# ERβ-mediated induction of cystatins results in suppression of TGFβ signaling and inhibition of triple negative breast cancer metastasis

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## Abstract

Triple negative breast cancer (TNBC) accounts for a disproportionately high number of deaths due to lack of targeted therapies and increased likelihood of distant recurrence. Estrogen receptor beta (ERβ), a well characterized tumor suppressor, is expressed in 30% of TNBCs and its expression is associated with improved patient outcomes. We demonstrate that therapeutic activation of ERβ elicits potent anti-cancer effects in TNBC through the induction of a family of secreted proteins known as the cystatins, which function to inhibit canonical TGFβ signaling and suppress metastatic phenotypes both *in vitro* and *in vivo*. These data reveal the involvement of

cystatins in suppressing breast cancer progression and highlight the value of ERβ targeted therapies for the treatment of TNBC patients.

**Significance Statement**

Triple negative breast cancer (TNBC) is the most aggressive form of breast cancer and patients exhibit high rates of recurrence and mortality in part due to lack of treatment options beyond standard of care chemotherapy regimens. In the subset of TNBCs that express estrogen receptor beta (ERβ), ligand-mediated activation of ERβ elicits potent anti-cancer effects. We report here the elucidation of the ERβ cistrome and transcriptome in TNBC and identify a novel mechanism whereby ERβ induces cystatin gene expression resulting in inhibition of canonical TGFβ signaling and blockade of metastatic phenotypes. These findings suggest that ERβ-targeted therapies represent a new treatment option for the subset of women with ERβ expressing TNBC.

**/body**

## Introduction

In the United States, breast cancer is the second most common cancer among women and is responsible for over 40,000 deaths each year (1). As with all cancers, breast cancer is a heterogeneous disease composed of genomically distinct subtypes defined clinically by the presence of three key biomarkers: estrogen receptor alpha (ERα), the progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (2, 3). Triple negative breast cancer (TNBC) accounts for 10-20% of all breast cancers and is defined by the absence of these three biomarkers (4). TNBC typically presents in younger women, is more prevalent in individuals of African-American and Hispanic ancestry, and is clinically unique due to its aggressive and metastatic nature (2, 4-7). The standard of care for early stage TNBC patients includes chemotherapy, most commonly delivered prior to surgery. For patients with residual disease after

chemotherapy, upwards of 50% will develop recurrent disease (8) and nearly all patients who develop metastases succumb to their disease. For these reasons, identifying novel therapeutic strategies for these patients is of critical importance.

Estrogen receptor beta (ERβ) is highly expressed in normal breast tissue; however, during the process of breast carcinogenesis, ERβ levels typically decrease (9-15). In cohorts of women with breast cancer, tumoral ERβ expression is associated with smaller tumor size, lymph-node negativity and lower histological grade (16, 17), supporting a tumor-suppressive role for this hormone receptor. ERβ has also been shown to decrease proliferation and to inhibit epithelial to mesenchymal transition (EMT) in TNBC cells (18-22).

Using an antibody validated in a number of laboratories (23-26), we and others have identified that 30% of TNBCs express ERβ (21, 27, 28). ERβ expression in TNBC is associated with prolonged disease free survival and overall survival relative to patients with ERβ negative disease (27). These findings indicate that therapeutically targeting ERβ is a relevant strategy that should be further explored for TNBC patients. However, the mechanisms by which ERβ elicits tumor-suppressive effects in TNBC is not well understood. Such information is critical for monitoring response to therapy and for identifying sub-sets of patients that are most likely to benefit from ERβ targeted therapies.

## Results

### Identification of the ERβ cistrome in triple negative breast cancer cells

Given our previous findings that approximately 30% of all triple negative breast cancers (TNBCs) express ERβ and that estrogen (E2) treatment of ERβ expressing TNBC cell lines

substantially inhibits cell growth (21), we first sought to assess the distribution of ERβ expression amongst the known molecular subtypes of TNBC: basal-like 1 and 2, mesenchymal and luminal androgen receptor (29). Using a cohort of TNBC patients extensively characterized at the DNA and RNA level (30), our data suggest that ERβ is expressed across all TNBC subtypes (SI Appendix, Table S1).

