Zucchini G, Armstrong AC, Wardley AM, Wilson G, Misra V, Seif M, Ryder WD, Cope J, Blowers E, Howell A, et al. A phase II trial of low-dose estradiol in postmenopausal women with advanced breast cancer and acquired resistance to aromatase inhibition. *Eur J Cancer*. 2015;51(18):2725–31. 8.028. PubMed PMID: 26597446.. (:. doi: 10.1016/j.ejca.2015.0.
32. Murphy CS, Pink JJ, Jordan VC. Characterization of a Receptor-negative, Hormone-nonresponsive Clone Derived from a T47D Human Breast Cancer Cell Line Kept under Estrogen-free Conditions. *Can Res*. 1990;50(22):7285–92.
33. Martin LA, Farmer I, Johnston SR, Ali S, Dowsett M. Elevated ERK1/ERK2/estrogen receptor cross-talk enhances estrogen-mediated signaling during long-term estrogen deprivation. *Endocr Relat Cancer*. 2005;12(Suppl 1):75–84. doi:10.1677/erc.1.01023. PubMed PMID: 16113101.
34. Sabnis GJ, Jelovac D, Long B, Brodie A. The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation. *Can Res*. 2005;65(9):3903–10. doi:10.1158/0008-5472.CAN-04-4092.
35. Santen RJ, Song RX, Masamura S, Yue W, Fan P, Sogon T, Hayashi S, Nakachi K, Eguchi H. Adaptation to estradiol deprivation causes up-regulation of growth factor pathways and hypersensitivity to estradiol in breast cancer cells. *Adv Exp Med Biol*. 2008;630:19–34. doi:10.1007/978-0-387-78818-0_2.
36. Masri S, Phung S, Wang X, Wu X, Yuan YC, Wagman L, Chen S. Genome-wide analysis of aromatase inhibitor-resistant, tamoxifen-resistant, and long-term estrogen-deprived cells reveals a role for estrogen receptor. *Cancer Res*. 2008;68(12):4910–8. doi:10.1158/0008-5472.CAN-08-0303. PubMed PMID: 18559539.
37. Hanamura T, Hayashi SI. Overcoming aromatase inhibitor resistance in breast cancer: possible mechanisms and clinical applications. *Breast Cancer*. 2018;25(4):379–91. doi:10.1007/s12282-017-0772-1. PubMed PMID: 28389808.
38. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst*. 2005;97(23):1746–59. doi:10.1093/jnci/dji400. PubMed PMID: 16333030.
39. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CG, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by

- global gene expression across time. *Proc Natl Acad Sci USA*. 2011;108(47):18879–86. doi:10.1073/pnas.1115188108. PubMed PMID: 22011582; PubMed Central PMCID: PMC3223472.
40. Obiorah IE, Fan P, Jordan VC. Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen-deprived state. *Cancer Prev Res (Phila)*. 2014;7(9):939–49. doi:10.1158/1940-6207.CAPR-14-0061. PubMed PMID: 24894196.
 41. Fan P, Tyagi AK, Agboke FA, Mathur R, Pokharel N, Jordan VC. Modulation of nuclear factor-kappa B activation by the endoplasmic reticulum stress sensor PERK to mediate estrogen-induced apoptosis in breast cancer cells. *Cell Death Discovery*. 2018;4:15. doi:10.1038/s41420-017-0012-7. PubMed PMID: 29531812; PubMed Central PMCID: PMC5841410.
 42. Song RX-D, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J. J. SR: Effect of Long-Term Estrogen Deprivation on Apoptotic Responses of Breast Cancer Cells to 17 β -Estradiol. *J Natl Cancer Inst*. 2001;93(22):1714–23. doi:10.1093/jnci/93.22.1714.
 43. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, Kalyana-Sundaram S, Wang R, Ning Y, Hodges L, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet*. 2013;45(12):1446–51. doi:10.1038/ng.2823. PubMed PMID: 24185510; PubMed Central PMCID: PMC4009946.
 44. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, Li Z, Gala K, Fanning S, King TA, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet*. 2013;45(12):1439–45. doi:10.1038/ng.2822. PubMed PMID: 24185512; PubMed Central PMCID: PMC3903423.
 45. Raj GV, Sareddy GR, Ma S, Lee T-K, Viswanadhapalli S, Li R, Liu X, Murakami S, Chen C-C, Lee W-R, et al: Estrogen receptor coregulator binding modulators (ERXs) effectively target estrogen receptor positive human breast cancers. *eLife* 2017, 6. doi:10.7554/eLife.26857.
 46. Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS. Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors. *Endocrinology*. 2004;145(7):3473–86. doi:10.1210/en.2003-1682. PubMed PMID: 15033914.
 47. Khongthong P, Roseweir AK, Edwards J. The NF-KB pathway and endocrine therapy resistance in breast cancer. *Endocr Relat Cancer*. 2019;26(6):R369–80. doi:10.1530/ERC-19-0087. PubMed PMID: 32013374.

