*Endocrinology*, 2022, **163**, 1–12
https://doi.org/10.1210/endocr/bqac172
Advance access publication 17 October 2022
**Research Article**

# Estrogen Receptor Beta 1: A Potential Therapeutic Target for Female Triple Negative Breast Cancer

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## Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer characterized by the absence of estrogen receptor alpha, progesterone receptor, and HER2. These receptors often serve as targets in breast cancer treatment. As a result, TNBCs are difficult to treat and have a high propensity to metastasize to distant organs. For these reasons, TNBCs are responsible for over 50% of all breast cancer mortalities while only accounting for 15% to 20% of breast cancer cases. However, estrogen receptor beta 1 (ERβ1), an isoform of the *ESR2* gene, has emerged as a potential therapeutic target in the treatment of TNBCs. Using an in vivo xenograft preclinical mouse model with human TNBC, we found that expression of ERβ1 significantly reduced both primary tumor growth and metastasis. Moreover, TNBCs with elevated levels of ERβ1 showed reduction in epithelial to mesenchymal transition markers and breast cancer stem cell markers, and increases in the expression of genes associated with inhibition of cancer cell invasiveness and metastasis, suggesting possible mechanisms underlying the antitumor activity of ERβ1. Gene expression analysis by quantitative polymerase chain reaction and RNA-seq revealed that treatment with chloroindazole, an ERβ-selective agonist ligand, often enhanced the suppressive activity of ERβ1 in TNBCs in vivo or in TNBC cells in culture, suggesting the potential utility of ERβ1 and ERβ ligand in improving TNBC treatment. The findings enable understanding of the mechanisms by which ERβ1 impedes TNBC growth, invasiveness, and metastasis and consideration of ways by which treatments involving ERβ might improve TNBC patient outcome.

**Key Words:** estrogen receptor beta, breast cancer, gene expression, cancer progression, metastasis

**Abbreviations:** CLI, chloroindazole; dox, doxycycline; EMT, epithelial mesenchymal transition; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; MMP, matrix metalloproteinase; NSG, NOD-SCID-gamma; PCR, polymerase chain reaction; TGF, transforming growth factor; TNBC, triple negative breast cancer; WT, wild type.

Triple negative breast cancers (TNBCs) are the most aggressive form of breast cancer because they are prone to therapeutic resistance and recurrence, often resulting in poor patient outcomes. One of the major causes of breast cancer–related deaths is metastasis to distant organs, which is also most prevalent in TNBCs (1). Hence, there is an urgent need to investigate new targets for breast cancer therapeutics for this subtype of breast cancer. While the role of estrogen receptor alpha (ERα) is well established in ERα-positive breast cancer, this key endocrine therapy target is absent in TNBCs, along with lack of HER2 and progesterone receptor. However, ERβ RNA and protein are present in most TNBCs (2, 3), which leads us to examine its potential as a therapeutic target in TNBCs.

In the human, the *ESR2* gene is transcribed and encodes 6 isoforms by alternative splicing (3, 4). RNA analyses indicate that the relative amounts of these isoforms differ in different tissues. Of note, isoforms 2 through 6 are altered in the C-terminal regions such that they shorten the region encoding the ligand binding domain. Hence, only the full length encoded protein, ERβ1 (530 amino acids), is capable of binding estrogen agonist and antagonist ligands (5–7). We and others have shown that ERβ1 suppresses the activity of ERα in hormone receptor–positive breast cancers (8–10), but the actions of ERβ in TNBC are much less well understood. Several studies with well-validated antibodies to human ERβ have documented the presence of ERβ protein in many human breast cancers by immunohistochemistry (11, 12). Also, recent large population-based studies examining thousands of primary breast cancers in the United States, Sweden, and elsewhere (13) showed that ERβ is expressed in over half of TNBCs (14–16), that expression is highest in the basal subtype of TNBCs, and that, in addition, patients with tumors expressing ERβ have more favorable overall survival, suggesting that ERβ might be available as a target for a new form of endocrine therapy for TNBC.

Previously, we documented a role for ERβ1 in restraining the growth and motility of TNBC cell lines in vitro (3). In the current study, we have explored the role of ERβ1 and an ERβ-specific ligand (chloroindazole, CLI) (17) in suppressing

**Received:** 5 August 2022. **Editorial Decision:** 11 October 2022. **Corrected and Typeset:** 8 November 2022

the growth and metastasis of TNBC in a preclinical animal model. Through analyses of the effects of ERβ1 on tumor growth, gene expression, and metastasis, we have delineated mechanisms underlying the tumor-suppressive actions of this receptor and evaluated the role of an ERβ1 agonist ligand in the inhibitory activity of ERβ1 in TNBC. These findings enable understanding of the mechanisms by which ERβ impedes TNBC growth, invasiveness, and metastasis, and consideration of ways by which treatments involving ERβ might improve TNBC patient outcome.

## Materials and Methods

### Cell Lines and Cell Culture Methods

Breast cancer cells were obtained from the American Type Culture Collection and were maintained and cultured as described (18, 19). MDA-MB-231 GFP-Luc cells transduced with a lentiviral vector and stably containing HA-tag-ERβ1 under a doxycycline (dox)-inducible promoter have been described previously (3). All cells were tested for mycoplasma using Mycosensor polymerase chain reaction (PCR) Assay Kit from Agilent Technologies (Santa Clara, CA) and found to be negative. The ERβ-selective agonist, CLI, was prepared in our laboratory as described (17).

### In Vivo Tumor Studies

All experiments involving animals were conducted in accordance with the National Institutes of Health (Bethesda, MD, USA) standards for the care and use of animals, with protocols approved by the University of Illinois IACUC (IACUC Protocol 20111).

For the tumor and metastasis studies, female NOD-SCID-gamma (NSG) mice, at 8 weeks of age (stock number 005557, from Jackson Laboratories, Bar Harbor, ME, USA) were used as detailed previously (20, 21). Animals were inoculated with $1 \times 10^6$cells of each cell type (parental MDA-MB-231 GFP-Luc; or MDA-MB-231 ERβ1 GFP-Luc) into the fourth inguinal mammary gland of each mouse on the right side. The details of the constructs have been described previously (3). Animals inoculated with control MDA-MB231 GFP-Luc cells, or dox-inducible ERβ1 cells received dox treatment for increased expression of ERβ1. Starting 1 week prior to cell injection, the animals received dox-containing water (at a concentration of 2 mg/L), provided in dark colored light-blocking bottles that were replaced twice weekly. The weights of the mice were measured weekly. In addition to the 2 ERβ1-expressing unliganded (control vehicle) groups (with and without dox), there were 2 additional groups of mice (also with and without dox) that received treatment with ERβ agonist ligand CLI. For the CLI treatment, starting 4 days before cell injection, a 20-mg cholesterol pellet containing 0.25 mg of CLI, prepared according to a method previously described (22, 23), was implanted into the dorsal side of each mouse by a small incision under anesthesia followed by suture. A new pellet was introduced every 2 weeks until the end of the study, alternating on either side of the host animal. The study was continued for 7 weeks during which time the tumor volumes were measured twice weekly using calipers (length × width$^2$/2). Tumors in the mammary gland and the extent of metastases were monitored by IVIS bioluminescence imaging over the time course of the study. At the end of the study, the animals were sacrificed and mammary tumors and lung tissues were collected for further processing as detailed below.

### IVIS Bioluminescence Imaging

Primary tumor growth and the extent of metastasis were followed using an IVIS spectrum computed tomography imaging system. Animals were injected intraperitoneally with D-luciferin (Regis Technologies, Morton Grove, IL, USA) at 150 mg/kg mouse body weight; luciferase activity was measured, and bioluminescence was quantified using Living Image software (PerkinElmer, Waltham, MA, USA) as previously described (21). To measure the metastatic burden, the primary tumor was covered with a black box to prevent signal saturation and inaccurate measurement of metastasis, and bioluminescence was monitored.