We then sought to elucidate the mechanisms by which ERβ elicits its tumor-suppressive effects in TNBC cells. As a first step, we utilized ChIP-Seq to delineated the ERβ cistrome in MDA-MB-231 cells that stably express ERβ following three hours of treatment with ethanol vehicle, 1 nM E2, or 10 nM LY500307, an ERβ-selective agonist. Results of these studies identified 2,911 ligand-independent ERβ binding sites in MDA-MB-231-ERβ cells (Figure 1A and B). A total of 26,896 and 10,059 sites were identified following E2 and LY500307 treatment, respectively (Figure 1A and B). ERβ binding sites were distributed throughout the genome with the majority of sites localizing within introns followed by intergenic regions and transcriptional start sites (Figure 1C). Very few ERβ binding sites were localized within exons (Figure 1C). Ligand-independent ERβ-bound chromatin regions showed significant enrichment of N-Myc and ER motifs, while the top two motifs enriched within both E2- and LY500307-induced ERβ binding sites were ER and AP1 response elements (Figure 1D). Comparison of all ERβ bound chromatin regions in vehicle, E2 and LY500307 treated cells demonstrated that nearly all of the ligand-independent binding sites were also identified in ligand treated cells (Figure 1E). As expected, nearly all of the LY500307-induced ERβ binding sites were conserved within the sites identified following E2 treatment (Figure 1E). However, 5,762 ERβ bound regions were only present

following E2 treatment (Figure 1E). Similar patterns were observed when comparing ERβ bound regions that were located specifically within gene promoters and enhancers (Figures 1F & G).

**ERβ mediated gene expression profiles in TNBC cells**

In parallel with the ChIP-Seq experiments, we sought to characterize changes in the gene expression profiles of MDA-MB-231-ERβ cells following E2 treatment. Microarray analyses were performed using Illumina HT-12 BeadChips and the complete list of genes that met a p-value $\leq 0.05$ and an absolute fold change of 1.5 cut off between vehicle and E2 treated cells is provided in SI Appendix (Table S2). In total, 976 genes were differentially expressed in MDA-MB-231-ERβ cells following estrogen treatment, with 578 genes being up-regulated and 398 genes down-regulated (Figure 2A). Heat map analysis of the top 20 most induced and repressed genes following E2 treatment are depicted in Figure 2B. Multiple interleukins and other inflammation-related factors were enriched in the group of genes shown to be the most inhibited by E2 treatment while four members of the cystatin superfamily of cysteine proteases were among the top 20 most estrogen-induced genes (Figure 2B). Confirmation of the microarray dataset was performed using genes chosen at random (SI Appendix, Figure S1). A Venn diagram was constructed to reveal the overlap between the ChIP-Seq and microarray results (Figure 2D). Once replicate genes (i.e. genes containing multiple ERβ binding sites) were removed from the ChIP-Seq data set, a total of 11,401 genes were assigned as being associated with at least one ERβ binding site. Of the 976 genes shown to be regulated by estrogen treatment, 585 were shown to have a nearby ERβ binding site (Figure 2D). Gene set enrichment analysis (GSEA) was performed using a pre-ranked gene list comprising the 976 genes identified in the microarray analysis and revealed significant associations with phenotypes pertaining to breast cancer grade

and cancer metastasis (Figure 2C). Specifically, genes induced by E2 in MDA-MB-231-ERβ cells were also shown to exhibit increased expression in non-basal like breast cancer, low-grade breast cancer and non-metastatic cancers (Figure 2C). All four of the cystatins shown to be induced by estrogen in ERβ-expressing cells were found at the leading edge of each of the gene sets identified by GSEA. A dendrogram depicting the relationship between cystatins 1, 2, 4, and 5 within the cysteine protease family is shown in Figure 2E as well as their expression levels in vehicle and estrogen treated MDA-MB-231-ERβ cells as determined by microarray analysis (Figure 2F). Regulation of cystatin gene expression by E2 was confirmed by RT-PCR in MDA-MB-231-ERβ cells, as well as in a second ERβ-expressing TNBC cell line (Hs578T), and compared to the effects elicited by the ERβ-selective agonist LY500307 following 5 days of treatment (Figure 2G and H). In both models, all 4 cystatins were significantly induced by estrogen and LY500307 and there were no appreciable differences between the two ligands. However, the fold-increase for all 4 cystatin genes was approximately 10-fold less in the Hs578T-ERβ cells (Figure 2G and H). To examine the basis for this difference, we first assessed the basal expression levels of the 4 cystatins between these two cell lines in the absence and presence of doxycycline (dox) treatment. As shown in SI Appendix (Figure S2A), the basal expression levels of the cystatins were extremely low in the absence of dox in both cell lines. Following dox treatment, the expression levels increased approximately 2-fold in MDA-MB-231-ERβ cells and 10-50-fold in Hs578T-ERβ cells. Interestingly, ERβ mRNA levels follow dox treatment were actually 10-fold lower in Hs578T-ERβ cells (SI Appendix, Figure S2B) suggesting that the apparent differences in cystatin induction by ERβ in these two models is explained by the more robust ligand-independent effects observed in the Hs578T-ERβ cell line.

