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## Progesterone receptor-B enhances estrogen responsiveness of breast cancer cells via scaffolding PELP1- and estrogen receptor-containing transcription complexes

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### Abstract

Progesterone and estrogen are important drivers of breast cancer proliferation. Herein, we probed ER-alpha and PR cross-talk in breast cancer models. Stable expression of PR-B in PR-low/ER+ MCF7 cells increased cellular sensitivity to estradiol and IGF1, as measured in growth assays performed in the absence of exogenous progestin; similar results were obtained in PR-null/ER+ T47D cells stably expressing PR-B. Genome-wide microarray analyses revealed that unliganded PR-B induced robust expression of a subset of estradiol-responsive ER-target genes, including *CathepsinD* (*CTSD*). Estradiol-treated MCF7 cells stably expressing PR-B exhibited enhanced ER Ser167 phosphorylation and recruitment of ER, PR, and the proline, glutamate and leucine rich protein 1 (PELP1) to an estrogen response element (ERE) in the *CTSD* distal promoter; this complex co-immunoprecipitated with IGF1R in whole cell lysates. Importantly, ER/PR/PELP1 complexes were also detected in human breast cancer samples. Inhibition of IGF1R or PI3K blocked PR-B-dependent *CTSD* mRNA upregulation in response to estradiol. Similarly, inhibition of IGF1R or PR significantly reduced ER recruitment to the *CTSD* promoter. Stable knockdown of endogenous PR or onapristone treatment of multiple unmodified breast cancer cell lines blocked estradiol-mediated *CTSD* induction, inhibited growth in soft agar, and partially restored

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tamoxifen-sensitivity of resistant cells. Further, combination treatment of breast cancer cells with both onapristone and IGF1R tyrosine kinase inhibitor AEW541 was more effective than either agent alone. In summary, unliganded PR-B enhanced proliferative responses to estradiol and IGF1 via scaffolding of ERalpha/PELP1/IGF1R-containing complexes. Our data provide a strong rationale for targeting PR in combination with ER and IGF1R in patients with luminal breast cancer.

## Keywords

Estrogen Receptor; Progesterone Receptor; PELP1; IGF1R; Cathepsin D; Breast Cancer

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## Introduction

Approximately 70% of newly diagnosed breast cancers are luminal breast cancers that are positive for estrogen receptor alpha (ER) and progesterone receptor (PR) expression (1). Although ER-targeted therapies are relatively successful in treating ER+ tumors, primary endocrine resistance occurs in up to a third of all breast cancers (2). Mechanisms of resistance to ER-targeted therapies are widely studied and include upregulation of growth factor pathways and increased ER cytoplasmic signaling, in addition to altered ER target gene profiles, enhanced ER phosphorylation, and deregulation of ER pathway components (3, 4). For example, the proline, glutamate and leucine rich protein 1 (PELP1), a growth factor receptor adaptor molecule and ER coactivator, is frequently upregulated in endocrine-resistant tumors (5). Endocrine-resistant tumors are characterized by increased aggressiveness and metastatic potential. Patients with recurrent endocrine-resistant tumors are generally treated with systemic chemotherapy.

PR expression in breast cancer predicts response to ER-targeted therapies, in part because ER regulates PR expression (3). Additionally, both ER and PR share similar signaling pathways (6) and exhibit substantial overlap in their transcriptomes (7). Recent mechanistic studies by our group and others have shown that PR is capable of driving breast cancer progression in both the absence and presence of progestin (8). Ligand-independent PR actions include scaffolding of growth factor pathways to enhance kinase signaling (9) and activation of pro-growth and pro-survival transcriptional programs in breast cancer cells (10–12). *In vitro* models of aromatase inhibitor-resistant breast cancer cells demonstrated sensitivity to the PR antagonist, CDB-2914, which inhibited cell cycle progression (13). Recently, ER has been shown to cooperate with PR in response to progestins (14). Additional evidence demonstrated that cells in the normal adult mammary gland (15) and in breast tumors (16) contain cell populations that express ER only, PR only, both receptors, or neither. A small population of SR+ (steroid receptor) cells in the normal mammary gland have been shown to secrete paracrine factors and thereby provide potent proliferative signals to adjacent SR- cells (17). Indeed, estradiol (E2) has been shown to drive the proliferative actions of PR-B, an ER target gene that is required for mammary gland alveologenesis (17). In addition, PR-B has been shown to induce breast cancer cell growth in soft agar assays, whereas PR-A does not (18). These data provide a strong rationale for the study of ER actions independently of PR and in conjunction with PR to better understand how these SRs

cooperate to modulate hormone responsiveness in normal and breast cancer tissues. Herein, we hypothesized that PR-B+/ER+ breast cancer cells have a heightened sensitivity to estrogen because of direct molecular cross-talk between ER and PR-B. This heightened sensitivity represents a potential mechanism by which resistance to endocrine therapy can develop.