### Western Blot Analyses

For Western blot analysis of lysates from cells or pulverized tissue, samples were resuspended in 1× TPER (Tissue Protein Extraction Reagent) buffer (Thermo Fisher) supplemented with 1× protease inhibitor cocktail (Millipore Sigma). The lysate was then sonicated 2 times in 10 second pulses while on ice followed by centrifugation at 14 000 rpm for 25 minutes, and the supernatant was collected and quantified using the BCA assay. Proteins were separated on 4% to 12% sodium dodecyl-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Western blotting used antibodies against the HA tag in the ERβ1 protein (3) (HA tag antibody from Cell Signaling Technology antibody ID RRID:AB_1549585 (24), used at 1:1000 dilution) and β-actin (Millipore-Sigma A2228 antibody ID RRID:AB_476697 (25), used at 1:20 000 dilution) as an internal loading control. Both IRDye 800 CW goat antirabbit secondary antibody (LI-COR, Cat# 926-32211 antibody ID RRID:AB_621843 (26)) and IRDye 680 CW goat antimouse secondary antibody (LI-COR, Cat# 926-68070 antibody ID RRID:AB_10956588 (27)) were diluted (1:5000) for incubation with the blots. Band intensities were analyzed with Licor Odyssey Image Studio 5.2 software that avoids saturation, eliminates comparison of multiple exposures, and allows digital analysis of bands of all intensities, with very accurate protein quantification over a broad linear range. All blots shown together were derived from the same experiment and were processed in parallel. Full uncropped images of blots are shown elsewhere (Fig. S1 (28)). Molecular weight markers were Chameleon Duo markers (IRDye800, IRDye680) from Licor (8-260 kDa).

### RNA Isolation and Real-time PCR

Total RNA was isolated from cells or pulverized tissues using TRIzol (Invitrogen) and reverse transcribed using MMTV reverse transcriptase (New England BioLabs). Real-time PCR was performed using SYBRgreen PCR Master Mix (Quantabio) as described (3, 29). Relative mRNA levels of genes were normalized to the housekeeping gene *36B4*, and fold change calculated relative to the vehicle treated samples. Results are the average ± SEM from the different animals in each group (n = 7 or 8) or from cells in triplicate assays. Primer sequences for the genes studied were obtained from the Harvard Primer Bank and are listed in Table 1.

**Table 1. Sequences of primers used in this study**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *ABCG2* | 5′-ACGAACGGATTAACAGGGTCA-3′ | 5′-CTCCAGACACACCACGGAT-3′ |
| *CCN5* | 5′-CCTGCGACCAACTCCACGTCT-3′ | 5′-TTCACCTCACAGCTGCTGTCGT-3′ |
| *CD24* | 5′-CTCCTACCCACGCAGATTTATTC-3′ | 5′-AGAGTGAGACCACGAAGAGAC-3′ |
| *CD44* | 5′-CTGCCGCTTTGCAGGTGTA-3′ | 5′-CATTGTGGGCAAGGTGCTATT-3′ |
| *CDH1* | 5′-GAGGGGTTAAGCACAACAGC-3′ | 5′-TTAGCCTCGTTCTCAGGCAC-3′ |
| *CDH2 (N-cadherin)* | 5′-GAGGCTTCTGGTGAAATCGC-3′ | 5′-AGAAGAGGCTGTCCTTCATGC-3′ |
| *CST1* | 5′-TAAGAGCCAGGCAACAGACCGTTG-3′ | 5′-AGAGCACAACTGTTTCTTCTGCAGT-3′ |
| *CST2* | 5′-CGAGCCAGGGAGCAGATCGTGGGC-3′ | 5′-AGAGCACAACTGTTTCTTCTGCAGT-3′ |
| *CST5* | 5′-AGTACTACAGCCGCCCTCTG-3′ | 5′-GGTTCGACCGAACTTCACAT-3′ |
| *ESR2, β1* | 5′-GTCAGGCATGCGAGTAACAA-3′ | 5′-GGGAGCCCTCTTTGCTTTTA-3′ |
| *FABP3* | 5′-GTGGAGTTCGATGAGACAACAGC-3′ | 5′-TGGTCTCTTGCCCGTCCCATTT-3′ |
| *KRT13* | 5′-GATGCTGAGGAATGGTTCCACG-3′ | 5′-AGCTCCGTGATCTCTGTCTTGC-3′ |
| *MMP7* | 5′-CAGGAAACACGCTGGCTCAT-3′ | 5′-GACTGCTACCATCCGTCCAG-3′ |
| *MPZL2* | 5′-TACACCTGCCAGGTGAAGAACC-3′ | 5′-TCAGTGCACAGGCAGAGCCAAT-3′ |
| *NANOG* | 5′-GTCTCGTATTTGCTGCATCGT-3′ | 5′-AACACTCGGTGAAATCAGGGT-3′ |
| *PCDHB2* | 5′-GAGCGCGTTCCGAAACAAAG-3′ | 5′-TCGGCCACTGAATAGTGCCTA-3′ |
| *POU5F1 (Oct3/4)* | 5′-GAGTAGTCCCTTCGCAAGCC-3′ | 5′-GAGAAGGCGAAATCCGAAGC-3′ |
| *ROBO1* | 5′-CGCCCCACACCCACTATTG-3′ | 5′-GAAGTCATCCCGAAGTATGGC-3′ |
| *RPLP0 (36B4)* | 5′-AGCCCAGAACACTGGTCT-3′ | 5′-ACTCAGGATTTCAATGGTGCC-3′ |
| *SERPINA5* | 5′-ATGCCCTTTTCACCGACCTG-3′ | 5′-TGCAGAGTCCCTAAAGTTGGTAG-3′ |
| *SMAD3* | 5′-CCATCTCCTACTACGAGCTGAA-3′ | 5′-CACTGCTGCATTCCTGTTGAC-3′ |
| *SNAI1 (Snail)* | 5′-TAGCGAGTGGTTCTTCTGCG-3′ | 5′-GTTAGGCTTCCGATTGGGGT-3′ |
| *SNAI2 (Slug)* | 5′-GAGCATACAGCCCCATCACT-3′ | 5′-CTCACTCGCCCCAAAGATGA-3′ |
| *TIMP2* | 5′-ACACGCAATGAAACCGAAGC-3′ | 5′-TTTGGGGTTGCCGCTGAATA-3′ |
| *VIM* | 5′-AAACTTAGGGGCGCTCTTGT-3′ | 5′-CGCTGCTAGTTCTCAGTGCT-3′ |

## RNA-seq Transcriptional Profiling and Gene Ontology and Pathway Signature Analyses

For gene expression analysis, total RNA was extracted from cells using TRIzol reagent and further cleaned using the Turbo DNase and RNAqueous kits (ThermoFisher, Waltham, MA, USA). Cells were treated with Veh (0.1% dimethyl sulfoxide) or with the compounds for the times indicated. Once the sample quality and replicate reproducibility were verified, samples from each group were subjected to sequencing. RNA at a concentration of 37.5 ng/μL in nuclease-free water was used for library construction. cDNA libraries were prepared with the mRNA-TruSeq Kit (Illumina, Inc., San Diego, CA, USA). In brief, the poly-A containing mRNA was purified from total RNA, the RNA was fragmented, double-stranded cDNA was generated from fragmented RNA, and adapters were ligated to the ends.