**Cystatins are highly induced by ERβ and correlate with relapse-free survival in TNBC**

Given the above findings, we next performed a time-course analysis of cystatin gene expression following E2 treatment of MDA-MB-231-ERβ cells. Cystatins 1, 2, 4 and 5 were shown to be significantly induced by E2 within 2-8 hours of exposure (Figure 3A), and continued to increase during extended treatment times of 1-5 days (Figure 3B). To confirm these findings at the protein level, immunohistochemistry for the most highly-induced cystatin, cystatin 5, was performed in MDA-MB-231-ERβ cell line pellets. Cystatin 5 staining was absent in vehicle treated cells, slightly positive following dox-induced ERβ expression and highly positive in the setting of E2 treatment (Figure 3C). These ligand-independent effects of ERβ on cystatin gene expression were confirmed at the mRNA level, where dox treatment of MDA-MB-231-ERβ cells was shown to significantly induce the expression of cystatins 1, 2, 4 and 5 relative to no dox treated cells even in the absence of a ligand (Figure 3D). In addition, the E2-mediated induction of cystatin 1, 2, 4, and 5 expression was completely abolished by the pure anti-estrogen, fulvestrant (ICI 182,780) (Figure 3E). To determine if the induction of cystatins by E2 was unique to ERβ, we also analyzed their expression levels in ERα+ MCF7 and T47D breast cancer cells after 24 hours of treatment (Figure 3F). Cystatins 1, 4 and 5 were completely undetectable by RT-PCR in both MCF7 and T47D cells while cystatin 2 demonstrated basal expression in T47D cells that was repressed with estrogen treatment (Figure 3F). Further, estrogen treatment of ERα-expressing MDA-MB-231 and Hs578T cells had no effect on the expression levels of cystatins 1, 2, 4 and 5 with the exception of a slight induction of cystatin 1 in the MDA-MB-231 model (SI Appendix, Figure S3). To determine the potential relevance of these cystatins in breast cancer, we examined their association with patient outcomes using the online Kaplan Meier plotter program in the breast cancer dataset (31). Using the multigene classifier for cystatins 1, 2, 4 and

5, high cystatin expression was significantly associated with improved relapse-free survival (RFS) in TNBC patients but not in $ER^+/PR^+$ patients (Figure 3G). Together these data indicate that cystatins are highly induced by E2 in an ERβ-specific manner and are positively correlated with improved RFS in TNBC patients.

**Direct regulation of cystatin gene expression by ERβ**

To identify the mechanisms by which ERβ induces cystatin gene expression, we interrogated the ChIP-Seq data and identified ERβ-bound regions within the promoter of all four cystatins as well as the first intron of cystatins 1, 4 and 5 (Figure 4A). An ERβ-bound region far upstream of the transcriptional start site of cystatin 2 was also indicated (Figure 4A). ERβ was shown to be associated with the promoter of cystatin 4 and 5 both in the absence and presence of a ligand, while its association with the cystatin 1 and 2 promoter was ligand-dependent (Figure 4A). ERβ association with intron 1 was also ligand-dependent for cystatins 4 and 5 but ligand independent for cystatin 1 (Figure 4A). These binding sites were centered over estrogen response elements (EREs) and a schematic showing the homology of a consensus ERE with the identified cystatin specific EREs are shown in Figure 4B. ChIP-PCR using the ERβ specific MC10 antibody was used to confirm ERβ association with selected EREs (Figure 4C). Given the nearly identical sequence homology between cystatins 1, 2 and 4, and their divergence from cystatin 5, we focused on cystatins 4 and 5 in future analyses. E2 treatment was shown to enhance ERβ association on the cystatin 4 and 5 promoters with only a trend towards increased binding in the setting of E2 on the ERE within intron 1 of cystatin 5 (Figure 4C).

