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Estrogen receptor β -mediated inhibition of actin-based cell migration suppresses metastasis of inflammatory breast cancer

Christoforos Thomas^{1,*,#}, Ilias V. Karagounis^{1,#}, Ratnesh K. Srivastava², Nicholas Vrettos³, Fotis Nikolos⁴, Noëlle Francois¹, Menggui Huang¹, Siliang Gong⁵, Qi Long⁵, Sushil Kumar², Constantinos Koumenis¹, Savitri Krishnamurthy^{6,8}, Naoto T. Ueno^{7,8}, Rumela Chakrabarti², Amit Maity^{1,*}

¹Department of Radiation Oncology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

²Department of Biomedical Sciences, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104

³Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104

⁴Cedars-Sinai Medical Center, Los Angeles, CA 90048

⁵Department of Biostatistics, Epidemiology and Informatics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

⁶Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030

⁷Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030

⁸Morgan Welch Inflammatory Breast Cancer Research Program and Clinic, The University of Texas MD Anderson Cancer Center, Houston, TX 77030

Abstract

Inflammatory breast cancer (IBC) is a highly metastatic breast carcinoma with high frequency of estrogen receptor α (ER) α -negativity. Here we explored the role of the second ER subtype, ER β , and report expression in IBC tumors and its correlation with reduced metastasis. Ablation

***Correspondence to:** Christoforos Thomas, Ph.D, Department of Radiation Oncology, University of Pennsylvania School of Medicine, 3400 Civic Center Blvd, Philadelphia, PA 19104, Phone: 2155733353, thomaschristoforos@gmail.com; Amit Maity, MD, PhD, University of Pennsylvania School of Medicine, Department of Radiation Oncology, TRC 2-West, 3400 Civic Center Boulevard, Philadelphia, PA 19104, Phone: 2156623759, Amit.Maity@uphs.upenn.edu.

#Equal Contribution

Author contributions

C. T. conceptualized and supervised the study, designed all experiments, performed experiments and participated in all experiments and analysis of all data, generated all stable cell lines and wrote the manuscript. A. M. supervised the study. I.K. performed *in vitro* experiments and participated in animal studies and data analysis. R. C. participated in design and execution of animal experiments. R. S and S. K performed experiments with mouse xenografts. N. V. assisted in sequencing analysis and generation of reporter constructs. F. N., F. N., M. H., performed experiments. S. G and Q. L. assisted the statistical analysis of patient data. C. K. provided helpful discussions and suggestions. N. T. U. provided human tissues and cell lines and participated in human data analysis. S. K. provided human tissues and evaluated the IHC staining. All authors discussed the results and commented on the manuscript.

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of ER β in IBC cells promoted cell migration and activated gene networks that control actin reorganization, including G-protein coupled receptors and downstream effectors that activate Rho GTPases. Analysis of preclinical mouse models of IBC revealed decreased metastasis of IBC tumors when ER β was expressed or activated by chemical agonists. Our findings support a tumor-suppressive role of ER β by demonstrating the ability of the receptor to inhibit dissemination of IBC cells and prevent metastasis. Based on these findings, we propose ER β as a potentially novel biomarker and therapeutic target that can inhibit IBC metastasis and reduce its associated mortality.

Keywords

Estrogen receptor β ; Inflammatory breast cancer; metastasis; cell migration; Rho GTPases

Introduction

Inflammatory breast cancer (IBC) is a rare but highly aggressive subtype of breast cancer accounting for about 10% of breast cancer-associated deaths in US. It is characterized by rapid progression, local and distant metastases and younger age of onset (1). Although application of multimodal therapy approach that includes systemic neoadjuvant chemotherapy, surgery and radiotherapy has significantly improved clinical outcomes, 5-year survival rates are still approximately 35-40% and much lower compared with other breast cancers (2). Poor prognosis is primarily due to the high metastatic potential of IBC tumors. One-third of patients have distant metastases at diagnosis, and about 40% of those with stage III disease at diagnosis that receive standard treatment, eventually experience distant recurrence (3). Despite the contribution of metastasis to the aggressive behavior of IBC, its biology is poorly understood. Because of the small number of tested samples and differences in the characteristics of patient groups, genomic studies have failed to discover distinct biological mechanisms and a specific gene expression signature in IBC (4). However, a number of preclinical studies have suggested changes in signaling pathways and molecules that regulate stemness, epithelial-to-mesenchymal transition (EMT), actin-based cell migration as well as inflammation and immune modulation including ALDH1, NF- κ B, EGFR, HER2 and Rho GTPases (5). Among the Rho GTPases that promote actin-based cell migration is the cytoskeleton remodeler RhoC (4). Although RhoC is upregulated in many IBC tumors and increases the invasiveness of IBC cells, it is considered an “undruggable” target and the pathways that activate RhoC are not fully defined (6,7). As of today, no specific therapeutic target for mitigation of IBC has been identified and there are currently no FDA-approved targeted therapies that are specific for the disease (1,8).

One notable biological difference between IBC and non-IBC is the higher frequency of estrogen receptor α (ER) α -negative status in IBC tumors. Approximately 60-70% of IBC tumors lose expression of ER α compared with about 25% of non-IBC cancers (8). Despite the lack of ER α expression from the majority of IBC tumors, estrogen has been proposed to affect these cancers through ER α -independent pathways (9). These may include the second ER subtype (ER β) that mediates effects of estrogen in normal and malignant breast (10). However, the expression of ER β in IBC tumors and its role in biology of the disease

have not been previously studied. It is generally accepted from studies in non-IBC, that ER β follows a different expression pattern than ER α . It has also been postulated that it elicits, as a transcription factor, distinct transcriptional responses and opposes ER α when both receptors are expressed in the same tumor (10–13). While ER α mediates the proliferative responses and is upregulated in luminal tumors, ER β declines in carcinoma *in situ* and further decreases in invasive lesions of non-IBC tumors (14,15). Conversely, as we and others have shown, enforced expression of ER β in mesenchymal-like non-IBC triple-negative breast cancer (TNBC) cells promotes epithelial transformation and decreases invasion by inhibiting drivers of epithelial to mesenchymal transition (EMT) including TGF β , EGFR and repressors of E-cadherin indicating the potential for ER β to function as an anti-metastatic factor (12,16–21).

The proposed anti-invasive activity of ER β in non-IBC settings prompted us to investigate whether the receptor is expressed in IBC and to test its impact on growth properties and metastatic potential. We report here the analysis of clinical specimens that reveals expression of ER β in IBC tumors and correlation of its expression with reduced metastatic phenotype. This is further supported by findings in preclinical models of inflammatory breast cancer where ER β and its agonists are associated with decreased burden of distant metastases. We link the anti-metastatic function of ER β to its ability to adjust the cytoskeleton architecture to inhibit cell migration by acting on Rho GTPases that are known mediators of IBC metastasis. Our findings indicate a novel role for ER β as a regulator of IBC metastasis and suggest that the receptor may prove useful as a prognostic marker for the disease as well as an attractive therapeutic target.