The single-end read data from the NovaSeq 6000 were processed and analyzed by using a series of steps. Base calling and demultiplexing of samples within each lane were conducted with bcl2fastq v2.20 Conversion Software (Illumina). Fastqc files containing raw RNA sequencing data were trimmed using Trimmomatic (version 0.38). Reads were pseudo-mapped to the Homo sapiens reference transcriptome (GRCh38; Gencode v34) using Salmon (v1.2.1) with the entire GRCh38 genome as the decoy sequence. Transcript-level counts were summed to the gene level while adjusting for effective lengths using tximport's (v1.16.1) "lengthScaledTPM" method. Gene-level counts underwent quality control and normalization in R (v4.0.2) using edgeR (Version 3.30.3). Genes were filtered out if they did not have expression levels above 0.25 CPM in at least 2 samples.

Heat mapping, hierarchical clustering, differential gene expression analysis, and pathway analysis were conducted by using iDEP (Integrated Differential Expression and Pathway analysis), a web tool for analyzing RNA-seq data that integrates R and Bioconductor packages (30). Packages include DESeq2, ggplot2, and limma for identifying differentially expressed genes, followed by Pathway analysis using Hallmarks from the MSigDB database. Heat maps were plotted and hierarchical clustering was performed using iDEP. The RNA-seq data has been uploaded to the Gene Expression Omnibus and is available as GSE accession number GSE210092.

## Immunohistochemistry

Tumors and metastases in host animal lungs were isolated from the animals and immediately fixed in formalin and then transferred to 70% alcohol after 24 hours. The fixed tissues were paraffin embedded and subsequently treated with antibodies under standardized conditions as described by us previously (31). Detection of luciferase in the breast cancer cells was by immunohistochemistry (antibody ID RRID: AB_2889835 (32) from Abcam), and quantitation of staining

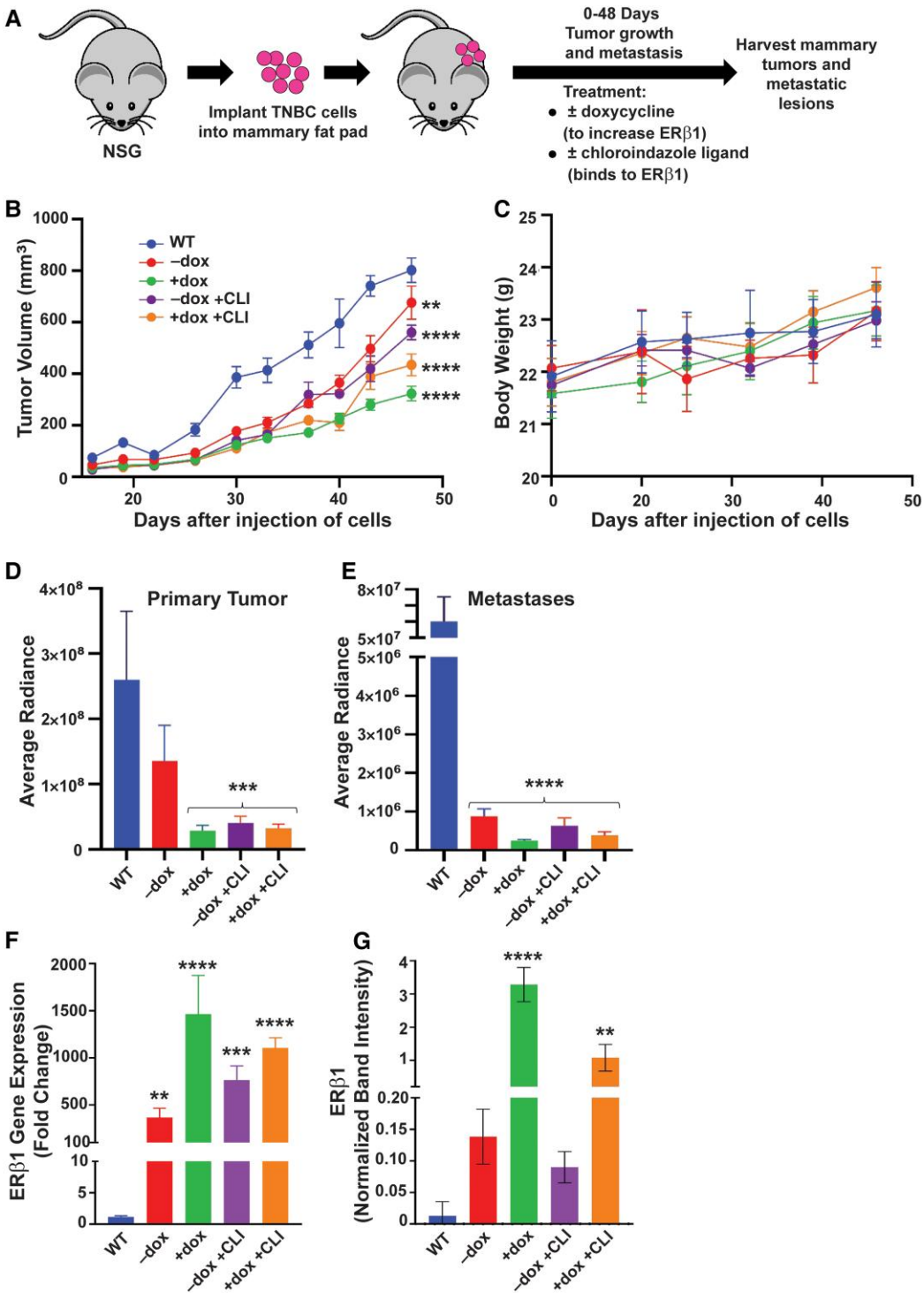



**Figure 1.** Effect of ERβ1 and ERβ ligand treatment on tumor growth and metastasis of TNBC in vivo. (A) Schematic of the overall experimental design of these studies. (B) Growth of MDA-MB-231 tumors in NSG mice over time, without and with dox induction of ERβ1 and treatment with ERβ1 ligand, CLI. Tumor volume determined by caliper measurements. Values are mean ± SEM with n = 7 mice per group. (C) Body weights of mice in different treatment groups over time show no statistically significant differences. (D, E) IVIS bioluminescence measurement of average radiance per mouse in each group at day 48 for primary tumors (n = 7 per group) and metastatic lesions in mice (n = 7 per group). (F) qPCR analysis of ERβ1 mRNA in primary tumors collected at day 48 in the 5 different groups of mice. Values are mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$. (G) ERβ1 protein in primary tumors in the 5 different groups of mice, determined from Western blot analyses. Band intensities are normalized relative to β-actin × $10^{-3}$. Values are mean ± SEM. $^{**}P < .01$, and $^{****}P < .0001$. Full gel blots are shown elsewhere (Fig. S1 (28)).

was done using microscopy (Leica Model DMI 40008) and Image J software analysis. Luciferase stained lung tissue analyses of metastases utilized 5 images per tissue sample and 6 or 7 samples per treatment group.