## Results

### PR-B enhances breast cancer cell proliferation in response to estradiol and IGF1

To address the question of whether ER and PR-B, the mitogenic PR isoform in the breast, work in concert to enhance estradiol sensitivity in breast cancer cells, we utilized the traditionally SR+ breast cancer cell model, MCF7. Beginning with a naturally occurring ER+/PR-low variant of MCF7 cells (19), we stably re-expressed either PR-B (pSG5-PR-B) or a vector (pSG5) control (Fig 1A). Cells expressing PR-B or vector-matched controls were then subjected to MTT proliferation assays and grown in soft agar. Notably, MCF7 cells lacking PR-B but containing abundant ER-alpha were not significantly responsive to estradiol as measured by MTT assays; however, MCF7 cells expressing PR-B displayed significantly heightened proliferation in response to estradiol (Fig S1A). PR-B-null MCF7 cells (empty vector) displayed a slight increase in colony formation in response to estradiol. MCF7 cells stably expressing PR-B displayed increased anchorage independent growth in response to estradiol relative to PR-null controls (Fig 1BC). Similar results were observed in ER+/PR-null T47D cells stably expressing PR-B relative to vector controls (Fig S1 and Fig 1C); interestingly, these cells were also more responsive to IGF1 treatment, a growth factor receptor pathway known to extensively crosstalk with ER signaling (20). In addition, we examined the estradiol-induced growth of unmodified MCF7L and BT474 breast cancer cell lines after treatment with the type II anti-PR drug onapristone. MCF7L cells are ER+/PR+ and express abundant levels of PR-A and PR-B (Fig 1E). BT474 cells are ER+/PR+/HER2+ and are considered relatively aggressive but still highly responsive to estradiol. Onapristone partially blocked (41%) estradiol-induced soft agar growth of MCF7L cells (Fig 1D) and dramatically inhibited (63%) estradiol-driven soft agar growth of BT474 cells (Fig 1E). Notably, onapristone treatment did not alter ER expression levels in either cell line (Fig 1F).

To complement these studies, we performed gene-silencing experiments in MCF7L cells that express abundant levels of PR-A and PR-B. ER+/PR+ MCF7L cells were stably transfected with shRNAs targeting GFP (control; shGFP) or PRs (shPR). Total PR mRNA (qRT-PCR) and PR protein (WB) levels were assessed to confirm PR knockdown in multiple clones (Fig S2A and not shown). ER expression remained relatively unchanged upon PR knockdown (Fig S2A). Soft agar assays demonstrated that cells expressing control shRNA exhibited basal colony formation that was significantly enhanced by estradiol treatment. In contrast, estradiol-induced soft agar colony formation was significantly blunted by shPR (Fig S2B) and partially blocked by onapristone (Fig 1D). Together, our data demonstrate a novel ligand-independent PR function in ER+ breast cancer cells, in which PR-B confers heightened responsiveness to estradiol and IGF1.

## Estradiol-treated PR-B+/ER+ breast cancer cells express a distinct gene signature associated with tumor progression

To determine whether unliganded PR-B participates in ER-dependent transcriptional responses, we performed gene array analyses to measure changes in the ER transcriptome. Serum-starved MCF7 cells stably expressing either vector or PR-B were treated with estradiol (6h), and mRNA was harvested for gene expression analyses. Unsupervised clustering of differentially regulated genes was visualized by heat map. As predicted (10, 12), PR-B expression alone profoundly altered hormone-independent (basal) gene expression (Fig 2A; lanes 1 and 3). Estradiol-stimulated gene expression profiles revealed 40 genes that were upregulated (>2 fold,  $P < 0.001$ ) and 49 genes that were downregulated (<2 fold,  $P < 0.001$ ) (Fig 2A, lanes 2 and 4) in PR-B-expressing cells compared to PR-null vector-matched controls (Table S1). IPA analysis revealed that the biological processes significantly associated with this PR-B-dependent, estradiol-induced gene signature included cancer, cell cycle, reproductive system disease, cellular movement, cell growth and proliferation, and cell death and survival (Fig S3A). Furthermore, Gene Set Enrichment Analysis (GSEA) showed that estradiol-induced genes in PR-B+ MCF7 cells were significantly ( $FDR < 0.05$ ) associated with genes upregulated in the luminal-B subtype of breast cancer (21) (Fig S3B), two independent tamoxifen-resistant signatures (22, 23), and *ESR1*-positive breast tumors relative to *ESR1*-negative tumors (24). Notably, luminal-B breast cancers are more likely to become endocrine resistant (25). These data indicate that PR-B and ER cooperate in the regulation of estradiol-induced gene expression programs associated with breast tumor progression.