To assess the activity of the identified EREs, a 500 base pair region centered on the ERβ binding site in the cystatin 4 and 5 promoter and intron were cloned into a luciferase reporter construct. With the exception of the cystatin 4 promoter construct, E2 treatment was shown to induce luciferase activity following transfection into MDA-MB-231-ERβ cells, effects that were completely abolished by ICI (Figure 4D). Site-directed mutagenesis was used to eradicate the EREs identified in the cystatin 5 promoter and cystatin 4 and 5 introns. Mutation of these EREs was also shown to abolish estrogen-induced luciferase activity (Figure 4E) confirming the involvement of these sites in ERβ-mediated induction of cystatin gene expression.

**Biological effects of cystatins in TNBC cells**

Given these findings, we sought to better understand the potential biological effects of cystatins in TNBC cells. Ingenuity pathway analysis of our microarray data revealed significant changes in multiple canonical signaling pathways (SI Appendix, Table S3). The top pathway identified pertained to fibrosis and consisted of numerous genes known to be involved in TGFβ signaling. Furthermore, upstream regulator analysis identified core components of the TGFβ signaling pathway including Smad4 and TGFβ1 (SI Appendix, Table S4) suggesting that ligand-mediated activation of ERβ impacts the TGFβ signaling pathway in TNBC cells. Based on these findings, we constructed a heat map consisting of known TGFβ pathway genes from the microarray data (Figure 5A). We also examined the effects of 24 hours or 5 days of estrogen treatment on the TGFβ signaling pathway using a TGFβ pathway PCR array. These data confirmed that estrogen treatment of ERβ expressing MDA-MB-231 cells significantly alters the expression of multiple genes within the canonical TGFβ pathway (Figure 5B). In order to determine if estrogen-mediated induction of cystatins impacted TGFβ signaling, we next analyzed the phosphorylation

levels of Smad2 and Smad3 following TGFβ stimulation in the presence and absence of a combination of recombinant cystatins 1, 2, 4 and 5. Pre-treatment of non-ERβ expressing parental MDA-MB-231 cells with recombinant cystatins resulted in blockade of Smad2 and Smad3 phosphorylation by TGFβ (Figure 5C). In parallel, recombinant cystatins were also shown to suppress the ability of TGFβ to induce a Smad-binding element (SBE) luciferase reporter construct (Figure 5D), demonstrating that cystatins inhibit TGFβ signaling. Interestingly, TGFβR2 and cystatin 5 were shown to co-localize in MDA-MB-231-ERβ cells following E2 treatment (Figure 5E). An interaction between cystatin 5 and TGFβR2 in the setting of estrogen treatment was confirmed using a duolink proximity assay as indicated by the punctate red staining (Figure 5F). Taken together, these data indicate that cystatins interact with TGFβR2 to inhibit canonical TGFβ signaling in TNBC cells.

**ERβ-mediated induction of cystatins inhibit TNBC cell invasion and migration**

In light of the invasive and migratory properties of TNBC, and the known roles of TGFβ signaling in driving breast cancer cell invasion and migration (32-35), we analyzed the impact of ERβ on these cellular properties. Invasion and migration of MDA-MB-231-ERβ and Hs578T-ERβ cells were analyzed using transwell assays following treatment with no dox vehicle control, dox vehicle, dox + 1 nM estrogen, or dox + 10 nM LY500307. Dox-induced expression of ERβ resulted in suppression of both MDA-MB-231-ERβ and Hs578T-ERβ cell invasion and migration, even in the absence of a ligand (Figure 6A-H). These inhibitory effects were significantly magnified following treatment with either E2 or LY500307 (Figure 6A-H).

Since cystatins are secreted proteins that are highly induced by ERβ and are capable of inhibiting canonical TGFβ signaling, we sought to determine their ability to suppress TNBC cell invasion. siRNA-mediated knockdown was optimized for each individual cystatin and all siRNAs were shown to block E2-mediated induction of the cystatins in MDA-MB-231-ERβ cells (Figure 6I). Combinatorial knockdown of all 4 cystatins was shown to block the ability of E2 to suppress cell invasion (Figure 6J). Treatment of parental MDA-MB-231 cells with recombinant cystatins was also shown to completely suppress TGFβ-mediated invasion, effects that were significantly greater than that of a TGFβ-specific inhibitor, SB431542 (Figure 7A and B). Combined, these data show that ligand-mediated activation of ERβ substantially inhibits the invasive and migratory properties of TNBC cells in part through the actions of cystatins and suppression of TGFβ signaling.