## Statistical Analyses

Statistics were calculated using analysis of variance, 2-way analysis of variance with multiple comparisons, or Student's

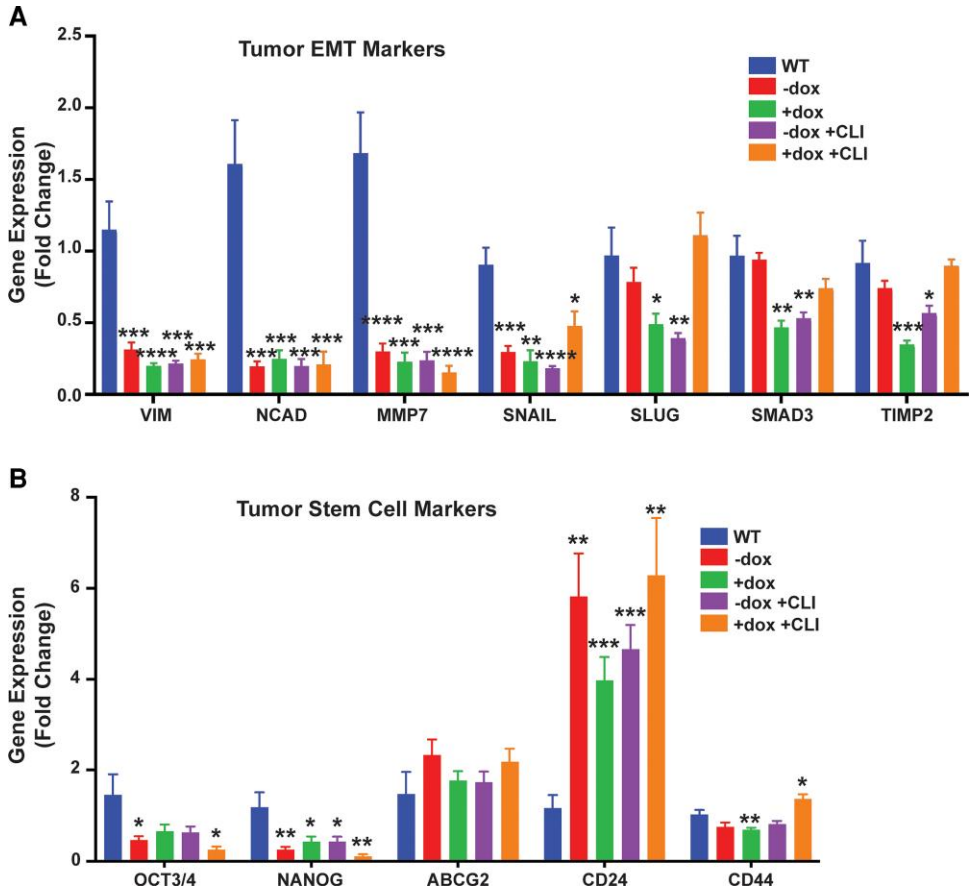



**Figure 2.** Expression for EMT and breast cancer stem cell marker genes in primary tumors and their modulation by ERβ1 and ligand. (A) qPCR analysis of mRNA expression of EMT markers in primary tumors expressing ERβ1 compared with WT tumors collected at day 48. (B) Gene expression analysis of breast cancer stem cell markers by qPCR in primary tumors. Values are mean ± SEM. Statistical analysis of individual genes was done by multiple t-test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

t-test, as appropriate, using GraphPad Prism 9.0 software. Significance was designated as $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

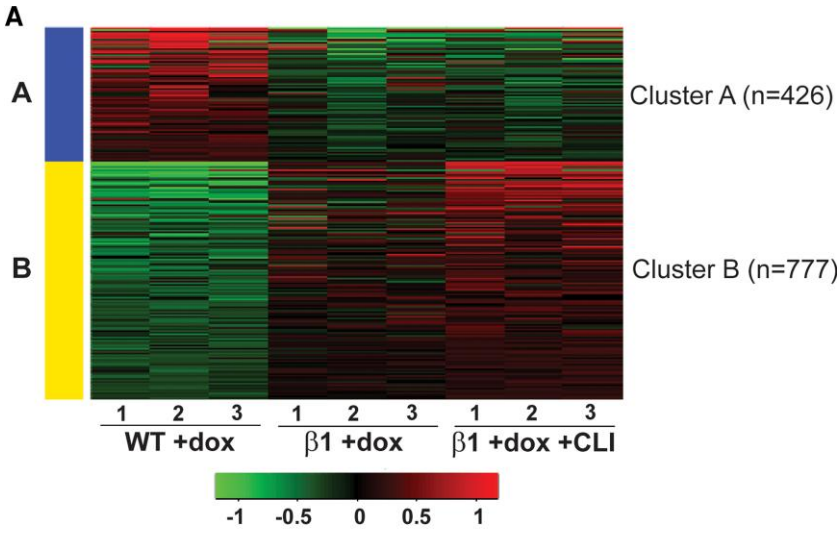
## Results

### Effect of ERβ1 on Tumor Growth and Metastasis in Vivo

To investigate the effect of ERβ1 on the progression of TNBC, we used a xenograft preclinical mouse model with human TNBC cells. We inoculated MDA-MB-231 TNBC cells expressing luciferase and ERβ1 (the latter under a dox-inducible promoter) into the mammary fat pad of immunocompromised female NSG mice and followed the growth of the primary tumor and lung metastases over a period of 7 weeks as schematically shown in Fig. 1A. Besides caliper measurements to follow tumor volume, we also evaluated the primary tumor burden and metastasis to distant organs, principally lung, by bioluminescence of the luciferase expressing ERβ1 cells using IVIS imaging.

To characterize how various biological and regulatory aspects depended on ERβ1 and ERβ agonist (CLI) ligand levels, we compared primary tumor growth, metastatic lesion development, and patterns of gene regulation under 5 conditions: (1) "no ERβ1" (empty vector, WT), (2) "low ERβ1" (vector without dox induction, –dox), or (3) "high ERβ1" (vector with dox induction, +dox), and in the last 2 cases animal treatment was also with the ERβ-specific ligand, CLI (4) "low ERβ1 + CLI" (–dox + CLI), and (5) "high ERβ1 + CLI" (+dox + CLI).

As seen in Fig. 1B, the parental TNBC cells (WT) showed the most rapid growth of primary tumors. Tumor growth rates were slowed by introducing ERβ1 (–dox) and more markedly by further upregulating ERβ1 (+dox). Although tumor growth varied greatly in the different treatment groups, no differences in host animal body weights were observed between the various animal groups over the course of the study (Fig. 1C). Suppressive effects from the elevation of ERβ1 were also evident in the greatly diminished bioluminescence signals from primary tumors and also from the TNBC lung metastases (Fig. 1D and 1E). CLI treatment of mice appeared to slightly reduce the primary tumor growth and bioluminescence signal when ERβ1 levels were elevated somewhat (–dox), but when ERβ1 expression was higher so that tumor growth was already greatly suppressed, treatment with CLI had no further inhibitory effect (Fig. 1B and 1D). The expression level of ERβ1 mRNA and protein was monitored in the tumors from the various treatment groups at day 48. Even in the absence of dox (–dox), there was some upregulation of ERβ1 mRNA and protein in tumors over the control WT level, suggesting some leakiness of the promoter, and the level of ERβ1 mRNA and protein in tumors was increased further in animals receiving dox (+dox) (Fig. 1F and 1G).




B

**Enriched Hallmark Pathways**

| Cluster | adj.Pval | nGenes | Pathways |
|---|---|---|---|
| B | 3.5e-09 | 30 | Estrogen Response Early |
| | 5.7e-08 | 27 | Estrogen Response Late |
| | 9.2e-07 | 23 | KRAS Signaling Up |
| | 4.6e-04 | 17 | Myogenesis |
| | 7.5e-04 | 19 | Epithelial Mesenchymal Transition |
| | 2.5e-03 | 15 | Inflammatory Response |
| | 7.4e-03 | 17 | TNFA Signaling via NFκB |

**Figure 3.** RNA-seq analysis of the effects of ERβ1 alone and together with ERβ agonist ligand, CLI, treatment on gene expression in breast cancer cells. (A) Heat map showing downregulation or upregulation of gene expression in MDA-MB-231 cells in the presence of elevated ERβ1 (+dox, 100 ng/mL doxycycline) or elevated ERβ1 and CLI treatment (100 ng/mL dox + 100 nM CLI) for 24 hours. K-means clustering reveals downregulated gene expressions (Cluster A) and upregulated gene expressions (Cluster B). (B) The Hallmark pathways most enriched in Cluster B are listed.