Materials and Methods

Cells, reagents and constructs

KPL4, SUM190, BCX-010, IBC3 and SUM149 cell lines were from The University of Texas MD Anderson Cancer Center (UTMDACC). FC-IBC02 cells were obtained from Fox Chase Cancer Center and MCF-7, MDA-MB-231, MCF-10A, ZR-75-1, T47D, UACC732, BT549 and HEK293 cells were from ATCC. All IBC cell lines were cultured in HAM's-F12 media (Corning) supplemented with 7% fetal bovine serum (FBS) (Sigma), 10 mM HEPES (Thermo Fisher), 5 μ g/mL insulin (Sigma), 1 μ g/mL hydrocortisone (Sigma) at 37°C in 5% CO₂. Non-IBC cell lines were cultured in DMEM (Corning) supplemented with 10% FBS with the exception of MCF-10A cells that were maintained in DMEM containing 10% FBS, 10 μ g/mL insulin, 0.5 μ g/mL hydrocortisone, 1 ng/mL cholera toxin (Sigma) and 10 ng/mL EGF (Sigma). Cells were also incubated in phenol red-free medium supplemented with 1-2% dextran-coated charcoal-treated FBS (Thermo Fisher) for 48-72 hours prior to treatment with the ER β agonist LY500307 (ApexBio), 17 β -estradiol (E2) or DMSO (Sigma) in the same media. Cell lines were used in experiments within three passages since thawing, were tested negative for mycoplasma and authenticated by short tandem repeat (STR) profiling. For the CRISPR knockout of ER β cells were transfected with pX330 vector containing the sgRNA hER β CRISPR-7: GGATTGACTGCAGTTGTAGG that was kindly provided by Dr. Li (George Washington University) with plasmid carrying puromycin resistance in a ratio 3:1. Following selection with puromycin resistant clones were isolated

and screened by sequencing and immunoblotting. For sequencing, genomic DNA was extracted with Genomic DNA Purification Kit (Thermo Fisher) and PCR amplified using the primers ATTATAATGACCTTTGTGCC and GGATATTCATGGTGGCTGTC to produce a 190 bp fragment flanking the region targeted by CRISPR gRNA. Amplicons were cloned in pJET1.2 vector (Thermo Fisher) and clones were sequenced. For stable expression of both firefly luciferase and GFP, lentivirus containing the pCDH-CMV-MCS-EF1 α -copGFP was produced by transfection and used to infect cells and the GFP-positive population was enriched by cell sorting. For stable expression of ER β , cells were infected with lentivirus containing the pLenti6/V5-FLAG-ER β 1 (FLAG at N-terminus) construct as previously described (16). Cells were also transiently transfected with the pIRESneo-ER β , pIRESneo-ER β 2, pIRESneo-ER β 5 and pIRESneo-ER α plasmids as previously published (22). For siRNA-knockdown, cells were transfected in 6-well plates with 60 nM ON-TARGETplus SMARTpool human ESR2, GPR141, RhoC, ELMO1 or HER3 siRNA pools or Non-targeting siRNA pool as control (Horizon Discovery) using Lipofectamine RNAiMAX (Thermo Fisher). The target sequences of siRNA pools are listed in Supplementary Table 1. For expression of GFP-RhoC, cells were transfected with the GFP-RhoC plasmid (Addgene) using an empty vector (pEGFP-C2) as control (23). For expression of ELMO1, the ELMO1-YFP plasmid was kindly provided by Dr. Jin (Laboratory of Immunogenetics, NIH, NIAID) and the pReceiver-M15 empty vector was used as control (GeneCopoeia) (24). Plasmids were transfected with Lipofectamine 2000 (Thermo Fisher) as previously published (16). All reagents are listed in Supplementary Table 2.

Migration and Invasion

In wound-healing assay, 7.5×10^4 cells were seeded in Culture-Insert 2 well in μ -Dish 35 mm (Ibidi) and allowed to form monolayer. The insert was then removed and the migration area in the wound was imaged and evaluated in an IncuCyte S3 Live-Cell Analysis System (Sartorius) constantly for up to 3 days. Invasion assay was performed as previously published (16). Cells ($2-2.5 \times 10^4$ for KPL4 and 3.5×10^4 for FC-IBC02) were plated in 6.5 mm Transwell chambers (Sigma). 6 to 18 h later, depending on the invasive potential of the cell line, the cells that had invaded through the filter and attached to its bottom surface were stained with crystal-violet, imaged and counted in five independent fields/well using a Nikon Eclipse TE2000-U microscope equipped with a DS-FI3 Camera and NIS-Element Software (Nikon).

Cell survival

2×10^3 cells were seeded in 96-well plates containing 100 μ l/well medium. 24 hours later, the alamarBlue Cell Viability Reagent (Thermo Fisher) was added in 1/10th volume directly to the cells and fluorescence was recorded in different time points using a fluorescence-based plate reader.

Microarray analysis

Total RNA was isolated from Control and ER β KO cells using Qiagen RNeasy Mini kit (Qiagen) and quantitated in NanoDrop 1000 spectrophotometer (Thermo Fisher). The quality of RNA was evaluated by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies). Microarray analysis was carried out in the University

of Pennsylvania Molecular Profiling Facility using Affymetrix Human Clarion D expression arrays (Thermo Fisher).

Biological Pathway Analysis

Differentially expressed genes that were identified by microarray analysis using a stringent (q-value <0.15 and fold change >2.5) or relaxed threshold (fold change >1.5) were analyzed using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems) and Metascape portal to identify enriched biological pathways.

Analysis of GPR141 promoter and luciferase Assays

A 3071 bp DNA fragment containing EREs and located upstream of GPR141 TSS was PCR amplified from human genomic DNA with forward 5-ACTGAGTTTTGCGCTTGTC-3 and reverse 5-CATTCATGTCTCCCCAAACC-3 primers. Amplicons were first cloned into pJET vector and then subcloned between XhoI and BglII sites into pGL4.10 (Promega). The pGL4.10 recombinant construct was used as template for amplification of primer-assisted mutagenesis fragments. One fragment lacked a 189 bp region centered the ERE and in the second fragment the ERE TGACCCAGCTGCCCT was mutagenized to TGcCtgAGCctggga by combining the forward primer with the reverse primers 5-AAGTGAATGTGTTCTAAGTTCCTCA-3 and 5-AATGCATGCAGAGCATGGAAATGCCTGAGCCTGGG-AGCTGTTGAAACAGAATCCTATT-3. Amplicons were cloned into the EcoRV site of pGL4.10. Cells were transfected using Lipofectamine 2000 with the reporter plasmids and a renilla luciferase construct to normalize for transfection efficiency. Relative light units were calculated as ratio of firefly luciferase to renilla luciferase activity. Luciferase activity was measured with Dual Luciferase Reporter Assay (Promega) as previously described (25).

Immunofluorescence analysis

Cells were plated on coverslips and 24 hours later were fixed with 3.7% paraformaldehyde (Sigma) in PBS, permeabilized with 0.3% Triton X-100 (Sigma) in PBS and blocked with 10% (v/v) FBS in PBS. For focal adhesion staining cells were incubated with anti-vinculin antibody (Abcam) overnight at 4°C and secondary antibody for 1 hour at room temperature. Actin filaments and nuclear DNA were stained with Alexa-Fluor 488-Conjugated phalloidin (Abcam) for 1 hour and 300 nM DAPI (Thermo Fisher), respectively. Fluorescent images were acquired using a Zeiss Observer.Z1 microscope through 60x lens with an AxioCam MRm camera and ZEN 2.3 Black edition (Carl Zeiss) software. Images were analyzed for focal adhesion density using NIS Elements Software (Nikon).

RhoC activation assay

RhoC activity was assessed with RhoC Activation Assay Kit (Cell Biolabs) according to manufacturer's instructions. Briefly, cells were incubated with lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% Glycerol) containing protease inhibitors for 20 min on ice and lysates were cleared by centrifugation for 10 min at 14,000 x g at 4° C. Lysates were incubated with Rhotekin RBD agarose beads with rotation at 4° C overnight and the beads were pelleted at 14,000 x g for 10 seconds. After proper

washing, proteins bound to beads were dissociated through resuspension in 2X SDS-PAGE sample buffer and analyzed by immunoblotting.