**ERβ activation prevents lung metastasis of MDA-MB-231 cells *in vivo***

A common site of TNBC metastasis is the lung; therefore, we sought to determine the effect of ERβ activation on the ability of TNBC cells to establish lung colonization *in vivo* as a model of metastasis. MDA-MB-231-ERβ-Luc cells were injected into the tail vein of ovariectomized athymic nude mice and randomized to one of three treatment arms: normal chow/placebo pellet, dox chow/placebo pellet or dox chow/estrogen pellet with eight mice in each group. Mice were monitored for the development of lung metastases via IVIS2000 xenogen imaging. Representative images from animals in each treatment arm are shown prior to sacrifice (**Error! Reference source not found.** 8A). Following sacrifice, metastatic lesions were quantitated in the lungs under a dissecting scope, and representative images of tumor nodules in the no dox placebo and dox placebo groups (10x magnification) are shown (Figure 8B). No macroscopic lung

nodules were observed in any of the animals in the dox estrogen group, effects that were confirmed following histological analysis for microscopic lesions (Figure 8C). Percent tumor incidence (Figure 8D), number of lung nodules (Figure 8E) and average tumor volume (Figure 8F) were also evaluated for each treatment group. These studies confirm the *in vitro* findings presented above and demonstrate that ligand-mediated activation of ERβ is capable of preventing the development of metastatic lesions *in vivo*.

**Discussion**

In this study we sought to characterize the biological effects of targeting ERβ in TNBC cells and to elucidate the mechanisms of action through which it functions in this form of the disease. For the first time, we have characterized the ERβ cistrome in TNBC cells and have determined the effects of E2 on the global gene expression profiles of MDA-MB-231-ERβ cells. Microarray analysis revealed estrogen-mediated induction of a family of genes known as cystatins and ChIP-Seq analysis identified ERβ binding sites within the promoter and intronic regions of these genes. These ERβ binding sites were confirmed to be occupied by ERβ and were shown to be transcriptionally active following exposure to ERβ agonists. Ligand-mediated activation of ERβ with estrogen or LY500307 resulted in decreased invasion and migration of TNBC cells *in vitro* and prevented the formation of lung metastasis *in vivo*. Taken together, we propose a novel mechanism through which ERβ elicits tumor-suppressive effects, particularly with regard to suppression of metastatic phenotypes, which is characterized by the induction of cystatins and the subsequent inhibition of canonical TGFβ signaling.

ChIP-Seq analysis identified nearly 30,000 ERβ binding sites across the genome of which the large majority were ligand-dependent. Estrogen treatment of MDA-MB-231-ERβ cells resulted

in significantly more enrichment of ERβ on DNA compared to the ERβ specific agonist LY500307. This discrepancy could be a result of differential receptor conformation when these two ligands are bound and/or alterations in co-factor recruitment. However, it is worth noting that nearly all of the LY500307-induced ERβ binding sites were conserved among the E2-induced binding sites suggesting that these differences could also be explained by a difference in the $IC_{50}$ for these two ligands. As is the case with ERα (36, 37), the large majority of ERβ binding sites were located in introns or intergenic regions of chromatin indicating that ERβ primarily occupies enhancer regions across the genome.

In parallel to the ChIP-Seq studies, microarray analysis was performed to identify genes differentially regulated by ERβ following estrogen treatment. Through this analysis, we identified over 900 genes that were significantly induced or repressed and demonstrated that genes whose expression was up-regulated by ERβ were associated with less aggressive breast cancer phenotypes and decreased metastatic potential. Of the most highly induced transcripts, four were members of a superfamily of genes known as the cystatins, specifically cystatins 1, 2, 4 and 5. These four genes were shown to be specifically induced by E2 in an ERβ-specific manner as their expression levels were either completely absent or repressed by E2 in ERα positive breast cancer cells and since they were not induced by estrogen in ERα-expressing MDA-MB-231 or Hs578T cells. Cystatins are small secreted proteins that have previously been shown to function as cysteine protease inhibitors (38-40). While extensive research has been performed on a closely related family member, cystatin 3 (41), relatively little is known about cystatins 1, 2, 4 and 5. Further, nothing is known about the expression levels or function of these four cystatins in breast cancer.