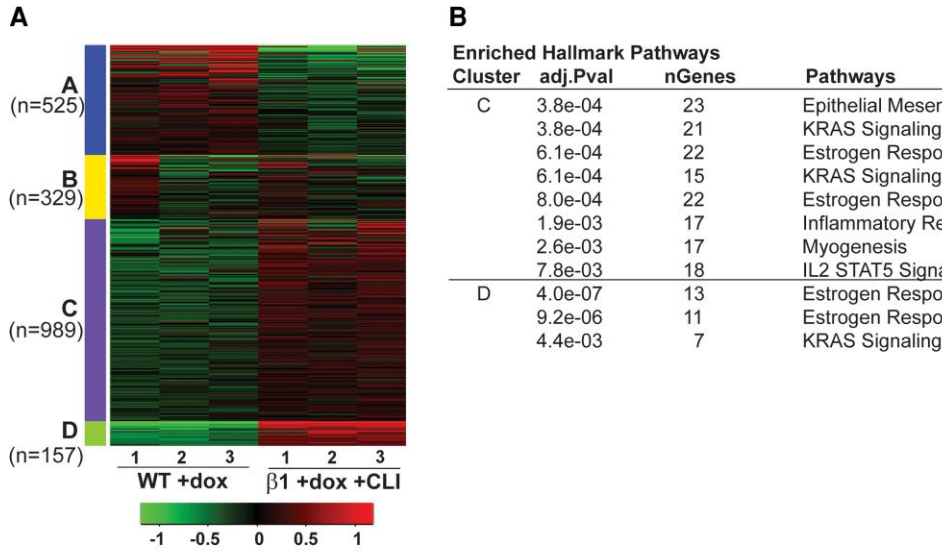
## Effects of ERβ1 on Epithelial to Mesenchymal Transition Markers and Breast Cancer Stem Cell Markers in TNBC tumors

Triple-negative tumors with elevated levels of ERβ1 showed reduction in epithelial to mesenchymal transition (EMT) markers and breast cancer stem cell markers, consistent with the ability of ERβ1 to impede primary tumor growth and metastasis of the cancer cells from the mammary gland (Fig. 2). In some cases, treatment with the ERβ1 agonist CLI further enhanced the inhibitory activity of ERβ1 on the expression of genes associated with EMT and cancer stem cells, supporting the beneficial actions of ERβ1. Figure 2A shows ERβ1 and CLI effects on the regulation of 7 genes known to play important roles in cell migration and signaling, and to serve as important markers of the EMT and metastatic progression in cancer. These include genes encoding Vimentin, NCAD/CDH2, and MMP7, all of which are elevated in many cancers and facilitate cell migration and metastasis. MMP7, a matrix metalloproteinase (MMP), and TIMP2, an MMP regulator, are involved in breakdown of the extracellular matrix, enabling cancer cells to migrate out of the primary tumor to form metastases. Although TIMP2 appears to generally function as an inhibitor of some MMPs, it can also be an activator in some contexts. The *SNAIL* and *SLUG* (*SNAI2*) genes encode Snail family transcription factors that downregulate E-cadherin, inducing EMT and the mesenchymal phenotype that facilitates invasiveness and cancer recurrence. *SMAD3* encodes a member of the SMAD family of proteins that can form a SMAD3/TGFbeta3 complex to regulate the TGF-beta signaling pathway, thereby enhancing EMT, tumor angiogenesis, and metastasis. As seen in Fig. 2A, ERβ1 downregulated expression of these EMT-associated genes, with greater suppression of some genes (*SLUG*, *SMAD3*, *TIMP2*) occurring with higher ERβ (green + dox vs red –dox group) and these showing further suppression with CLI treatment, when ERβ1 was more moderately elevated (–dox) (purple vs red bars).

As seen in Fig. 2B, it is notable that several genes encoding proteins with key roles in maintaining cancer stem cell pluripotency and self-renewal and which predict a worse prognosis for patients with cancer were found to be under regulation by ERβ1. OCT3/4 and NANOG are highly expressed in cancer stem cells and play related roles where they are thought to function as oncogenes in promoting cancer cell proliferation and tumorigenicity. Therefore, it is of note that ERβ1 strongly reduced expression of *OCT3/4* and *NANOG* in the tumors. Likewise, ERβ1 reduced the cancer stem cell population, as seen in the reduced *CD44/CD24* gene expression ratio, associated with greatly elevated expression of the *CD24* gene. By contrast, there was no significant change in the expression of a gene, *ABCG2*, associated with drug efflux from cancer cells.

## Gene Regulation by ERβ1 and ERβ1 Ligand in TNBC cells

In order to gain further insight into gene networks and pathways regulated by ERβ1 and ERβ ligand, we did RNA-seq




B

Enriched Hallmark Pathways

| Cluster | adj.Pval | nGenes | Pathways |
|---|---|---|---|
| C | 3.8e-04 | 23 | Epithelial Mesenchymal Transition |
| | 3.8e-04 | 21 | KRAS Signaling up |
| | 6.1e-04 | 22 | Estrogen Response Late |
| | 6.1e-04 | 15 | KRAS Signaling dn |
| | 8.0e-04 | 22 | Estrogen Response Early |
| | 1.9e-03 | 17 | Inflammatory Response |
| | 2.6e-03 | 17 | Myogenesis |
| | 7.8e-03 | 18 | IL2 STAT5 Signaling |
| D | 4.0e-07 | 13 | Estrogen Response Early |
| | 9.2e-06 | 11 | Estrogen Response Late |
| | 4.4e-03 | 7 | KRAS Signaling Up |