Immunoblotting

Cells were lysed in RIPA buffer (Thermo Fisher) including the following protease and phosphatase inhibitors; Complete Mini, EDTA free (Roche), Protease Inhibitor cocktail (Sigma), Halt™ Phosphatase Inhibitor cocktail (Thermo Fischer) and phosphatase inhibitor cocktails 2 (Sigma) and 3 (Sigma). Proteins were separated in SDS-PAGE and transferred onto nitrocellulose membrane (Thermo Fisher). Membranes were blocked for 1 hour with 5% dry milk in TBS containing 0.05% Tween-20 at room temperature. Membranes were probed with primary antibodies overnight at 4° C and proteins were visualized with ECL detection kit (GE Healthcare) as previously described (16). ERβ was immunoblotted with two rabbit polyclonal (Millipore, GeneTex) and the 14C8 antibody (GeneTex) and recombinant ERβ protein (Thermo Fisher) was loaded to ensure specificity. Antibodies against RhoC, ERα, pAKT1, AKT1, GST, ELMO1, ROCK1, HER3 and GAPDH were from Cell signaling. Anti-β-Actin antibody was from Abcam and anti-GPR141 was from Thermo Fisher. All antibodies are listed in Supplementary Table 2.

Immunocytochemistry

Cells were fixed in 10% formalin for 30 minutes, resuspended in 0.8% agarose and cell suspension was applied in donut-shape agarose mold that was paraffin embedded. 5 μm paraffin sections were immunostained using a Leica BOND RXm automated platform with the Bond Polymer Refine Detection kit (Leica). Briefly, following dewaxing and rehydration, sections were pretreated with the epitope retrieval BOND ER2 high pH buffer (Leica) for 20 minutes at 98°C. Endogenous peroxidase was inactivated with 3% H₂O₂ for 10 minutes and non-specific tissue-antibody interactions were blocked with PowerVision IHC/ISH Super Blocking solution (Leica) for 30 minutes. Sections were then incubated with the ERβ 503 antibody in the same blocking solution at a concentration of 1/300 for 45 minutes at RT. Immunoreactivity was revealed with diaminobenzidine (DAB) chromogen reaction and slides were counterstained in hematoxylin, dehydrated, cleared and permanently mounted with a resinous mounting medium (Thermo Fisher).

Chromatin immunoprecipitation

Protein-DNA complexes were crosslinked with formaldehyde solution (50mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1mM EDTA, 0.5 mM EGTA, 11% Formaldehyde) for 10 min at room temperature. Cells were harvested and cell suspensions were centrifuged. For nuclei purification, cell pellets were washed in PBS, snap-frozen in liquid nitrogen and consecutively incubated in ice-cold hypotonic buffer (20 mM HEPES-KOH, pH 7.5, 20 mM KCl, 1 mM EDTA, 10% Glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) including protease inhibitors for 10 min at 4° C and lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100, 1mM DTT, 0.1 mM PMSF) with protease inhibitors. Nuclei was washed in ice-cold wash buffer (10 mM Tris-HCL, pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1mM DTT, 0.1 mM PMSF). Chromatin in nuclear extract was sheared by sonication, clarified by centrifugation and the supernatant was used for immunoprecipitation. Chromatin-bound FLAG-tagged ERβ was

incubated with a monoclonal anti-FLAG M2 antibody (Sigma) or mouse IgG (Santa Cruz) and precipitated with protein G magnetic beads (Thermo Fisher). Protein-DNA complexes were eluted, decrosslinked at 65° C overnight and after RNase (0.2 mg/ml) and proteinase K (0.2 mg/ml) treatment the DNA was purified and the DNA enrichment was measured by real-time PCR using promoter-specific primers (Supplementary Table 3).

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. In the orthotopic model, 6-week NCG old female mice (Charles River) were anaesthetized and injected with 5×10^5 tumor cells into the mammary fat pad. In the experiments with the ligand treatment, NCG mice were ovariectomized at 4 week of age (Charles River) and injected with 5×10^5 tumor cells into the mammary fat pad three weeks later. Pellets containing LY500307 (4.9 mg/pellet, 49-day release) or vehicle tablets (Innovative Research of America) were implanted subcutaneously in the dorsal neck region of mice at the time of cell injection. Mice were weighed and mammary tumors were measured once a week until sacrifice. Tumor volume (mm^3) was calculated as $V = (L \times W^2)/2$. Once tumors reach $\sim 500 \text{ mm}^3$ mice were anaesthetized and tumors were resected. Mice were also injected intravenously with 5×10^5 tumor cells. Animals were monitored for signs for morbidity, body weight loss and tumor ulceration and euthanized at the endpoint by cervical dislocation according to IACUC guidelines. Primary tumors and organs were harvested for analysis.

For BLI, following anesthesia with 2.5% isoflurane, mice were injected retro-orbitally with 100 μl of 15 mg/ml Firefly D-Luciferin substrate (Gold Biotechnology). *In vivo* images of mice and *ex vivo* images of harvested lung tissues were acquired 5 min following injection with D-Luciferin using the IVIS Spectrum system (Caliper Life Sciences). Data were analyzed with the Living Image 3.0 software (Caliper Life Sciences) and expressed as total photon flux.

For histological analysis, harvested lungs were perfused and fixed in 10% buffered formalin (Sigma) for 24 hours. Lungs were then processed and embedded in paraffin, sectioned and stained with H&E. Metastases were quantified microscopically by a pathologist in blinded fashion by counting the number of neoplastic cell aggregates (more than 5 cells large) in 15 randomly selected 200x microscopic fields. Bones were fixed in 10% formalin at 4°C under constant agitation for 3 days, soaked in 30% sucrose in PBS at 4°C overnight and embedded in OCT (Sigma). 5 μm thick frozen sections were cut and examined using the Zeiss Observer.Z1 microscope.

Patient Information

In the first cohort of breast cancers, a total of 40 inflammatory breast carcinoma tissue samples were collected from 2008 to 2015. The majority of these patients underwent modified radical mastectomy and received neoadjuvant chemotherapy (Taxol/Adriamycin). Histological types of the total 40 samples were defined into grade II (12) and III (26). Clinical stage was defined according to WHO classification criteria (2007). IHC staining for ER β was performed on total of 40 cancer specimens. In the second cohort, patients with IBC

(25) and non-IBC (58) tumors received similar neoadjuvant chemotherapy (anthracycline- and taxane-based chemotherapy) followed by surgery at MD Anderson Cancer Center. Patients with IBC also had a similar histological classification (12% grade II and 88% grade III) with those with non-IBC (22.5% grade II and 77.5% grade III).

Immunohistochemistry (IHC)

Briefly, sections were deparaffinized by two washes in xylene and rehydrated in a gradient ethanol series (2× 100%, 95% and 70%). Antigen retrieval was performed in a sodium citrate buffer (pH 6.0) for 25 minutes in sub-boiling temperature followed by a 20-minute incubation on ice. Sections were blocked and permeabilized with a 0.1% NP-40 / 3% BSA solution for 10 minutes at room temperature followed by incubation with the 503 anti-ER β antibody (1:50) at 4°C overnight (15,26,27). Stained sections were washed in TBST and incubated with secondary biotin-conjugated anti-Chicken antibody (Thermo Fisher) for 30 minutes and ABC system was used to visualize staining (DAKO). Sections were counterstained with hematoxylin, dehydrated and mounted. Staining was assessed by the IBC pathologist blinded to the origination of samples and patient outcome. Staining scores were generated with the German semi-quantitative scoring system. Each specimen was assigned a score based on intensity of nuclear staining (no staining = 0; weak staining = 1, moderate staining = 2, strong staining = 3) and proportion of stained cells (0% = 0, 1–24% = 1, 25–49% = 2, 50–74% = 3, 75–100% = 4). The final immunoreactive score was estimated by multiplying the intensity score with the score of the % of stained cells ranging from 0 (minimum score) to 12 (maximum score). For ER β , we defined 0-8 as negative and 9-12 as positive. This is similar to previously published cutoffs (28,29).