ERβ has previously been shown to be a prognostic factor in TNBC, as high expression of this receptor is associated with improved overall survival (OS), disease-free survival (DFS), and distant metastasis-free survival (DMFS) (27). Given our findings that ligand mediated activation of ERβ results in substantial increases in cystatin gene expression in TNBC, we analyzed the association of cystatin 1, 2, 4 and 5 expression levels with relapse-free survival of TNBC patients. Intriguingly, high expression of all four cystatins correlated with improved relapse-free survival, but only in TNBC patients and not in breast cancer patients expressing ERα and PR. These findings are in agreement with the results of the present study implicating important tumor-suppressive effects of cystatins in TNBC. Furthermore, these data suggest that monitoring cystatin expression levels may have prognostic and/or predictive value in this form of the disease.

Utilizing our ChIP-Seq dataset, we were able to identify ERβ binding sites within the promoter and intronic regions of each of the four cystatin genes shown to be induced by E2 treatment in TNBC cells. These binding sites encoded functional EREs that were activated by ERβ in a ligand-dependent manner. ERβ was also shown to occupy some of these binding sites in a ligand independent fashion. This likely explains our findings that expression of ERβ alone, even in the absence of a ligand, is capable of causing a slight induction of cystatin gene expression. These findings demonstrate that ERβ directly associates with these regulatory elements to enhance cystatin gene expression in TNBC cells.

Although the cystatins were among the most highly regulated genes following E2 treatment of TNBC cells, it was not immediately obvious as to what effect, if any, they may have on TNBC cell biology. We therefore returned to our gene expression dataset and identified alterations in the TGFβ signaling pathway through the use of Ingenuity Pathway Analysis. Furthermore, these analyses predicted that our gene expression signature correlated with decreased TGFβ ligand activity. TGFβ signaling is a known driver of metastasis, disease progression, and resistance to chemotherapy in TNBC (42, 43) and activation of TGFβ signaling is associated with worse outcomes for breast cancer patients (33, 44). Given that a previous study has linked the closely related family member, cystatin 3, with decreased TGFβ pathway activity (45) we speculated that increased expression of cystatins by ERβ may result in suppression of TGFβ signaling in TNBC cells. Indeed, we demonstrated that cystatins block canonical TGFβ signaling in breast cancer cells likely due to their ability to directly interact with the TGFβR2 resulting in inhibition of TGFβ ligand occupancy.

Given that activation of the TGFβ pathway drives invasiveness in TNBC (42, 43), and in light of our findings that ERβ induces the expression of cystatins capable of inhibiting canonical TGFβ signaling, we sought to determine the effects of ERβ on TNBC cell invasion and migration. Our data demonstrate that the presence of ERβ, even in the absence of a ligand, suppresses both invasion and migration of TNBC cells, effects that are dramatically magnified by treatment with E2 or the ERβ selective agonist, LY500307. ERβ-mediated inhibition of invasion and migration is dependent on the biological activities of the cystatins since suppression of cystatin gene expression reverses these effects. Furthermore, we demonstrated that cystatins are capable of blocking TGFβ-induced cell invasion, effects that were even stronger than a TGFβ inhibitor.

These *in vitro* effects were confirmed in an *in vivo* metastatic mouse model, where ligand mediated activation of ERβ was shown to completely block lung colonization of TNBC cells.

## Conclusion

In conclusion, our data elucidate the ERβ cistrome in TNBC and identify and characterize the functional roles of cystatins in this disease. The present report builds upon prior data that ERβ is expressed in approximately 30% of TNBC patients and that ligand mediated activation of ERβ suppresses TNBC cell proliferation (21) and tumor progression (18, 46). Our results lend further support to the notion that therapeutic targeting of ERβ may elicit clinical benefit for TNBC patients, and that this effect is dependent on ERβ to induce tumoral expression of cystatins. These results lay the foundation for future studies aimed at analyzing the anti-tumor activity of estrogen and ERβ selective agonists in ERβ positive TNBC patients.

## Materials and Methods

Detailed information regarding the cell lines, chemical, reagents and procedures utilized in these studies are described in SI Appendix, SI Materials and Methods.

## Authors' contribution

Concept and design: JMR, MS, JNI, MPG, and JRH. Collection and assembly of data: JMR, ESB, VJS, AWN, IC, MS, MPG, and JRH. Data analysis and interpretation: JMR, ESB, VJS, AWN, IC, DGM, JSC, MPG, and JRH. Manuscript writing: JMR and JRH. All authors read and approved the final manuscript.