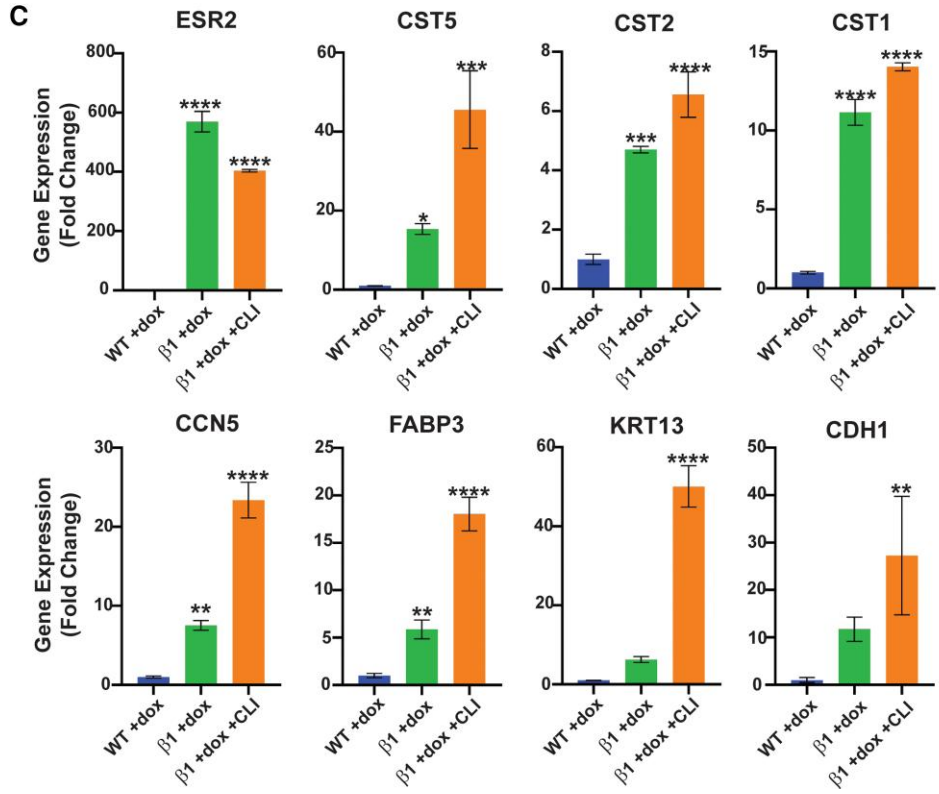



**Figure 4.** RNA-seq analysis of the effects of ERβ1 together with ERβ agonist ligand, CLI, treatment on gene expression in breast cancer cells. (A) Heat map showing downregulation or upregulation of gene expression in MDA-MB-231 cells in the presence of elevated ERβ1 and CLI treatment (100 ng/mL dox + 100 nM CLI) for 24 hours. K-means clustering of the 2000 most variable genes reveals 4 clusters (Clusters A-D) with distinct patterns of gene expression. (B) The Hallmark pathways enriched in Cluster C and D are listed. The genes comprising the 3 most significantly regulated Hallmark pathways (Estrogen Response Early; Estrogen Response Late; and KRAS Up) are listed elsewhere (Table S1 (28)). (C) Genes with expression upregulated by ERβ1 and more highly upregulated by ERβ1 with agonist ligand CLI treatment. MDA-MB-231 cells, WT or containing dox-inducible stable ERβ1 were exposed to 100 ng/mL doxycycline in the presence of control vehicle (+dox) or 100 nM CLI (+dox + CLI) for 24 hours. RNA was then harvested from the cells and analyzed by qRT-PCR for the genes indicated. The expression of ESR2 (top left) was monitored to show the marked increase in ESR2 RNA with doxycycline in the presence of vehicle or CLI. n = 3 per group (WT; +dox; +dox + CLI). Values are mean ± SEM (n = 3 per group) with $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

analyses on these breast cancer cells. As shown in the heat map in Fig. 3A, K-means clustering of the 2000 most variable genes comparing the WT + dox, ERβ expressing (β1 +dox), and ERβ expressing plus CLI ligand treated cells (β1 +dox + CLI) revealed 2 clusters, with Cluster A encompassing genes downregulated by the presence of ERβ1 and Cluster B containing genes upregulated by ERβ1 and, in some cases, further upregulated with the addition of CLI. When analyzing these results

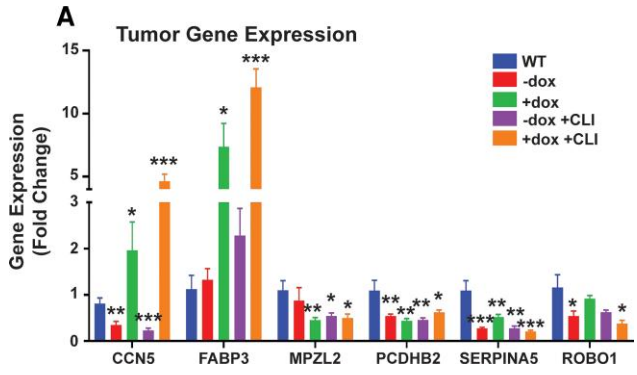



**Figure 5.** Expression of important genes in TNBC primary tumors and their regulation by ERβ1 and the ERβ ligand CLI. qPCR analysis of target gene mRNA expression in primary tumors with or without dox inducible ERβ1 in the presence or absence of the ERβ agonist ligand CLI. Tumors were harvested at day 48. Values are mean ± SEM. $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

using Hallmarks: MSigDB, cluster B showed several enriched Hallmark pathways involved with estrogen response, EMT, and inflammation (Fig. 3B).

To further investigate and compare the role of the ERβ ligand, K-means clustering was employed to compare ERβ1 + CLI with WT. Results (Fig. 4A and 4B) show 4 clusters with cluster C, as expected, closely resembling Cluster B in the RNA-seq heat map in Fig. 3 and being genes upregulated by ERβ1 or ERβ1 +CLI, but with a clear Cluster D emerging in the ERβ1 + CLI compared to the WT heat map of genes more acutely regulated by ERβ plus CLI than in Cluster C. Hallmark pathway analysis showed pathways with statistical significance involving hormone responsiveness and KRAS signaling represented in Clusters C and D. Some genes more strongly upregulated with ERβ ligand treatment in these cells (*CCN5*, *FABP3*, *KRT13*, *CDH1*, *CST1*, *CST2*, and *CST5*) are shown in Fig. 4C. The specific genes in the Estrogen Response Early, Estrogen Response Late, and KRAS Signaling Up Pathways defined by Cluster D in Fig. 4B are listed elsewhere (Table S1 (28)). They include some known estrogen-responsive genes such as *CCN5/WISP2*, *ELF3*, *GREB1*, *KRT13*. *PDZK1, PTGS2* (33–35) and several factors such as *SEMA3B* (semaphorin 3B), and *SERPINA3* with known roles in axonal guidance and development that may have similar cytoarchitectural effects in tumor cells that could impact invasiveness and metastasis (36, 37). We also examined the effect of dox alone (in the absence of ERβ1) on gene expression. In WT cells with no added ERβ1, we saw a very limited effect of dox on gene expression, whereas the heat maps (Fig. S2 (28), and Figs. 3 and 4) revealed a more robust effect of ERβ1 and CLI on many gene expressions when ERβ1 was elevated by dox exposure (ERβ1 +dox vs ERβ1, no dox).

### Regulation in TNBC tumors of Genes Highly Regulated in TNBC cells

Based on these findings in cells, we examined some of these genes for regulation in the primary tumors. As shown in Fig. 5, we found that ERβ1 altered the expression of several notable genes with critical roles in breast cancer and other cancers. The expression of genes associated with migration and invasion (*MPZL2*, *PCDHB2*, *SERPINA5*, and *ROBO1*) was suppressed by ERβ1 and CLI, while expression of genes associated with inhibition of cell proliferation and invasiveness was increased (*CCN5/WISP2* and *FABP3/MDGI*).

Of note, the ERβ1 upregulated expression of the transcriptional repressor CCN5/WISP2 is thought to reduce tumor progression and invasion by inhibiting TGF-beta signaling (38, 39). *FABP3* (fatty acid binding protein 3, also known as mammary derived growth inhibitor), a known tumor suppressor and inhibitor of mammary epithelial cell growth (40–42), was also increased by ERβ1. Both *CCN5* and *FABP3* responses were enhanced by CLI treatment in cells (Fig. 4C) and in tumors (Fig. 5), so that the elevated gene expression might be beneficial. *MPZL2* (Myelin Protein Zero Like 2) was downregulated by ERβ1, as was expression of the cadherin-like adhesion protein–encoding gene, *PCDHB2*, and also *SERPINA5* gene expression (Fig. 5), which could inhibit plasminogen activator–dependent tumor cell invasion and metastasis by inactivating membrane associated serine protease activities. Less is known about the function of ROBO1, but it is thought to regulate axon navigation in the brain and might regulate cell migration also in tumors (43). Therefore, suppression of MPLZ1, PCDHB2, SERPINA5, and ROBO1 by ERβ1 might play a role in impeding TNBC migration, invasion, and metastasis seen in these studies.

### Reduction of Metastases and Metastatic Gene Expression by ERβ

Immunohistochemical quantification of metastatic lesions in the lung by luciferase staining confirmed that metastases were greatly reduced by ERβ1 (Fig. 6A and 6B). This can be seen by comparing tissues from control mice that show extensive metastases and mice inoculated with ERβ1-expressing TNBC cells that show few metastatic lesions (Fig. 6A and 6B). Likewise, expressions of most EMT-associated genes and stem cell marker genes in the metastatic lesions from ERβ1-expressing TNBC tumors were changed in the same directions as observed in the primary tumors (shown in Fig. 2), with reduction in EMT and stem cell marker gene expression with these often being more suppressed with ERβ ligand treatment (Fig. 6C). A model schematizing our findings is presented in Fig. 7. The model shows that ERβ1 acts as a suppressor of TNBC tumor growth and metastasis, with inhibitory effects of ERβ1 and agonist ligand on associated gene regulations that reduce EMT and the cancer stem cell–like population to restrain cancer progression.