Statistics and reproducibility

Group comparisons were conducted by Student *t* test or Mann-Whitney test, with the mean values and the SD calculated for each group. Kaplan–Meier survival curves were calculated using overall survival and metastasis-free survival time for each patient from each group, with the log-rank test used to test significant differences between the groups. The Cox proportional hazards model was used to assess the effect of ER β expression while controlling for age, no. of nodes and grade.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Patient-related clinical data are included as Supplementary tables. Microarray data have been uploaded to GEO repository under the accession code GSE149852.

Results

ER β is expressed in inflammatory breast cancer tumors and is inversely associated with metastasis

The decline of ER β expression in invasive lesions on breast cancer and the association of the receptor with decreased migration of breast cancer cells have been viewed as evidence of anti-metastatic function (13,14,17,19–21). However, a clear demonstration of

the anti-metastatic activity of ER β in patients with breast cancer is lacking. To this end, we focused on IBC because ER β has not been previously studied in this highly metastatic carcinoma. Moreover, since patients with IBC have different status of metastasis at diagnosis and a proportion of those without initial metastases eventually show disease progression, we set out to investigate whether differences in the expression of ER β may account for the different metastatic potential of IBC tumors. We analyzed 40 archived tumor samples that were derived from post-neoadjuvant chemotherapy surgical specimens of mastectomy from IBC patients that belong to all clinical breast cancer subtypes (see Methods). The clinicopathological characteristics of the patients are summarized in Supplementary Table 4. To determine the expression levels of ER β we performed immunohistochemistry (IHC) in IBC samples using the 503 antibody that is specific for both the human and mouse proteins. Its specificity for mouse ER β was previously tested in the mammary gland of conditional ER β knockout mice (27). Specificity for human ER β was validated with various methods including pre-incubation of the antibody with excess of recombinant human ER β protein (15,26). To further ascertain its specificity for human ER β , we performed immunocytochemistry in HEK293 cells transiently transfected with wild-type ER β , ER α or the ER β splice variants ER β 2 (also known ER β cx) and ER β 5 using the empty vector-transfected cells as negative control. ER β isoforms have been reported to elicit contrasting actions in non-IBC breast cancer cells and associated with clinical outcome in non-IBC (30,31). As shown in Supplementary Figure 1A and B, strong nuclear staining was detected in ER β -expressing cells, no staining in empty vector- and ER α -transfected cells and modest nuclear signal in ER β 2- and ER β 5-expressing cells suggesting that the 503 antibody is specific for ER β and appears to have higher affinity for the wild-type receptor compared with its splice variants. This finding indicates that ER β staining in human IBC tissues is specific for ER β and may, in some extent, reflect expression of splice variants. ER β staining was not evaluated in 3 out of 40 clinical specimens due to absence of tumor tissue. We observed strong nuclear staining in tumor cells in 11 out of 37 inflammatory breast carcinoma samples (29%) (Figure 1A). Of those tumors that were stained positive for ER β , 90% were ER α and progesterone receptor (PR) positive and 45% were HER2 positive. To assess the clinical importance of ER β , we analyzed the survival and metastasis follow-up information of the same patients. From the 37 patients with known ER β expression, those with high ER β levels in tumors were significantly associated with better metastasis-free survival (MFS) than the ones with low ER β tumors (5-year MFS: 50.0% vs 17.2%, $P=0.02$, Figure 1B). Similarly, patients with high expression levels of tumor ER β showed improved overall survival (OS) compared with those with low ER β tumors ($P=0.1$). (Supplementary Figure 1C). Moreover, we observed inverse correlations between the expression of ER β and adverse pathologic features including tumor size, grade and number of lymph nodes (Supplementary Figure 1D). Notably, given the small patient sample size, these associations were not statistically significant. However, these trends and the correlation of ER β with better MFS hold that tumors that express higher levels of ER β are associated with favorable prognosis. To corroborate the inverse association of ER β with metastasis, we simultaneously evaluated the effect of ER β expression, age and grade on MFS by using the Cox proportional hazards model. As shown in Supplementary Figure 1E, ER β positivity remained an independent favorable prognostic factor for MFS (hazard ratio (HR)=0.88, $P=0.025$). Moreover, we analyzed a dataset with available gene expression and

clinical information from 25 patients with IBC and 58 patients with non-IBC tumors that received similar treatment using an ER β specific probe (211119_at) (Supplementary Table 5) (8). Although no significant difference in the expression of ER β was observed between IBC and non-IBC tumors, high tumor mRNA levels of the receptor were significantly associated with longer OS in patients with IBC but not in those with non-IBC tumors (Figure 1C–E). Analysis using Cox regression model confirmed the ER β as favorable prognostic factor for OS in IBC patients (HR=0.22, P =0.0053) (Supplementary Figure 1F). To investigate whether the observed lack of association between ER β expression and clinical outcome in non-IBC occurs only in this available cohort of patients, we analyzed publicly available datasets (TCGA, WANG and TRANSBIG) with non-IBC patients. As shown in Supplementary Figure 2, ER β is associated with better survival only in ER α -positive and HER2-positive patients from the TCGA dataset. In contrast, no significant associations were identified in WANG and TRANSBIG datasets as well as in TNBC patients from the TCGA dataset. Taken together, these results indicate that IBC tumors with high ER β expression exhibit lower propensity to metastasize compared to ones with low levels of the receptor, implying a potentially protective role of ER β against disease progression and its associated mortality.

ER β impacts the phenotype of inflammatory breast cancer cells

To corroborate the inverse clinical association of ER β with metastasis, we analyzed preclinical models of IBC. We, and others, previously associated the induction of ER β expression in mesenchymal-like non-IBC TNBC cells with epithelial transformation and decreased invasion (12,16–18,20). In the present study we assessed for the first time the expression and function of ER β in a panel of human IBC cell lines. Consistent with the presence of ER β protein in IBC tumors (Figure 1), we observed expression of endogenous receptor in IBC cell lines (Figure 2A). We specifically detected significant expression in triple-negative (FC-IBC02, SUM149) and HER2-positive (UACC732, KPL4) IBC cell lines and low levels in BCX-010, IBC3 and SUM190 cells (Figure 2A) (32–34). We initially observed an inverse association between the endogenous amount of ER β and the migratory morphology of IBC cell lines. FC-IBC02 and KPL4 cells with the highest ER β levels look epithelial-like with polygonal scheme whereas SUM190 and BCX-010 cells with the lowest expression display a morphology with membrane protrusions and decreased adhesion (Figure 2B). To confirm this association, we generated clones of KPL4 cells with CRISPR/CAS9-mediated knockout of ER β (ER β KO). As shown in Supplementary Figure 3A, sequencing analysis of two ER β KO clones revealed insertions or deletions close to the protospacer adjacent motif (PAM) that led to premature stop codon near the beginning of the open reading frame and knockout of the targeted gene that was confirmed by the absence of the protein in immunoblots (Figure 2C and D). Unlike ER β , the low, yet detectable levels of ER α in these IBC cells did not decrease by the same CRISPR/CAS9 system (Supplementary Figure 3B) (33). Moreover, we stably re-expressed ER β in one of the ER β knockout clones (ER β KO+ER β) (Figure 2D). We examined the morphology of cells in colonies of the same size to ensure equal plating density among cell cultures with different ER β levels. Cells were also kept in FBS-containing media suggesting that ER β in control cells may be activated by the estrogen that is present in serum. In line with our initial observation, we have seen decreased adhesion and enlarged protrusions in two different

clones of ER β knockout IBC cells whereas re-expression of the receptor in knockout cells rescued the more compact and cobblestone morphology of control cells (Figure 2E). Similar to CRISPR-mediated knockout, knockdown of ER β with small interference RNA (siRNA) resulted in a more migratory phenotype in both KPL4 and FC-IBC02 cells (Figure 2F and Supplementary Figure 3C). In contrast, knockdown of ER β in cultures with FBS-containing media or its activation by the selective agonist LY500307 in estrogen-depleted media did not significantly alter the morphology of SUM149 cells that express reduced endogenous ER β levels compared to KPL4 and FC-IBC02 cells and display a mixed epithelial and mesenchymal phenotype (Supplementary Figure 3D) (35).