Figures

Figure 1

E2 promoted the growth of SC31 and inhibited the growth of GS3. (A) Tumor growth curve of SC31/GS3-PDX with E2 (1 mg) or placebo treatment. Tumors and pellets were implanted at the same time or pellets were implanted in mice after tumor volume reached approximately 150–200mm³. (B) Hematoxylin and eosin staining and immunohistochemistry of SC31/GS3 tumors treated with placebo or E2 (1mg). Scale bar represents 100 μm. Error bars represent SEM.

Figure 2

E2 suppressed the expression of ER α and cell cycle proliferation genes in GS3. (A) Cell viability of 104 organoids (isolated from GS3 tumor) treated with varying concentrations of E2 in vitro (* $P = 0.000025$, ** $P < 0.000001$ on Day 14). (B) Cell viability of organoids (isolated from GS3 tumor) co-treated with E2 and ER α antagonist (MPP) or Er β antagonist (PHTPP) in vitro. (C) ER α expression of GS3 tumors treated with placebo, 7-day E2, 28-day E2 and intermittent E2 by western blotting. (D) Protein expression levels of GS3 tumors treated with 5-, 10-, 53-day E2, and control samples as determined by reverse phase protein array analysis (* $P < 0.05$, ** $P < 0.01$). (E) ESR1 and PGR expression levels of GS3-organoids treated with varying concentrations of E2 in vitro (* $P < 0.05$, ** $P < 0.01$). (F) Fold changes of gene expression in GS3 tumors treated with 5-, 10-, 53-day E2, and control sample as determined by Bulk RNAseq. Each treatment included two samples. (G) Gene expression levels of GS3-organoids isolated from GS3 tumors with 5- and 10-day E2, and control sample (** $P < 0.0001$). E2-INT, intermittent E2 treatment. Error bars represent SEM.

Figure 3

SC31 and GS3 tumors were analyzed by scRNAseq. (A) UMAP of cells from both placebo- and E2-treated SC31-PDX, and color-coded according to clusters, treatments, and cell cycle phases. (B) UMAP of cells from both placebo- and E2-treated GS3-PDX, and color-coded according to clusters, treatments, and cell cycle phases. (C) Heat map using the top 10 differentially expressed genes in SC31. (D) Dendrogram showing cluster relationship to other clusters based on gene expression data in SC31. (E) Heat map using the top 10 differentially expressed genes in GS3. (F) Dendrogram showing cluster relationship to other clusters based on gene expression data in GS3.

Figure 4

Gene expression comparison of cells from tumors treated with E2 vs. placebo. (A) Selected feature plots of cells expressing *ESR1*, *PGR* and *MKI67* in UMAP separated by treatments in SC31. (B) Selected feature plots of cells expressing *ESR1*, *PGR* and *MKI67* in UMAP separated by treatments in GS3. (C) Distribution of cells staying at the G1, S, or G2/M phase in *ESR1*⁺ cells and *ESR1*⁻ cells separated by treatments in SC31. (D) Distribution of cells in G1, S, or G2/M phase in *ESR1*⁺ cells and *ESR1*⁻ cells separated by treatments in GS3.

Figure 5

E2 treatment of tumors altered the expression of IL24 in individual cells. (A) Feature plot of cells expressing IL24 in UMAP from scRNAseq in SC31 and GS3. (B) Distribution of cells expressing ESR1 in IL24+ cells or IL24- cells from scRNAseq in GS3. (C) Distribution of cells staying at the G1, S, or G2/M phase in IL24+ cells or IL24- cells from scRNAseq in GS3. (D) IL24 expression levels in GS3 tumors with different treatments including E2 and ICI (*P < 0.05, **P < 0.01). Error bars represent SEM. E2-7d, E2 treatment for 7 days; E2-28d, E2 treatment for 28 days; ICI, ICI treatment for 28 days (once weekly, four times); E2-INT, intermittent E2 treatment.

Figure 6

Model for estrogen regulation in ER+ breast cancer. (A) Tumor growth curves of intermittent E2 (1 mg) treatment which was cycled every 28 days (E2 pellet on/off every 4 weeks, three times) after tumor volume reached approximately 200mm³. (B) Hematoxylin and eosin staining and immunohistochemistry of GS3 tumors treated with placebo, E2 (4 weeks), or intermittent E2. Scale bar represents 100 μ m. (C) Hierarchical clustering using 1,312 differentially expressed genes from bulk RNAseq.

E2 induced estrogen-regulated genes in both *ESR1*⁺/*ESR1*⁻ cells

Figure 7

Characterization of estrogen regulation in ER+ breast cancer. After E2 treatment, E2 regulated ESR1+ cells but ESR1- cells were also behaved in the same manner as ESR1+ cells (potential interactions). The percentage of estrogen-regulated gene-positive cells increased in ER+ breast cancer regardless of the tumors in which growth are promoted or suppressed by E2. On the contrary to the common ER+ breast cancer, decreasing the number of ESR1+ cells and G2/M phase cells following the E2 treatment were observed in a part of ER+ AI resistance breast cancer. Elimination of ER+ cells played a crucial role for E2 suppress tumor growth. More importantly, E2 increased the percentage of cells expressing IL24, which was known as a tumor suppressor gene.

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