Known ER-target genes among the 40 genes upregulated in PR-B+/ER+ MCF7 cells relative to PR-null/ER+ vector-matched controls were examined by qRT-PCR. PR-B expression significantly enhanced estradiol responsiveness to *SLC7A5* (Fig 2B, upper panel), a gene known to be regulated by ER and IGF-1(26). Notably, a query of TCGA (The Cancer Genome Atlas) breast cancer data (27) found that *SLC7A5* mRNA was expressed at higher levels in invasive breast carcinoma than in normal mammary tissue (Fig 2B), lower panel). In addition, PR-B expression significantly increased estradiol sensitivity to induced ER-target genes *WISP2* and *PTGES*, (Fig 2C). Similar to PR-dependent but estradiol-induced genes, numerous genes were also downregulated by estradiol in PR-B-expressing cells relative to PR-null (vector) cells, including *maoa* (Fig 2D, upper panel), *lxn*, and *tmprss2* (Fig 2E); *maoa* expression is significantly downregulated in invasive breast carcinoma compared to normal breast tissue, as determined by analysis of TCGA (Fig 2D, lower panel).

Notably, PR-B expression exclusively enhanced estradiol sensitivity for the classical ER-target gene *cathepsin-D* (*CTSD*) (Fig 3A). Estradiol-induced *CTSD* expression in PR-B+ MCF7 cells was not significantly affected by the addition of progestin or IGF1 (Fig S4A–B). The expression of *TFF1* (*pS2*), another well-characterized ER-target gene, was not affected by PR-B expression (Fig 3A). As expected, expression of *SGK*, a classical PR target gene, was increased in PR-B+ MCF7 cells treated with the synthetic progestin, R5020 (Fig 3A). Analysis of the TCGA breast cancer data set revealed that *CTSD* expression was also significantly higher in invasive breast cancers than in normal tissues (Fig 3B). Interestingly,

analysis of *PR* and *CTSD* mRNA expression demonstrated a tendency toward co-occurrence in invasive breast carcinoma in the TCGA data set, whereas *PR* and *TFF1* expression were not associated. These data demonstrate that PR-B cooperates with ER to drive the expression of a subset of ER-target genes.

PR-A and PR-B isoforms are co-expressed in normal and neoplastic cells; however, the ratio of PR-A to PR-B frequently varies amongst breast tumors (28). To determine whether PR-A also cooperates with ER to drive *CTSD* expression, we utilized previously characterized PR-null T47D cells stably expressing either PR-A or PR-B ((11) and Fig S5A). Similar to our results in MCF7 cells, PR-null/ER+ T47D cells did not induce *CTSD* expression in response to estradiol, whereas PR-B+/ER+ T47D cells did induce *CTSD* expression (Fig 4A). In contrast to PR-B expressing cells, PR-A+/ER+ T47D cells failed to increase the expression of *CTSD* in response to estradiol (Fig S5B). These data suggest that PR-B is the primary mediator of cross-talk with ER in the upregulation of *CTSD*.

### PR DNA-binding domain is essential for *CTSD* expression

*CTSD* is widely used as a measure of estradiol responsiveness in breast cancer models (29). We therefore focused on the regulation of this gene as a model for probing mechanistic interactions between ER and PR-B. To test the requirement for the PR DNA-binding domain in estradiol-induced *CTSD* regulation, we again utilized PR-null T47D cells engineered to stably express either wild type (wt) PR-B or a DNA-binding domain mutant of PR-B (DBM PR-B) (18). Importantly, ER protein levels in T47D cells stably expressing either wt or DBM PR-B cells were comparable; however, PR-B protein levels were higher in T47D cells expressing DBM PR-B (Fig 4B, inset), perhaps due to deficient turnover of this mutant protein. T47D cells expressing DBM PR-B failed to appreciably induce *CTSD* upon estradiol stimulation relative to the significant response observed in T47D cells expressing wt PR-B (Fig 4B). The classical ER-target gene, *TFF1*, was significantly upregulated in both cell lines upon estradiol treatment (not shown). These data show that the PR DNA-binding domain is required for ER/PR-B co-regulation of *CTSD* expression.

### PR-B and ER are recruited to the *CTSD* promoter in response to estradiol

ER binds to both proximal (30) and distal (29) sites in the *CTSD* promoter in response to estradiol. We focused on the distal ER-binding site as the primary site for estradiol-mediated *CTSD* upregulation (29). ChIP (chromatin immunoprecipitation) assays were performed on MCF7 cells expressing PR-B or vector-matched controls. Cells were treated (1hr) with vehicle or estradiol prior to assessment of ER recruitment to the *CTSD* distal promoter. Interestingly, ER binding to the distal region of the *CTSD* promoter was negligible in PR-null, vector-matched control cells, whereas it was robust in PR-B expressing cells treated with estradiol (Fig 4C). Previously published estradiol responsive elements (EREs) in the promoter region of a control gene (*TFF1*) were also examined for ER binding under these conditions (30); ER was equally recruited to the *TFF1* promoter upon estradiol stimulation in vector and PR-B expressing MCF7 cells (not shown).