ER β inhibits migration and decreases the invasiveness of inflammatory breast cancer cells

Guided by the inverse association of ER β with the migratory morphology of IBC cells, we next examined whether the receptor can inhibit migration and decrease the invasiveness of the same cells when they grow in FBS-containing media. We initially analyzed wound healing as an indicator of cell migration in incuCyte live cell imaging system. As shown in Figure 3A, knockout of ER β enhanced migration in KPL4 cells as it is indicated by the faster wound closure in ER β knockout cells, whereas re-expression of the receptor in knockout cells restored their collective motion to the speed of control cells. This effect was not due to higher proliferation of ER β knockout cells as demonstrated by the results of a proliferation assay (Supplementary Figure 4A). Moreover, overexpression of ER β in control cells further decreased migration, underscoring the crucial role of ER β expression in controlling migration in IBC cells (Supplementary Figure 4B). Consistent with the increased migration in the absence of ER β , a significantly higher number of ER β KO IBC cells invaded the membrane in Transwell chambers compared with ER β -proficient cells (Figure 3B). Conversely, activation of the receptor by its selective agonist LY500307 decreased the invasiveness of ER β proficient but not ER β knockout cells in estrogen-depleted media, corroborating both the anti-invasive function of ER β and the specificity of the ligand (Figure 3C and Supplementary Figure 4C). Similar to KPL4 cells that express low levels of ER α , downregulation of ER β enhanced invasion in ER α -negative FC-IBC02 cells (Figure 3D and Supplementary Figure 4D). Although ER β may elicit its anti-migratory activity in KPL4 cells by heterodimerizing with ER α , our findings suggest that ER β can inhibit invasion in IBC cells irrespective of the ER α status (36). In contrast, and consistent with its effects on cell morphology, knockdown of the receptor in SUM149 cells altered migration and invasion at significantly less extent compared with KPL4 and FC-IBC02 cells (Supplementary Figure 4E and F).

ER β inhibits metastasis of inflammatory breast cancer tumors

We next examined whether the anti-migratory activity of ER β had an impact on metastasis of inflammatory breast cancer tumors *in vivo*. For this, we stably infected control, ER β KO and ER β KO+ER β cells with lentivirus containing firefly luciferase and green fluorescent protein (GFP) reported genes and injected the cells either orthotopically in the mammary fat pad or by tail vein in immunodeficient mice. In the lung colonization model, mice were analyzed by *in vivo* bioluminescence (BLI) and their harvested lungs by *ex vivo* BLI and histology four weeks after the inoculation of cells. As shown in Figure 4A, B and C and Supplementary Figure 5A, mice that they were injected with cells lacking ER β had

a higher number of micrometastases in lungs compared with mice injected with control or ER β knockout cells in which ER β was re-introduced. In orthotopic model, mice and selected organs were evaluated four weeks following resection of primary tumors. Similar to the lung colonization model, mice with orthotopically-injected ER β knockout tumors had a higher number of micrometastases in lungs and bones compared with the mice bearing ER β -proficient tumors (control and ER β KO+ER β) as it is demonstrated by the intensity of BLI signal and the abundance of GFP-positive cells in bone marrow (Figure 4D, E and F). Assessment of the primary tumor volume indicated that the higher metastatic burden in mice with ER β KO tumors was not due to their higher growth rate (Supplementary Figure 5B). To ascertain the effect of activating endogenous ER β on metastasis, we treated ovariectomized immunodeficient mice with orthotopically-injected IBC tumors with the ER β agonist LY500307 by using implanted pellets (5mg/kg/day) for 7 weeks. As shown in Figure 4G, harvested lungs from mice that were treated with LY500307 displayed reduced BLI signal compared with those that were exposed to vehicle pellets indicative of decreased number of metastasis further supporting the repressive effect of ER β on metastasis.

ER β regulates genes that enable migration and cytoskeleton remodeling in IBC cells

We next set out to explore the mechanism that is employed by ER β to elicit its anti-metastatic activity. We focused on pathways that regulate migration and factors that are known to be specifically altered in inflammatory breast cancer. We carried out microarray analysis to identify differentially expressed genes between control and ER β knockout KPL4 cells that grow in regular media. Using an absolute fold-change of 1.5 cut off and q-value of 0.1 we identified 482 altered genes with 284 upregulated and 198 downregulated (Figure 5A and Supplementary Table 6). Analysis of the differentially regulated genes using the Metascape portal showed enrichment of several biological processes. Among the highest ranking ones, was the regulation of cell migration that is linked to the observed anti-migratory effect of ER β and the GO term that is related to response to steroid hormone that results from the knockout of ER β (Figure 5B). Heat map analysis of the genes annotated to cell migration revealed well known regulators of EMT and invasion (Figure 5C). To acquire a more comprehensive picture of the altered pathways in ER β knockout cells, we used a stringent threshold in selecting differentially regulated genes. Applying 2.5 as cut off for fold-change the GO term that is associated with regulation of actin cytoskeleton reorganization was enriched (Supplementary Figure 6A). Genes in this ontology group included both Rho GTPases (RhoD, RAC3) that drive actin-associated cell migration and factors (ARHGDIB) that regulate their activity (Supplementary Figure 6B) (6,7,37). Upregulation of genes that are involved in this type of cell migration was previously described as IBC-specific molecular alteration (4).

ER β regulates actin cytoskeleton re-organization in inflammatory breast cancer cells

Increased formation of stress fibers through polymerization of actin (F-actin formation) and transmission of force through focal adhesions are common features of motile cells (38). Given the motile phenotype of ER β knockout cells and the enrichment of genes that are involved in actin re-organization, we examined whether the receptor represses the formation of actin stress fibers and decreases the number of focal adhesions in IBC cells. As shown in Figure 5D and E, increased formation of actin stress fibers and focal adhesions was observed

in ER β knockout cells as it is indicated by the immunofluorescence staining of polymerized actin with FITC-conjugated phalloidin and vinculin, a member of focal adhesion complexes. In contrast, re-expression of the receptor in ER β knockout cells restored the cytoskeletal structure seen in control cells. Analysis of the images revealed that enrichment of ER β knockout cells in actin filaments and membrane protrusions is accompanied by a significant enlargement in size (Figure 5D). Because Rho GTPases promote both formation of actin stress fibers and focal adhesions and RhoC has been reported to increase the invasiveness of IBC cells, we examined whether ER β affects the expression and activity of cytoskeleton remodelers in these cells (6,7). Using a RhoC activation assay, we initially observed accumulation of active (GTP-loaded) RhoC in ER β knockout cells (Figure 5F). Analysis of mRNA levels indicated that elevated RhoC activity is not due to increased transcription in these cells (Supplementary Figure 7A). In contrast to knockout of ER β , treatment of IBC cells with the ER β agonist LY500307 in estrogen-depleted media decreased the active form of RhoC (Figure 5G). This effect was not due to ER α because LY500307 did not alter the expression of this receptor in IBC cells (Supplementary Figure 7B) (33). To evaluate the contribution of RhoC to the migratory phenotype of ER β knockout cells, we knocked down RhoC in these cells using siRNA. Downregulation of RhoC reversed the increased invasion of ER β knockout cells at a significant degree suggesting that RhoC inhibition is essential for the anti-migratory activity of the receptor (Figure 5H).