## Discussion

Our findings document that ERβ1 restrains TNBC tumor growth and metastasis, suggesting that ERβ1 and ERβ1-regulating ligands might be of potential therapeutic benefit in suppressing breast cancer growth and metastasis. Using gene knockdown and dox-inducible lentiviral systems in TNBC cells in culture, we have previously shown that ERβ1 suppressed cell proliferation, cell migration and invasion, reduced survivin, and increased expression of the tumor suppressor E-cadherin. Treatment with the ERβ agonist, CLI (3, 17, 23, 44), increased ERβ1 upregulation of cystatins and reduced TNBC cell invasiveness (3). The current studies expand our understanding of ERβ1 receptor and ligand

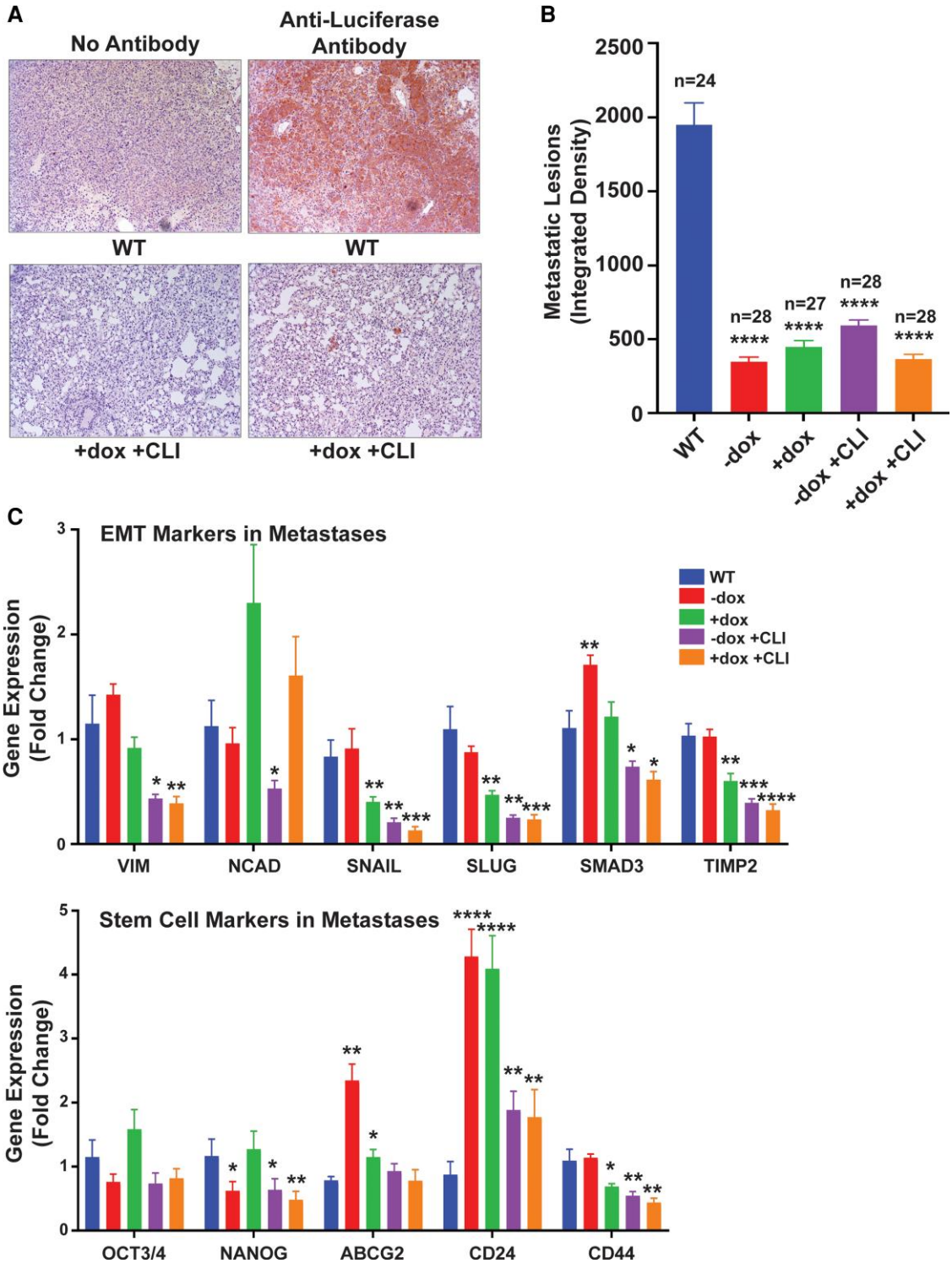



**Figure 6.** Immunohistochemical analysis of breast cancer metastases in the lungs of host mice with WT or ERβ1 containing TNBC mammary tumors, and effects of ERβ1 and CLI ligand on expression of EMT and stem cell marker genes in breast cancer lung metastases. Animals received water with or without doxycycline for induction of increased ERβ1 and also treatment with control vehicle or ERβ agonist ligand CLI. Lungs were harvested at day 48 and prepared for immunohistochemistry with staining of tissues as described in "Materials and Methods." Representative tissue sections are shown. (A) Vehicle-treated control lung from animals with lung metastases derived from mammary tumors with WT TNBC breast cancer cells show extensive metastatic lesions, whereas lung tissue from animals with breast tumors expressing ERβ1 + CLI shows greatly reduced metastatic lesions. Magnification is 100×. (B) Integrated density showing quantitative assessment of metastatic lesions in the different treatment groups. Overall quantitative analysis of multiple metastatic lesions in tissue sections (n = 6) and fields (n = 5/section) is presented. Values are mean ± SEM with n = 24-28 evaluations per group. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$ by multiple t-test. (C) q-PCR gene expression analyses of EMT and stem cell marker genes in lung metastases. These genes were also studied in the primary tumor (Fig. 2). Values are mean ± SEM (n = 7 per group). **** $P < .0001$ by multiple t-test.

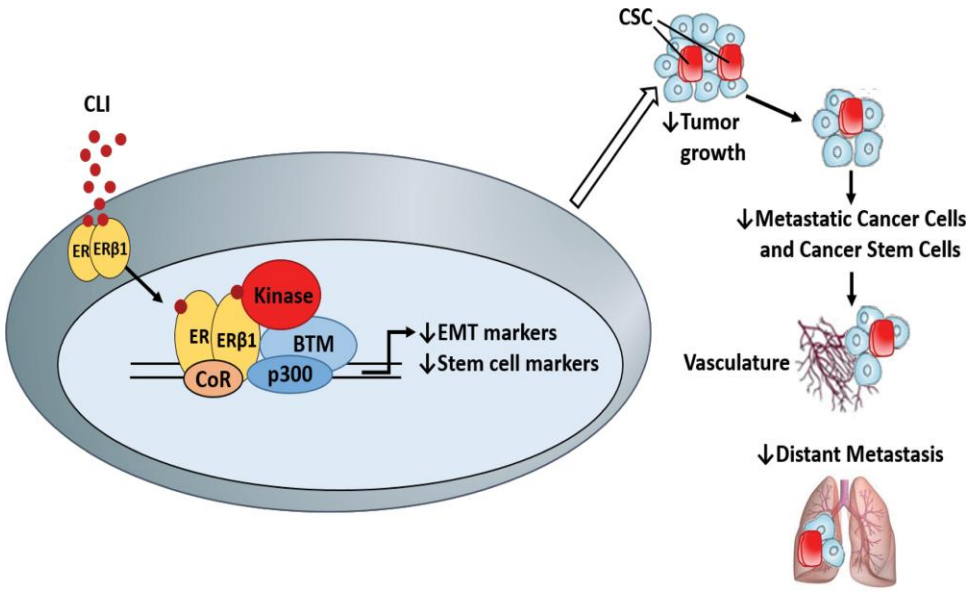



**Figure 7.** Schematic model depicting the suppressive effects of ERβ1 and the ERβ ligand CLI on TNBC tumor growth, EMT markers, the cancer stem cell–like population, and metastasis. Binding of CLI ligand to ERβ1 may change the shape of ERβ1, affecting receptor interaction with kinases, coregulators, and components of the transcription complex. ERβ1 activity reduces tumor growth and distant metastasis. CoR, coregulator; CSC, cancer stem cells; EMT, epithelial mesenchymal transition.

regulation by documenting their marked restraining of TNBC primary tumor growth and distant metastasis, as well as their regulation of patterns of gene expression in tumors and metastatic lesions that provide critical support for their ability to inhibit the malignant character of these cancer cells.