To determine whether ER/PR-B complexes drive estradiol-dependent transcription of *CTSD*, we again performed ChIP assays in PR-B expressing MCF7 cells treated with vehicle or

estradiol (40min) using antibodies specific for either ER or PR. As above, ER recruitment was again observed upon estradiol stimulation of PR-B containing cells (Fig 4D). In addition, PR recruitment to the *CTSD* promoter was detected after estradiol stimulation of MCF7 cells (Fig 4D), though at lower levels than ER recruitment. Similar results were observed in T47D cells stably expressing PR-B (not shown). Although PR recruitment to the *CTSD* promoter was not as robust as ER recruitment, the PR antagonist onapristone diminished estradiol-induced ER binding to the *CTSD* promoter (Fig 4E) and blocked estradiol-induced *CTSD* mRNA expression (Fig 4F) in MCF7L (PR high) cells. These studies further indicate that unliganded PR-B facilitates ER in the regulation of select estradiol-induced genes.

### **Unliganded PR-B facilitates phosphorylation of specific ER residues in the presence of estradiol or growth factors**

Previous reports demonstrated that PR-B and ER interact with c-Src as part of tyrosine kinase-associated signaling complexes that function to initiate rapid activation of downstream kinase pathways in response to steroid hormones (6, 19). One function of SR-containing rapid signaling complexes is to ensure appropriate phosphorylation of ligand-bound steroid hormone receptors that in turn regulate SR-dependent genomic events (8, 31). To examine whether PR-B regulates ER phosphorylation, MCF7 cells expressing vector or PR-B were stimulated with estradiol for 1 hour prior to Western blotting with phospho-specific antibodies. IGF-1 treatment was included as a positive control for ER Ser167 phosphorylation (26). *IRS1*, a PR-B target gene and IGF1R adaptor molecule, exhibited slightly higher expression in PR-B expressing cells. Interestingly, MCF7 cells expressing PR-B exhibited higher basal (Fig 5A, lanes 1 versus 4) and estradiol-stimulated (Fig 5A, lanes 2 versus 5) Ser118 phosphorylation of ER relative to controls. IGF1, but not estradiol, induced ER phospho-Ser167 in MCF7 cells lacking PR-B. However, when PR-B was expressed, basal phosphorylation levels of ER Ser167 increased (lanes 1 and 4). In addition, this site was appreciably phosphorylated in response to both estradiol and IGF1 (lanes 5 and 6). These data suggest that in the absence of added progestin, PR-B expression serves to increase the ability of estradiol or growth factors (IGF1) to initiate ER-specific phosphorylation events, perhaps via scaffolding of localized cytoplasmic or membrane-associated signaling complexes.

### **PELP1 is part of ER/PR-B-containing transcriptional complex and is recruited to the *CTSD* promoter**

Estradiol-bound ER participates in cytoplasmic signaling complexes that include protein kinases (c-Src, PI3K), growth factor receptors (IGF1R, EGFR), and scaffolding molecules (PELP-1 [MNAR]) (18, 20, 32, 33). PELP1 functions to modulate ER activity by acting as a scaffolding molecule for effective cytoplasmic signaling and as an ER transcriptional coactivator in the nucleus (32). We speculated that PELP1 might enhance local kinase signaling (ie, ER phosphorylation). To determine whether PR-B participates in a signaling complex with ER and PELP1, we performed co-immunoprecipitation assays in MCF7 cells transiently transfected with PR-B and treated cells for 10 min with vehicle, estradiol, or IGF-1. PR-specific immunoprecipitates, but not IgG controls, contained abundant PELP1 and low levels of estradiol-induced IGF1R (Fig 5B). IGF1R and ER were also weakly

detected in PELP1 immunoprecipitates from PR-B expressing MCF7 cells (Fig S6A). Co-immunoprecipitation of ER and PR, recently published (14), was confirmed in our system (not shown). In addition, PELP1, ER, and PR displayed robust binding to the *CTSD* distal promoter in response to estradiol treatment but not vehicle treatment (Fig 5C). Interestingly, we also consistently detected weak recruitment of IGF1R to the same region of the *CTSD* promoter. However, this signaling complex (ER, PR, PELP1, and IGF1R) was not detected on the *TFF1* promoter (not shown). These data suggest that cytoplasmic signaling complexes containing ER, PR, PELP1, and IGF1R appear to directly participate in *CTSD* gene regulation as part of transcriptional complexes associated with chromatin.