ER β alters actin cytoskeleton dynamics in IBC cells by regulating Rho GTPase activators

We next examined how ER β regulates the activity of RhoC. RhoC cycles between an inactive GDP-bound and an active GTP-bound state. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, while GTPase activating proteins (GAPs) promote GTP hydrolysis (39). In addition, AKT1 phosphorylation and activation of RhoC is associated with actin reorganization and invasion in IBC cells (40). Rho GTPases are also activated downstream of G-protein coupled receptors (GPCRs) (41). GPCRs activate Rho GTPases by binding through the heterotrimeric G proteins to and activating GEFs (41). They can also transactivate growth factor receptors or recruit scaffolding proteins leading to activation of downstream effectors including AKT1 (41). One of the complexes that serves as GEF and is activated downstream of GPCRs is the ELMO1/DOCK. ELMO1 interacts with both heterotrimeric G proteins and Rho GTPases and is required for GPCR-mediated migration of breast cancer cells (24). Our microarray analysis revealed upregulation of ELMO1 and the member of rhodopsin family of GPCRs GPR141 in ER β knockout cells (Supplementary Table 6), which was confirmed by qPCR (Figure 6A and B). The repressive effect of ER β was specific for GPR141 since knockout of the receptor did not alter the mRNA of GPR30, another GPCR that is expressed in IBC tumors and is associated with metastasis in breast cancer (Figure 6A) (9). Moreover, co-immunoprecipitation experiments showed interaction between GPR141, ELMO1 and RhoC suggesting that ELMO1 operates downstream of GPR141 in IBC cells (Supplementary Figure 7C). As in KPL4 cells, knockdown of ER β with siRNA significantly increased the mRNA of GPR141 and ELMO1 in FC-IBC02 cells (Figure 6C). Similar to mRNA, the protein levels of GPR141 and ELMO1 were also inversely correlated with that of ER β in IBC cells (Figure 6D). Moreover, the downstream effector of Rho ROCK1 and the phosphorylated form of AKT1 were upregulated in absence of ER β suggesting that AKT1 may be activated downstream of

GPR141 contributing to RhoC activation in ER β KO cells (Figure 6D and Supplementary Figure 7D). Similar to the expression of ER β , its activation by the agonist LY500307 decreased the levels of GPR141 in control cells in estrogen-depleted media (Supplementary Figure 7E). On further examination of the involvement of ELMO1 and GPR141 on the effects of ER β in IBC cells, we observed that their downregulation by siRNA reversed the migratory morphology of ER β knockout cells and substantially decreased invasion (Figure 6E and F). Treatment of the same cells with pertussis toxin (PTX) that inhibits GPCRs by disrupting their interaction with heterotrimeric G proteins, also significantly reduced the number of invading cells (Figure 6G). In contrast, overexpression of ELMO1 in control cells promoted the formation of membrane protrusions and increased their invasive potential (Figure 6H). Consistent with the inhibitory effect on invasion, knockdown of GPR141 completely reversed the activation of RhoC and AKT1 in ER β knockout cells. Inhibition of GPR141 with PTX had a similar effect on RhoC activity but it did not alter the phosphorylated AKT1 suggesting that AKT1, although it functions downstream of GPR141, is not direct effector of heterotrimeric G proteins (). Downregulation of ELMO1 also decreased the activity of RhoC in ER β knockout cells, albeit to a less extent than GPR141 (Figure 6I) (42). Conversely, upregulation of ELMO1 in control cells dramatically increased the activity of RhoC (Figure 6I). In contrast, knockdown of HER3, which can be transactivated by GPCRs and is overexpressed in IBC tumors, did not substantially reduce the activity of RhoC in ER β knockout cells. Taken together, these findings underscore the importance of GPR141 and ELMO1 in migration of IBC cells and their involvement in the anti-migratory effect of ER β .

GPR141 and ELMO1 are ER β direct repressed genes

Genomic analysis of the GPR141 locus revealed that GPR141 and ELMO1 are located near each other on chromosome 7 and in opposite orientation. This finding strengthens the possibility that their expression is regulated by direct binding of ER β to nearby regulatory elements (Figure 7A). To investigate this, we carried out chromatin-immunoprecipitation (ChIP) experiments to examine whether ER β interacts with sites that contain estrogen response elements (ERE) within the promoter and close to the first exon of GPR141 and ELMO1 (Figure 7A). We also analyzed sites from the promoter of ACTB that do not contain ERE. Due to lack of validated ChIP-specific ER β antibodies, we compared the DNA-ER β binding in regular media-growing ER β KO and ER β KO+ER β cells that stably express FLAG-tagged ER β using an anti-FLAG antibody for immunoprecipitation. As shown in Figure 7B, a strong binding of ER β to ERE-containing sites of GPR141 and ELMO1 was observed in cells expressing ER β . To corroborate the interaction of ER β with the promoters of GPR141 and ELMO1 and demonstrate the capacity of activating ligands to induce the DNA binding activity of the receptor in IBC cells, we assessed the association of ER β with the same regulatory elements in ER β KO+ER β cells following estrogen depletion and treatment with different concentrations of the ER β agonist LY500307 or estrogen. We included in the same assay a site that was previously found to be exclusively bound by ER β (43). We observed a substantial increase in the binding of ER β to GPR141-associated site following treatment with LY500307 or estrogen that was similar to the binding to the previously validated region (Fig. 7C). We also saw increased association of ER β with the regulatory element of ELMO1 with the higher concentration of LY500307, however, this

was lower compared with that of GPR141-related site (Fig. 7C). To assess the functionality of the identified sites, we cloned a region containing the wild-type or mutated versions (base substitution and deletion) of the ER β binding site of GPR141 into a luciferase reporter construct and assessed the activity in luciferase reporter assays. We observed that knockout of ER β increases the expression of luciferase that is driven by the promoter with wild-type but no mutated ERE confirming the contribution of this regulatory region to the repressive effect of ER β on GPR141 expression (Figure 7D).

GPR141 and ELMO1 are selectively upregulated in inflammatory breast cancer

Given that GPR141 and ELMO1 were not previously studied in IBC, we examined their expression and function in IBC cells. We initially assessed the expression of each protein in a panel of breast cancer cell lines including five IBC and five non-IBC luminal and TNBC cell lines. We observed selective expression of ELMO1 in IBC because four out of five IBC cell lines presented with significant levels of ELMO1 and only one out of five non-IBC cell lines had comparable expression. GPR141 appeared to have a similar expression pattern with that of ELMO1 (Figure 7E). Similar to protein, the mRNA levels of ELMO1 and GPR141 were higher in IBC cell lines compared with the non-IBC cells (Figure 7F). Interestingly, ER β displayed an opposite mRNA expression pattern with higher levels in non-IBC cell lines proposing an inverse association between its expression and those of ELMO1 and GPR141 (Supplementary Figure 8A). This is further supported by the fact that SUM190 cells that have the lowest protein expression of ER β and the most migratory phenotype among the IBC cell lines (Figure 2A and B) express the highest mRNA and protein levels of ELMO1 and GPR141 (Figure 7E and F). It is also consistent with the increase in the expression of GPR141 and ELMO1 following knockout of ER β in IBC cells (Figure 6A–D). Unlike GPR141 and ELMO1, RhoC mRNA levels did not substantially differ between IBC and non-IBC cells (Supplementary Figure 8B). It is also interesting to note that SUM149 cells whose migration was slightly affected by ER β downregulation was the only IBC cell line that did not express ELMO1 and GPR141 further supporting the involvement of both proteins in the anti-migratory mechanism of ER β action in IBC cells (Figure 7E and F and Supplementary Figure 4E and F). Because of the absence of GPR141 and ELMO1 in these cells, we examined whether ER β can act directly on RhoC. Although immunoblotting showed that SUM149 cells indeed express higher levels of RhoC than FC-IBC02 and KPL4 cells, downregulation of ER β was not able to increase the mRNA levels of RhoC in these cells replicating the effect of ER β in KPL4 cells (Figure 7G and Supplementary Figure 7A and 8C).