Of note, we found that ERβ1 suppressed the expression of EMT- and metastasis-associated genes (eg, *SNAIL*, *SLUG*, *VIMENTIN*, *N-CADHERIN*, *MMP7*, and *TIMP2*), with some being further suppressed in the presence of the ERβ-selective ligand CLI. Likewise, breast cancer stem cell markers (eg, *OCT3/4*, *NANOG*) were markedly reduced by ERβ1 in tumors, whereas expression of *CD24* was markedly upregulated by ERβ1, consistent with ERβ1 reducing the breast cancer stem cell population.

Of interest, we observed effects of ERβ1 level and presence of ligand, as well as ERβ1 constitutive activity on different genes. It is notable that the effects of low vs high levels of ERβ1 (ie, ERβ/–dox vs ERβ/+dox), as well as the additional effect of CLI were not uniform across all genes. It is not surprising that individual ERβ1-regulated genes have different patterns of response to cellular ERβ1 level. Nevertheless, we know from the pronounced effect of ERβ1 on the growth of the tumors and metastases that the integrative effect of both low and high ERβ1 is antiproliferative and antimetastatic. That the effectiveness of CLI can vary with low vs high ERβ1 levels might be related to the well-known, substantial constitutive activity of unliganded ERβ1 (8–10, 45, 46). In some cases, a high dose of ERβ1 (ERβ/+dox) might be effecting a maximum response due to its constitutive activity, as seen with *VIM*, *NCAD*, *OCT3/4* and *NANOG*. This would obviate any further effect of adding an ERβ ligand such as CLI, whereas an effect of ligand might be apparent when ERβ1 levels are lower (ERβ/–dox), as seen for *SNAIL*, *SLUG*, *SMAD3*, and *TIMP2* gene expressions. In other cases, higher levels of ERβ1 might be needed for an effect of CLI to be apparent, as evident with *CCN5*, *FABP3*, *KRT13*, and cystatin (*CST1, 2, and 5*) gene expressions. Further, by ligand modulation of the conformation of ERβ1, the ERβ1-CLI complex as shown in the Fig. 7 model might have different interactions with coregulators, kinases and components of the transcription complex, and thereby alter gene expression at steps determined by factors beyond the level of ERβ1 alone.

Our in vivo studies, and our extensive RNA-seq studies, utilized MDA-MB-231 cells, which are a mesenchymal subtype of TNBC (47, 48). Since there are other subtypes of TNBC, we do not know how broadly the findings extend to other subtypes of TNBC, and this remains an important aspect for future investigation. We have done some examination of gene regulations by ERβ1 and CLI in MDA-MB-436 cells, another mesenchymal TNBC line but with BRCA1 mutation, and in basal TNBC MDA-MB-468 cells. These limited studies, that need expansion in the future, showed similar regulation of some gene expressions but also some differences, supporting evidence for heterogeneity among TNBCs in their responsiveness to different agents (47) that needs to be investigated and further delineated.

In this study, we have compared the effects of ERβ1 and ERβ ligand on cells in short-term culture, and on tumor progression and metastasis in vivo. Some of the findings on gene regulation seen in the cancer cells, such as the effects on EMT and stem cell markers, no doubt contribute in the suppression of tumor growth and metastasis to lung observed over the 7 weeks of the in vivo study in animals. Of interest, the in vivo study revealed that even low levels of ERβ1 expression could substantially reduce tumor development and outgrowth of metastatic lesions. Notably, high or low ERβ1 greatly suppressed metastases and ligand did not afford further suppression, whereas ERβ ligand did reduce primary tumor growth and some gene expressions. The findings highlight the critical roles of the tumor microenvironment and the metastatic tissue environment in the impact of this nuclear receptor and its state of ligand occupancy on the beneficial inhibitory impact of ERβ1, as modeled in Fig. 7. In this

schematic, we show that ERβ1 impedes primary tumor growth and reduces metastases, at least in part by suppressing EMT gene expression and the expression of stem-like cancer cell markers. In future studies, it will be important to examine the effectiveness of ERβ1 and ERβ ligand not only in the suppression of metastatic formation and growth, as done in this study, but also in the suppression of already present metastatic lesions themselves, as would be the case in treatment of patients with established triple negative metastatic breast cancer.

While these studies and those of others (49–51) have indicated that increasing the level of intracellular ERβ is required to obtain the suppressive effects of ERβ, our findings here show that even modest elevation of ERβ1 may enable very beneficial suppressive effects on TNBC tumor growth and distant metastases. Epigenetic therapeutic approaches are now in use in a few cancers and are being further investigated (52, 53). Hence, it might be possible to increase ERβ1 in TNBC as done already in high-grade serous ovarian cancer and in mesothelioma using histone deacetylase inhibitors and/or DNA methyltransferase inhibitors (54–56). However, it is difficult to postulate with the current state of knowledge what level of ERβ1 might be needed to achieve effective metastatic suppression of TNBC. Further studies are needed to establish these levels.

Currently, a Phase II Clinical Trial (organized by the Mayo Clinic) is underway enrolling ERβ-positive TNBC patients with advanced or metastatic disease to test the efficacy of ERβ stimulation by estradiol. To date, no results have been reported but it will be of interest to know the patient clinical outcomes (NCT03941730 "Estradiol in Treating Patients With ER Beta Positive, Triple Negative Locally Advanced or Metastatic Breast Cancer", expected completion date of April 30, 2024, https://clinicaltrials.gov/ct2/show/NCT03941730?term=estrogen+receptor+beta&cond=breast+cancer&draw=4&rank=2). While much more needs to be explored regarding a potential therapeutic benefit of ERβ1 and ERβ-regulating ligands in suppressing triple negative breast cancer growth and progression, our findings provide insight into the mechanisms and effects of this nuclear receptor that underlie and support its antitumor activity.

## Acknowledgments

We thank Alvaro Hernandez of our Roy J. Carver Biotechnology Center for assistance with running RNA-seq samples, and Jenny Drnevich and Jeffrey Haas for assistance with data processing. We also thank Blake N. Plotner for assistance in conducting some assays.

## Funding

This work was supported by grants from the Breast Cancer Research Foundation (BCRF-083 and BCRF-084 to JAK and BSK), the National Institutes of Health (1R01 CA220484 to J.A.K. and B.S.K.) and the Julius and Mary Landfield Cancer Research Fund at the University of Illinois (to B.S.K.).

## Disclosures

All authors declare no conflicts of interest.

## Data Availability

RNA-seq data have been uploaded to the Gene Expression Omnibus and are available as GSE accession number GSE210092. The authors declare that data supporting the study are available within the paper and its supplementary information (28). Other original data files and material will be available from the corresponding author (B.S.K.) upon reasonable request.

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