### IGF1R- and AKT-dependent signaling events are required for *CTSD* regulation

Previous findings have shown that cytoplasmic PELP1 is an enhancer of both MAPK and AKT signaling, leading to increased ER phosphorylation on Ser167 and Ser118 and enhanced transcriptional activity (34). Using a previously characterized NLS PELP1 mutant that is localized primarily to the cytoplasm (34), we found that *CTSD* mRNA expression was higher in cells expressing cytoplasmic NLS PELP1 than in cells expressing wt PELP1 or cells expressing vector alone (Fig S6B). These data indicate that PELP1-dependent signaling (ie, heightened rapid signaling events) may enhance specific transcriptional responses mediated by ER, PR, and PELP1-containing complexes.

To determine whether PELP1-associated kinase activities are required for estradiol-induced *CTSD* expression, PR-B+ MCF7 cells were pre-treated with inhibitors of IGF1R (AEW541), PI3K (LY-294002), MAPK (U0126), and c-Src (PP2). Estradiol-mediated activation of *CTSD* expression was then examined by qRT-PCR. None of these kinase inhibitors affected basal *CTSD* mRNA expression in PR-B+ MCF7 cells. PI3K or IGF1R inhibition blocked estradiol-induced *CTSD* expression (Fig 5D). In addition, IGF1R inhibition specifically blocked phosphorylation of ER Ser167 but not Ser118 (inset). In contrast, neither PI3K nor IGF1R inhibition affected estradiol-mediated *TFF1* expression (Fig 5D). MAPK inhibition with U0126 blocked estradiol-induced *CTSD* and *TFF1* expression (not shown). Surprisingly, c-Src inhibition with PP2 did not significantly alter estradiol-induced *CTSD* or *TFF1* expression, but it did inhibit *CCND1* expression, a Src-dependent (positive control) gene (not shown) (35). In ChIP assays, IGF1R inhibition with AEW541 blocked estradiol-induced ER binding to the *CTSD* promoter; however, it did not affect estradiol-induced ER recruitment to the *TFF1* promoter (Fig 5E). Together, these data support a role for ER/PR-B/PELP1 complexes in activation of IGF1R and downstream PI3K/AKT signaling in the regulation of *CTSD* expression.

### Dual PR and IGF1R blockade inhibits growth of tamoxifen-resistant breast cancer cells

As shown above, blockade of either PR or IGF1R partially reduced estradiol-induced ER binding to the *CTSD* promoter and blocked *CTSD* mRNA expression. To determine the effect of dual blockade of PR and IGF1R, we performed soft agar assays in tamoxifen-sensitive (MCF7L) and -resistant (MCF7 1GX) cell variants. PR+/IGF1R+ tamoxifen-resistant MCF7 1GX cells were engineered to overexpress RAF1 and passaged once as xenografts in mice (36). Estradiol-induced colony formation was lower among MCF7L cells treated with onapristone and AEW541 than among cells treated with either agent alone (Fig

6A). In the presence of tamoxifen, estradiol-treated MCF7 1GX cells exhibited robust colony formation; however, onapristone significantly decreased basal and estradiol-induced growth in soft agar (Fig 6B). Importantly, estradiol-induced colony formation was completely blocked among MCF7 1GX cells treated with onapristone and AEW541 (Fig 6C). These data suggest that PR-B and IGF1R expression alter ER transcriptional programs to enhance breast cancer growth even in the context of tamoxifen resistance, perhaps by re-sensitizing cells to tamoxifen.

### ER/PR/PELP1 complexes are detectable in human breast tumors

To determine whether the ER/PR/PELP1 signaling complex is present in breast cancer *in vivo*, we obtained human tumor samples and performed co-immunoprecipitation studies. Nine tumor samples, clinically determined to be ER+/PR+, expressed PELP1, PR, ER, and actin (loading control) in Western blots (Fig 7A). Pull-down of PELP1 revealed that PELP1 had a strong association with ER and a variable, yet persistent, association with PR. In addition, ER and PELP1 co-immunoprecipitated with PR in breast tumors, and PELP1 and PR co-immunoprecipitated with ER (not shown). Together, these data demonstrate that ER/PR/PELP1 form complexes in human breast cancers, the consequence of which is likely more aggressive proliferative responses to estrogens.