Discussion

Although the functional characterization of ER β has improved our understanding about the mechanism of estrogen receptor action and the regulation of estrogen signaling in normal and malignant tissues, controversy still surrounds its clinical importance in breast cancer. The progressive decline of ER β in invasive lesions combined with its anti-invasive and pro-differentiative effects in pre-clinical models of breast cancer, lend support for a role as suppressor of both breast cancer progression and metastasis (12,16–19). However, most of these associations were observed during the analysis of breast cancer cells with transfected

ER β and a convincing link between the loss of ER β function and increased metastasis in breast cancer has been lacking. Besides, other publications have challenged the tumor-repressive properties of ER β (44). To clarify the exact role of ER β in metastasis, we focused on inflammatory breast cancer, a highly aggressive carcinoma with high rate of metastasis and poor prognosis (3). Since lack of targeted therapy is recognized as one of the causes of poor prognosis, and IBC tumors were not previously characterized for the presence and function of ER β , we analyzed clinical specimens from patients that underwent mastectomy (2). We observed significant levels of ER β in about 30% of tumors, and in support of our initial hypothesis, interrogation of clinical information revealed associations between high expression of ER β and less metastatic potential. The same correlation is recapitulated in our xenograft models of IBC where the occurrence of distant metastases increased by the loss of ER β function but declined by treatment with a selective ER β agonist. It is likely that the anti-metastatic activity of ER β reflects its ability to inhibit single cell-dissemination or the hybrid E/M phenotype that is implicated in collective cell migration that enables the formation of highly metastatic clustered lymphatic emboli (45). Analysis of preclinical models of IBC supports both mechanistic explanations since ER β was found to promote epithelial differentiation and decrease the invasiveness of IBC cells.

The increased metastasis of IBC has been associated with the overexpression of genes that are involved in actin-based cell migration including members of the Rho family of GTPases (4). Rho GTPases play an important role in cytoskeletal reprogramming which by increasing cell motility controls morphogenesis and cancer cell movement during metastasis. Rho GTPases bind to and activate ROCK1 that in turn induces actin polymerization and actin-myosin contraction. Overexpression of RhoA and ROCK1 is implicated in breast cancer metastasis and upregulation of RhoC is believed to drive metastasis in IBC tumors (6,46). Our experiments in IBC cell lines demonstrate strong repressive effects of ER β on RhoC activity. Examination of upstream signaling that includes the direct activators GEFs and GPCRs that activate GEFs identified two novel direct target genes of ER β , the GPCR GPR141 and the GEF-interacting protein ELMO1 (Figure 7H) (24,41). The selective expression of these genes in IBC cells and their strong pro-invasive activity in the same tissues underscore their importance both for the biology of IBC metastasis and as potential targets for therapeutic intervention. The possibility of targeting these molecules is strengthened by the fact that GPR141 belongs to the rhodopsin family of GPCRs that respond to high variety of natural ligands and small molecule inhibitors (47) and ELMO1 functions downstream of GPCRs (24).

We believe that our study helps to clarify the confusion that surrounds the presence and function of ER β in breast cancer (48–50). First, our findings connect the loss of function of endogenously expressed ER β with the increased metastasis in breast cancer. Therefore, our study stands out from previous investigations that have associated the artificially introduced receptor in breast cancer cells with decreased invasion (13,17,19–21). Second, our results demonstrate the capacity of ER β and its agonists to exert anti-metastatic actions in the highly aggressive IBC tumors and provide with mechanistic insights as to how the receptor signals in IBC cells. Further, our clinical investigations provide with associations that support a protective effect of ER β against disease progression. In light of these associations and because of the lack of biomarkers to identify IBC patients who benefit from standard

therapy, our research proposes ER β as a potential predictor of clinical outcome (3). It is also of equal importance that the expression of ER β in IBC tumors combined with the anti-metastatic effects of ER β agonists highlight the possibility of using ER β ligands as a novel treatment to combat IBC metastasis and its associated mortality. On the other hand, the association of high ER β expression with favorable outcome raises the question as to whether the receptor can be activated by endogenous ligands or act in a ligand-independent manner as it was previously suggested (10). Although this may be valid when the receptor is expressed at high levels, low expression of ER β that occurs in the majority of tumors may require a potent and selective agonist to enhance its anti-metastatic activity. Another question of equal significance stems from the variable expression of ER β in IBC tissues, which suggests that there are tumors that can retain and others that lose the ability to express functional ER β . Therefore, it is of paramount importance to identify what signaling pathways in tumor cells and the tumor microenvironment allow the expression of ER β and why this occurs. Answers to these questions will be useful to establish ER β as an important factor in both the prognosis and treatment of breast cancer metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Findings demonstrate the capacity of ER β to elicit anti-metastatic effects in highly aggressive Inflammatory breast cancer and propose ER β and the identified associated genes as potential therapeutic targets in this disease.

Figure 1. ER β expression is inversely associated with metastasis in IBC patients.

(A) Representative staining of ER β in two cases of IBC tumors (scale bar, 100 μ m).

Images on the right display enlargement of cancer epithelium with ER β -positive (black arrows) and negative cells (white arrows) (scale bar, 50 μ m). (B) Kaplan-Meier estimates of metastasis-free survival (MFS) of patients with IBC (n=37). The plot compares groups of patients with high (score >8) and low (score $\leq$ 8) ER β protein levels. Marks represent censored data. P -value refers to two-sided log-rank test. (C) Plot illustrates comparison of tumor mRNA levels of ER β (ESR2) between IBC and non-IBC patients using two-tailed

Student's t-test. **(D-E)** Kaplan-Meier estimates of overall survival (OS) of patients with IBC (n=25) and non-IBC (n=58). Comparison was made between the group of patients with high (>2.68) and low (≤ 2.68) ESR2 expression. *P*-value was generated by two-sided log-rank test.

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Figure 2. ERβ is associated with less migratory morphology in IBC cells.

(A) ERβ levels in breast cancer cell lines. The expression was quantified and normalized to Actin-β. Relative band intensities were normalized to that of H1299 cells stably expressing ERβ that is set to 1. Recombinant short ERβ protein (477 amino acids (aa), 53.4 kDa vs. 530 aa of full length ERβ, 59 kDa) was loaded to demonstrate specificity of the ERβ antibody. (B) Morphology of IBC cell lines with varying ERβ expression. The more migratory morphology the cells have the less ERβ express (scale bar, 100 μm). (C) Protein levels of ERβ in control and different ERβ knockout clones. (D) ERβ levels in control, ERβ overexpressing, ERβKO knockout and ERβKO knockout after stable re-expression of ERβ KPL4 cells. (E) Morphology of KPL4 cells with different ERβ levels (scale bar, 100 μm). (F) ERβ levels and morphology of FC-IBC02 cells following knockdown of ERβ with siRNA pools (scale bar, 100 μm). Arrows indicate migratory cells.

Figure 3. ER β inhibits migration and decreases the invasiveness of IBC cells.

(A) Wound healing was assessed in KPL4 cells with different ER β levels in IncuCyte live-cell imaging system. Wound healing assay was performed twice independently with similar results. The graph shows the wound area in different times following wound formation.