**Acknowledgements**

We would like to thank Molly Nelson-Holte for her expertise in assisting with the animal work, Dr. Ed Leof for generously supplying the phospho-Smad3 antibody, Drs. Steve Johnsen and Vivek Mishra for their insight and help with the GSEA, and Dr. Rani Kalari for the statistical analysis from the BEAUTY project. Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under through the Mayo Clinic Breast Cancer SPORE: P50CA116201 (MPG and JRH), as well as the Prospect Creek Foundation (JRH), the Eisenberg Foundation (JRH), the Mayo Graduate School (JMR), the Medical Research Council: MR/L00156X/1 (AWN), The Urology Foundation Scholarship: RESCH1302 (AWN) and a grant from the NIH: R01 AR068275 (DGM). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or other funding agencies. The authors declare no conflict of interest.

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## Figure Legends

**Figure 1. Identification of the ERβ cistrome in MDA-MB-231 TNBC cells.**

(A) Heat map of ERβ tag densities in MDA-MB-231-ERβ cells treated with vehicle, estrogen or LY500307 for 3 hours. (B) Total peaks called for each treatment. (C) Global distribution of binding sites among treatments. (D) Top 2 motifs identified, and their peak distributions, for each condition. Venn diagrams indicating overlap of all binding sites (E), promoter region-specific binding sites (F) and enhancer regions (G) between treatment conditions.

**Figure 2. ERβ regulated gene expression signatures in TNBC cells.**

(A) Pie chart depicting the number of estrogen regulated genes in MDA-MB-231-ERβ cells following 5 days of treatment. (B) Heat map depicting expression levels of the top 20 most highly up- and down-regulated genes following estrogen treatment. (C) Gene Set Enrichment Analysis of the microarray data indicating associations of ERβ specific gene signatures with multiple cancer related phenotypes. (D) Venn diagram indicating overlap between genes identified as having nearby ERβ binding sites via ChIP-Seq and genes significantly regulated by estrogen treatment from the microarray data sets. (E) Dendogram indicating relationship of cystatins identified to be significantly up-regulated by ERβ in response to estrogen treatment. (F) RT-PCR confirmation of the microarray data for cystatins 1, 2, 4 and 5 in response to estrogen treatment of MDA-MB-231-ERβ cells. (G & H) Independent RT-PCR confirmation of cystatin induction following 1 nM E2 or 10 nM LY500307 treatment for 5 days in MDA-MB-231-ERβ and Hs578T-ERβ cells. Data are represented as average ± SEM. *denotes p-value $<0.05$ relative to vehicle treated control cells.

**Figure 3. ERβ specific regulation of cystatins in TNBC cells.**

(A-B) RT-PCR analysis of cystatin 1, 2, 4 and 5 expression levels following estrogen treatment of MDA-MB-231-ERβ cells for indicated times. (C) Immunohistochemistry analysis of cystatin 5 protein levels in MDA-MB-231-ERβ cell pellets treated as indicated for 5 days. (D) mRNA expression levels of cystatin 5 as detected by RT-PCR in MDA-MB-231-ERβ cells treated as indicated for 5 days, images taken at 40x magnification. (E) RT-PCR analysis demonstrating blockade of estrogen induced cystatin gene expression by the pure anti-estrogen, ICI. (F) RT-PCR analysis of cystatin expression levels in ERα positive MCF7 and T47D cells following vehicle and estrogen treatment for 24 hours. (G) Kaplan-Meier plots depicting relapse-free survival (RFS) as a function of high and low cystatin 1, 2, 4 and 5 expression levels in ER/PR positive breast cancer vs TNBC. Data are represented as average ± SEM. * denotes p-value <0.05 relative to vehicle control treated cells. δ denotes p-value <0.05 between indicated treatments.

**Figure 4. Identification of ERβ regulatory elements responsible for mediating estrogen induced expression of cystatins 1, 2, 4 and 5.**

(A) Screen shots from the UCSC genome browser of ERβ signals from ChIP-Seq experiments on the promoters and first introns of indicated cystatin genes in MDA-MB-231-ERβ cells. (B) Comparison of a consensus ERE half-site to the EREs identified in the promoters and first introns of cystatins 1, 2, 4 and 5 via ERβ ChIP-Seq. (C) ChIP-PCR confirmation of ChIP-Seq data for indicated sites following pulldown with an ERβ-specific antibody (MC10) in MDA-MB-231-ERβ cells. (D) Luciferase assays indicating basal and estrogen induced (24 hours) activity of indicated cystatin promoter and intronic regions encoding identified ERβ binding sites in MDA-MB-231-ERβ cells. (E) Luciferase assays depicting the estrogen mediated activity of indicated cystatin promoter and intronic elements following site-directed mutagenesis of the identified

EREs relative to wild type controls. Data are represented as average ± SEM. * denotes p-value <0.05 compared to vehicle treated control cells. δ denotes p-value <0.05 compared to estrogen treated cells.