## Discussion

The classical definition of PR function is that the receptor is a ligand-activated transcription factor whose promoter selection is driven by rapid cytoplasmic phosphorylation events (8). Herein, we extend this definition by showing that unliganded PR-B acts as a molecular scaffold for the formation of an ER/PELP1-containing transcription complex at newly defined ER/PR-B-target genes. We found that, in the absence of added progestin, the PR-B isoform activated a subset of ER-target genes in estradiol-mediated pathways to enhance breast cancer cell proliferation and anchorage independent growth in multiple cell line models (MCF7, T47D, and BT474). In this context, PR-B associated with ER and PELP1 *in vitro* and *in vivo* to form a transcriptional complex on select ER-target genes, including *CTSD*. The ER/PR-B/PELP1 complex facilitated the phosphorylation of specific ER sites and activation of IGF1R downstream PI3K/AKT signaling in the regulation of *CTSD*. We also detected IGF1R at the same region of the *CTSD* promoter. Inhibition of IGF1R, PR, or both blocked estradiol-dependent breast cancer cell proliferation. Importantly, our gene profiling studies indicated that PR-B cross-talk with ER results in more aggressive proliferative responses to estrogens and shifts the ER transcriptome towards the luminal-B phenotype, which is more likely to become tamoxifen resistant. Taken together, our data support a model (Fig 7B) in which PR-B/ER/PELP1 complexes formed at, or near, the membrane with growth factor receptors (ie, IGF1R) are capable of initiating signaling events (ie, ER phosphorylation) that direct these complexes to specific promoters (ie, *CTSD*) and thereby activate particular gene sets and their biological programs. Novel ER/PR/PELP1 target genes may provide useful biomarkers for selection of patients likely to respond to antagonism of components of this transcriptional complex or its related signaling pathways.



### PR-B DNA-binding domain was required for ER recruitment to target genes

Our study shows that unliganded PR-B facilitates estradiol-induced ER interactions with kinases (eg, IGF1R, AKT) perhaps in part due to increased PR-B driven IRS1 expression (37). However, the specific function of unliganded PR-B in the ER/PELPI/IGF1R transcriptional complex is likely multifold. We found that the PR-B DNA-binding domain was required for ER recruitment to the *CTSD* promoter. Direct PR binding to ERE-containing DNA enhancer regions has been reported (38), though it is also possible that PR-B interacts with a PRE located on a distant region of DNA that loops back to the *CTSD* region and thereby brings together regulatory regions across large expanses of DNA (39). Related to this idea, we identified numerous PRE half sites in close proximity to the *CTSD* distal ERE, but PR-B recruitment to these sites was not specifically detected in our ChIP assays (not shown). Notably, the ER/PR cooperative mechanism described herein appears distinct from previous descriptions of ER/PR crosstalk upon stimulation of breast cancer cells with progestin (14). In our study focused on ligand-independent PR actions, ChIP assays failed to detect ER/PR/PELPI complexes in association with either the *MYC* or *CCND1* genes in response to estradiol (not shown).

### IGF1R/PI3K pathway contributes to estradiol-induced, ER/PR-B-mediated breast cancer growth

ER/PR/PELPI-mediated activation of growth-promoting transcriptional programs described herein required IGF1R and PI3K/AKT activation. These findings underscore the interest in PI3K as an attractive target for endocrine-resistant patients (40). Indeed, activating mutations in PI3K or AKT occur in up to 25% of breast cancers (41). Additionally, phosphorylation of Ser167, the AKT site on ER-alpha, reduces sensitivity to tamoxifen *in vitro* (42, 43). Similarly, hyperactive IGF signaling is associated with resistance to endocrine therapies (44). Notably, blockade of IGF1R with dalotuzumab significantly decreases estradiol-stimulated growth in MCF7L (PR+) xenografts (45). We found that the combination of AEW541 and onapristone completely abolished estradiol-dependent soft agar colony formation in similar MCF7L and tamoxifen-resistant MCF7 1GX cells. However, even though onapristone blocks PR-containing transcriptional complexes, it leaves upstream (AKT) signaling intact. These findings suggest that combining IGF1R and PR antagonists may provide a means to block endocrine-resistant, ER+/PR+ tumors via targeting these distinct but highly cooperative pathways.

### Breast cancer therapy and ER/PR crosstalk

As a classical ER-target gene, PR expression is used as a clinical marker of functional ER in breast tumors and thus predicts the likelihood of effective response to ER-targeted therapies. Our data linking heightened estrogen sensitivity to PR scaffolding activity lend additional mechanistic support to these long-held clinical findings. Indeed, our studies underscore the need to consider PR is an active member of ER-containing transcriptional complexes. Interestingly, clinical assessment of PR expression by IHC in breast tumors does not distinguish between PR-A and PR-B isoforms. PR-A expression is highly estrogen-dependent, while PR-B expression is also regulated by other factors (46). Notably, the scaffolding activity of PR-B described herein occurs in the absence of added progestin, and

ER/PR/PELP1-target genes are unresponsive to added progestin. These genes clearly contribute to breast cancer growth and survival phenotypes. We were surprised that both luminal-B and tamoxifen-resistant gene sets were enriched in estradiol-treated MCF7 cells expressing PR-B relative to PR-null cells. While more studies are needed, these provocative data suggest that in the presence of estrogen, PR-B may in fact be required for transition of luminal-A breast cancer cells to the luminal-B sub-type.