(B) Invasion was assessed in Transwell chambers. Representative images of the cells that invaded to the lower compartment of the chamber are shown (scale bar, 200 μ m). Cells were quantified in five independent fields and the graph indicates the mean (cell number per field) $\pm$ s.d. of three independent experiments, two-tailed Student's t-test. (C) Invasion of control and ER β KO cells following treatment with vehicle (DMSO) or different concentrations of the ER β agonist LY500307 for 16 hours. Graph indicates the mean of invaded cells per field $\pm$ s.d. of three independent experiments, two-tailed Student's t-test. (D) FC-IBC02 cells were treated with control or ER β -specific siRNA pools for 48 hours. Cells were then seeded

in Transwell chambers and allowed to invade for 16 hours. Representative images of invaded cells are shown (scale bar, 100 μm). Graph illustrates the mean of cell number per field $\pm$ s.d. of three independent experiments, two-tailed Student's t-test.

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Figure 4. ER β inhibits metastasis of IBC tumors.

(A) 5×10^5 control, ER β KO and ER β KO+ER β KPL4 cells expressing firefly luciferase and GFP were delivered in NCG mice by tail-vein injection. Whole-body bioluminescence images are shown. Graph indicates total photon flux per second from lungs *in vivo* at the endpoint that was normalized to the signal at the day of cell injection $\pm$ s.d., two-tailed Student's t-test. (B) *Ex vivo* bioluminescence images of lungs from NCG mice that were intravenously injected with KPL4 cells with different ER β levels. (C) Representative H&E images of lungs of NCG mice that were injected through tail-vein with control and

ER β KO KPL4 cells. Encircled tissue contains a metastatic cell aggregate (scale bar, 50 μ m). Graph shows the mean of lung metastatic cell aggregates that are more than 5 cells large and counted in 15 randomly microscopic fields $\pm$ s.d., two-tailed Student's t-test (n=3). **(D)** Firefly luciferase- and GFP-expressing (5×10^5) control, ER β KO and ER β KO+ER β KPL4 cells were injected into mammary fat pad of NCG mice. *In vivo* bioluminescence signal from lungs of indicated groups is shown and the graph presents the mean of total photon flux per second $\pm$ s.d., two-tailed Student's t-test. **(E)** *Ex vivo* images of lung metastasis from mice with orthotopically injected cells using bioluminescence imaging. **(F)** Fluorescence images of bone sections. GFP-expressing tumor cells that were injected into the fat pad of NCG mice infiltrated the bone marrow (blue, nuclei of bone marrow cells) (scale bar, 50 μ m). Graph indicates the mean of GFP-positive KPL4 cells per field $\pm$ s.d., two-tailed Student's t-test. **(G)** *Ex vivo* lung metastasis in ovariectomized NCG mice 7 weeks following orthotopic injection with control KPL4 cells and pellets containing vehicle or LY500307. Graph depicts the mean of total photon flux per second $\pm$ s.d., two-tailed Student's t-test.

Figure 5. ERβ regulates genes that control cytoskeleton reorganization.

(A) Pie chart showing the number of ERβ-regulated genes in ERβKO KPL4 cells. (B) Graph of enriched GO terms across input gene lists with p-values. Of note, the third top-ranked GO term is associated with response to steroid hormone due to knockout of ERβ. (C) Heat map illustrating expression levels of up-regulated and down-regulated genes annotated to the enriched GO term that is associated with regulation of cell migration. (D) Control, ERβKO and ERβKO+ERβ KPL4 cells were stained with FITC-phalloidin to detect F-actin stress fibers (green) and DAPI for nuclei. Cell area was quantified based on

F-actin staining. Data are shown as mean $\pm$ s.d., n=50 cells, Mann-Whitney t-test. **(E)** KPL4 cells with different ER β levels were stained with FITC-phalloidin, anti-vinculin antibody for focal adhesions and DAPI. The number of focal adhesions per cell was determined based on vinculin staining. Data are shown as mean $\pm$ s.d., n=50 cells, Mann-Whitney t-test. **(F)** Active (GTP-loaded) RhoC that interacts with the GST-fused Rho binding domain of Rhotekin in KPL4 cells with different ER β levels. **(G)** Active RhoC in KPL4 cells following treatment with the ER β agonist LY500307 for 24 hours in estrogen-depleted media. **(H)** Following treatment with control or RhoC siRNA pools ER β KO cells were seeded in Transwell chambers and allowed to invade for 16 hours. Representative images of invaded cells are shown. Graph depicts the mean of invaded cells per field $\pm$ s.d. of three independent experiments, two-tailed Student's t-test.

Figure 6. GRP141 and ELMO1 are essential for the migratory phenotype of ERβKO cells.

(A) mRNA expression of GPR141 and GPR30 in control, two clones of ERβKO and ERβKO+ERβ KPL4 cells. Values are normalized to control cells that are set to 1 and represent the mean $\pm$ s.d. of at least three independent experiments, two-tailed Student's t-test. (B) mRNA levels of ELMO1 in KPL4 cells with different ERβ levels. Graph depicts the mean $\pm$ s.d. of three experiments, two-tailed Student's t-test. (C) mRNA of ELMO1, GPR141 and ERβ in FC-IBC02 cells following knockdown of ERβ with siRNA pools. Expression is shown as mean $\pm$ s.d., two-tailed Student's t-test. (D) Protein levels of

GPR141, ELMO1, ROCK1 and ER β in KPL4 cells with different ER β levels. The band intensities of GPR141 and ELMO1 were quantified and normalized to Actin- β . **(E-G)** Morphology and invasion of ER β KO KPL4 cells following treatment with siRNA against GPR141 or ELMO1 or 0.2 μ g/ml pertussis toxin (PTX) in medium for 6 hours (scale bar, 100 μ m). Graphs illustrate the % of migratory cells per field (mean from at least 3 different fields) (left) and the mean of invaded cells per field (right) $\pm$ s.d. of three independent experiments, two-tailed Student's t-test. **(H)** Morphology and invasion of control cells stably transfected with empty vector or ELMO1 recombinant plasmid (scale bar, 100 μ m). Levels of ELMO1 in control and ELMO1-overexpressing KPL4 cells are also indicated. Graphs show the % of migratory cells per field and the mean of invaded cells per field $\pm$ s.d., two-tailed Student's t-test. **(I)** Levels of active RhoC in control and ELMO1-overexpressing KPL4 cells, and in ER β KO KPL4 cells after treatment with siRNA against GPR141, ELMO1, HER3 or 0.2 μ g/ml PTX for 6 hours.

Figure 7. ERβ directly regulates the expression of GPR141 and ELMO1 that are selectively expressed in IBC cells.

(A) Map illustrating the location and orientation of GPR141 and ELMO1 on chromosome 7 and their ERE-containing regulatory sites. (B) ERβ ChIP followed by qPCR at the GPR141 and ELMO1 regulatory elements in ERβKO and ERβKO+FLAG-tagged ERβ KPL4 cells following pull-down with an anti-FLAG antibody (n= 3 technical replicates). ChIP was performed twice independently with similar results. (C) ChIP-qPCR analysis showing interaction of ERβ with regulatory elements of GPR141 and ELMO1 as well as a previously

validated ER β -specific binding site that was served as positive control (POS) following treatment with E2 or LY500307 in estrogen-depleted media. **(D)** Luciferase assay showing expression of luciferase in control and ER β KO cells that is driven by GPR141 regulatory region containing wild-type, mutated or no ER β -binding site (Mut, mutated; Del, deleted). Data are represented as mean $\pm$ s.d., two-tailed Student's t-test. **(E)** ELMO1 and GPR141 protein levels in IBC and non-IBC breast cancer cell lines and normal mammary epithelial MCF-10A cells. **(F)** mRNA levels of GPR141 and ELMO1 in IBC and non-IBC breast cancer cell lines. Values are normalized to the expression of MCF-7 cells and represent the mean $\pm$ s.d., two-tailed Student's t-test. **(G)** ELMO1 and RhoC protein levels in IBC cell lines. **(H)** Schematic illustration of the mechanism employed by ER β to inhibit metastasis in IBC.