**Figure 5. Estrogen and cystatin mediated regulation of canonical TGFβ signaling in TNBC cells.**

(A) Heat map analysis of known TGFβ pathway genes shown to be differentially regulated by estrogen treatment in MDA-MB-231-ERβ expressing cells. (B) Heat maps generated from a TGFβ qPCR array indicating the effects of 24 hours or 5 days of estrogen treatment on indicated genes in MDA-MB-231-ERβ cells. (C) Western blot for phospho-Smad2, phospho-Smad3, total Smad2/3 and tubulin in MDA-MB-231 cell extracts. Cells were pre-treated with a combination of recombinant cystatins 1, 2, 4 and 5 for 6 hours followed by TGFβ (2ng/mL) or vehicle treatments for 30 minutes. (D) Luciferase assays indicating the activity of a Smad Binding Element reporter construct in parental MDA-MB-231 cells treated as indicated. (E) Immunofluorescent analysis of TGFβR2 (red) and cystatin 5 (green) proteins in MDA-MB-231-ERβ cells treated as indicated for 24 hours. Representative images are shown at 20x magnification. (F) Proximity based duolink assay indicating interaction between cystatin 5 and TGFβR2 proteins (red dots) in MDA-MB-231-ERβ cells treated as indicated for 24 hours. Representative images are shown at 20x magnification.

**Figure 6. Cystatins mediate ERβ suppression of TNBC cell migration and invasion.**

Cell migration and invasion assays were performed with MDA-MB-231-ERβ and Hs578T-ERβ cell lines. Representative images following indicated treatments are shown (A, C, E and G) and quantification of triplicate experiments ± SEM are indicated (B, D, F, H). (I) RT-PCR analysis indicating the efficacy of cystatin 1, 2, 4 and 5 siRNAs with regard to suppressing estrogen

induced cystatin gene expression in MDA-MB-231-ERβ cells. (J) Effects of siRNA mediated silencing of cystatin 1, 2, 4 and 5 expression on MDA-MB-231-ERβ cell invasion in the presence of indicated treatments. Data are represented as average ± SEM. * denotes p-value ≤ 0.05 relative to -Dox/Veh controls. ** denotes p-value ≤ 0.01 relative to Dox/Veh cells following adjustment for multiple comparisons.

**Figure 7. Cystatins inhibit TGFβ-induced invasion in TNBC cells.**

(A) Representative images of parental MDA-MB-231 cell invasion assays following treatment with TGFβ ligand (2ng/mL), TGFβ + recombinant CST proteins (125 ng/ml of each recombinant cystatin), or TGFβ + SB431542 (10 μM/ml), a TGFβ specific inhibitor. (B) Quantification of triplicate invasion experiments ± SEM following indicated treatments. * denotes p-value ≤ 0.05 relative to vehicle control treated cells. ** denotes p-value ≤ 0.01 relative to TGFβ treated cells following adjustment for multiple comparisons.

**Figure 8. Estrogen treatment inhibits lung colonization of ERβ-positive TNBC cells.**

MDA-MB-231-ERβ-Luciferase cells were injected into ovariectomized nude mice via the tail vein and randomized to indicated treatments. (A) IVIS2000 xenogen imaging indicating the presence of lung metastasis in mice randomized to the no dox placebo and dox placebo groups, but not in the dox estrogen group. (B) Gross images of lung nodules from representative animals in the indicated treatment groups (10x magnification). (C) Representative images depicting histological analysis of FFPE mouse lungs from animals in each treatment group following H&E staining and IHC analysis for ERβ at 40x magnification. Quantification of the incidence of lung nodules (D), the number of lung nodules (E) and the tumor volume of lung nodules (F) in mice randomized to the indicated treatment groups. * denotes p-value ≤ 0.01 relative to between indicated treatment groups following adjustment for multiple comparisons.