In sum, inclusion of PR-targeted therapies as part of the modern endocrine arsenal seems warranted and timely. Notably, over half of acquired endocrine-resistant tumors retain PR expression (47, 48). Furthermore, PR activates many of the same genes as ER (7), and PR is a major driver of proliferative and pro-survival pathways in the breast and in breast cancer (8). Herein, we show that PR-B contributes to estrogen responsiveness via direct ER/PELP1 cross talk at classical estrogen-dependent genes. Thus, combination treatment with both PR- and ER-targeted therapies may provide significant clinical benefit. Indeed, in rat models, additive effects of tamoxifen and onapristone were observed in mammary cancers induced by DMBA or MNU (reviewed in (49)). Additionally, onapristone (and to a lesser extent RU486) inhibited tumor growth and estrogen stimulation in the MXT mouse model of breast cancer (49). Similarly, treatment of MCF7 and T47D cells with either tamoxifen or RU486 blocked estrogen-mediated cell proliferation (49). It is thus tempting to speculate that PR-B in PELP1/ER complexes may function to compensate for ER inhibition in tumors that progress under estrogen- or ER-blocking therapies. As such, we conclude that antiprogestins, perhaps given early as intermittent or cyclical treatments, in combination with current endocrine therapies may prevent or delay endocrine failure in patients with luminal tumors expressing novel ER/PR-B-target gene biomarkers.

## Materials and Methods

### Cell lines and reagents

T47D-Y (PR-null) and MCF7 (PR-low) (19) cells were stably transfected with pSG5 or pSG5-PR-B and pSV-Neo or pIRES or pIRES-PR-B using FuGENE reagent (ThermoFisher) to generate multiple vector matched clones. Cells were cultured as previously described (12, 19). Additional MCF7 cells were obtained from ATCC (PR-A/B+), cultured as indicated, and stably infected with retrovirus expressing LXS<sub>N</sub>, LXS<sub>N</sub>-wtPELP1, or LXS<sub>N</sub>-NLS PELP1 (34). MCF7C4-12 (PR-null), MCF7L (PR-A/B+), BT474 (PR-A/B+), and MCF7 1GX (PR-A/B+) cells were cultured as described (12, 26, 36, 50), MCF7L PR knockdown cells were generated using lentivirus shRNAs targeting PR (5 sequences) or GFP in pLKO (ThermoFisher).

Cells were treated with estradiol (1nM), tamoxifen (100nM), and ICI 182,780 (1uM) obtained from Sigma-Aldrich (St. Louis, MO); LY-294002 (10uM), U0126 (10uM), and PP2 (10uM) obtained from Calbiochem (Darmstadt, Germany); R5020 (10nM) purchased from NEN Life Science Products (Boston, MA); and IGF1 (5nM) from GroPep Bioreagents (Thebarton SA, Aus).

### Proliferation assays

MTT and soft agar assays were performed as previously described (with three biological replicates of each condition per experiment) and results presented are representative of two to five experimental repeats (12, 18). Student's T-test was performed to determine statistical significance between treatment groups in each experiment.

### Western blots (WB) and Immunoprecipitation (IP) Assays

WB and co-IPs were performed as previously described (12). The following specific antibodies were employed: PR-A and -B ab-8 (ThermoFisher), PELP1 (Bethyl Laboratories), PR-A and B H190, IGF1R $\beta$ , ER (SantaCruz Biotechnologies, Santa Cruz, CA), actin (Sigma-Aldrich), ER phospho-167, ER phospho-118, and Erk1/2 (Cell Signaling, Beverly, MA).

### qRT-PCR

RNA was isolated from cells in triplicate wells treated with ethanol (vehicle) or estradiol (1nM) for 24 hours using TriReagent (Roche). Data shown is representative of two to nine replicates of each qPCR experiment which was performed as previously described (12). Student's T-tests were performed to determine statistical significance between treatment groups in each experiment.

### Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using ChIPit express kits (ActiveMotif, Carlsbad, CA) according to the manufacturer's protocol as previously described (12) and antibodies specific for PR (ab-8) and ER (Santa Cruz). Briefly, MCF7 ( $7 \times 10^6$ ) or T47D ( $15 \times 10^6$ ) cells were plated in 150mm dishes, starved in unsupplemented phenol red free IMEM (24h), and treated for 40min or 1hr with ethanol or estradiol. Relative recruitment was determined by qPCR of purified ChIP and input DNA in triplicate. The results presented are representative of two to eight independent experimental repeats.

### Whole genome expression analysis

Vector matched MCF7 cells stably expressing pSG5 or pSG5-PR-B were serum starved in modified IMEM (Gibco) for 1day and treated with estradiol or ethanol (6h) before RNA extraction using a RNeasy kit (QIAGEN). RNA samples in triplicate were prepared for expression analysis using the Illumina HT-12v4 bead chip platform according to the manufacturer's protocols. Gene expression normalization and analysis was completed as previously described (12). Heatmap genes were significantly regulated >3 fold, Benjamini and Hochberg adjusted  $P$  value < 0.001. All gene expression data is available in the NCBI Gene Expression Omnibus (GEO) database, accession number: GSE45643 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=prknleoqgaqgkxc&acc=GSE45643>).

Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)) was used to investigate genes up or downregulated after estradiol treatment in MCF7 cells expressing PR-B as previously described (12); genes with fold change >2.8 were included in

the analysis (BH adjusted  $P < 0.01$  in the sample comparison: [PR E2/PR vehicle] versus [vector E2/vector vehicle]).

### Human breast cancer tissue studies

De-identified frozen breast cancer tissues were obtained from patients with known ER+ PR+ breast tumors from the UT Southwestern tissue core. Immunohistochemical staining of matching specimens revealed that ER/PR expression in these tumor samples was largely restricted to the tumor with little to no expression in the surrounding stroma. Total tissue protein was extracted using lysis buffer [50mM HEPES (pH 7.5), 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM MgCl<sub>2</sub>] containing protease inhibitor cocktail. Lysates (100µg) were subject to immunoprecipitation.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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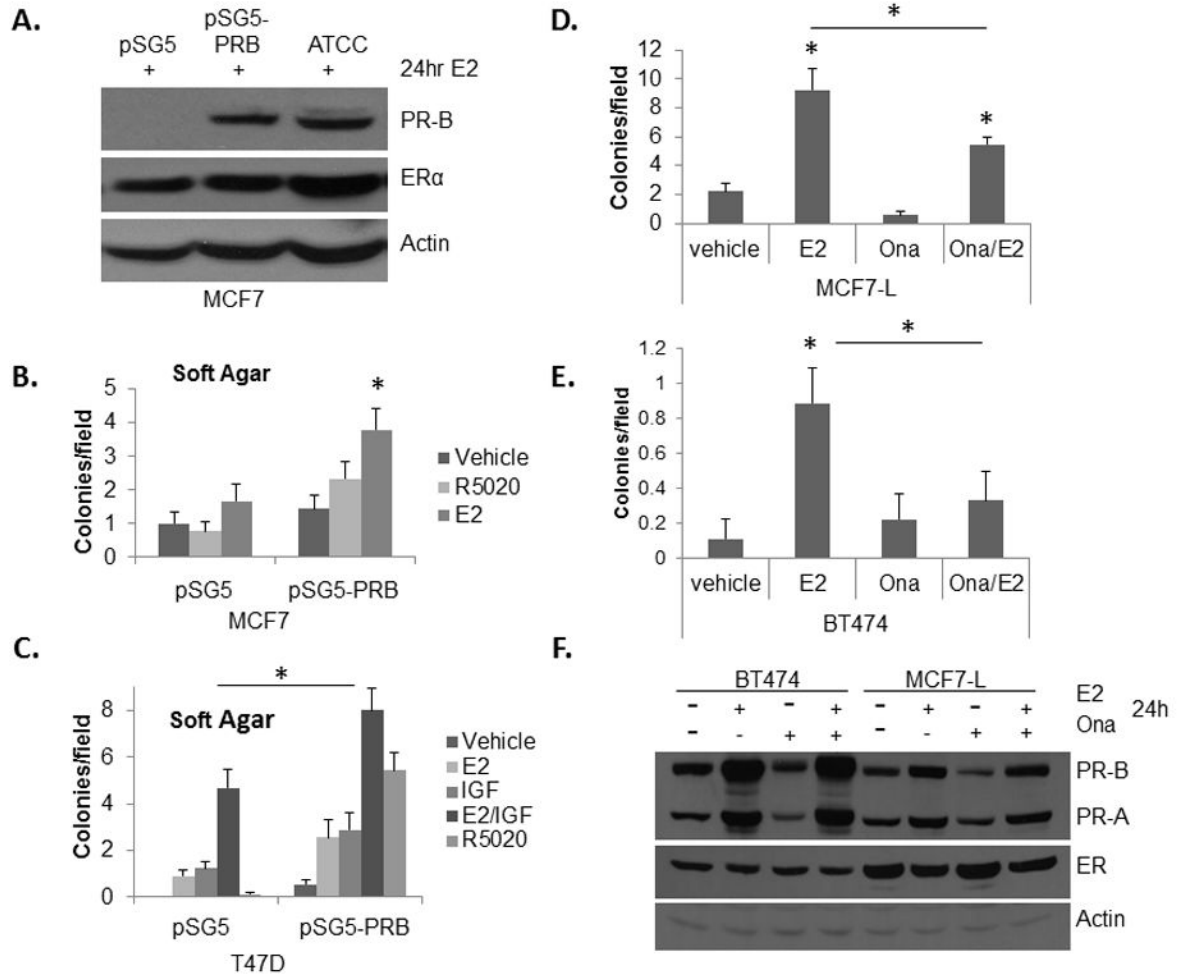
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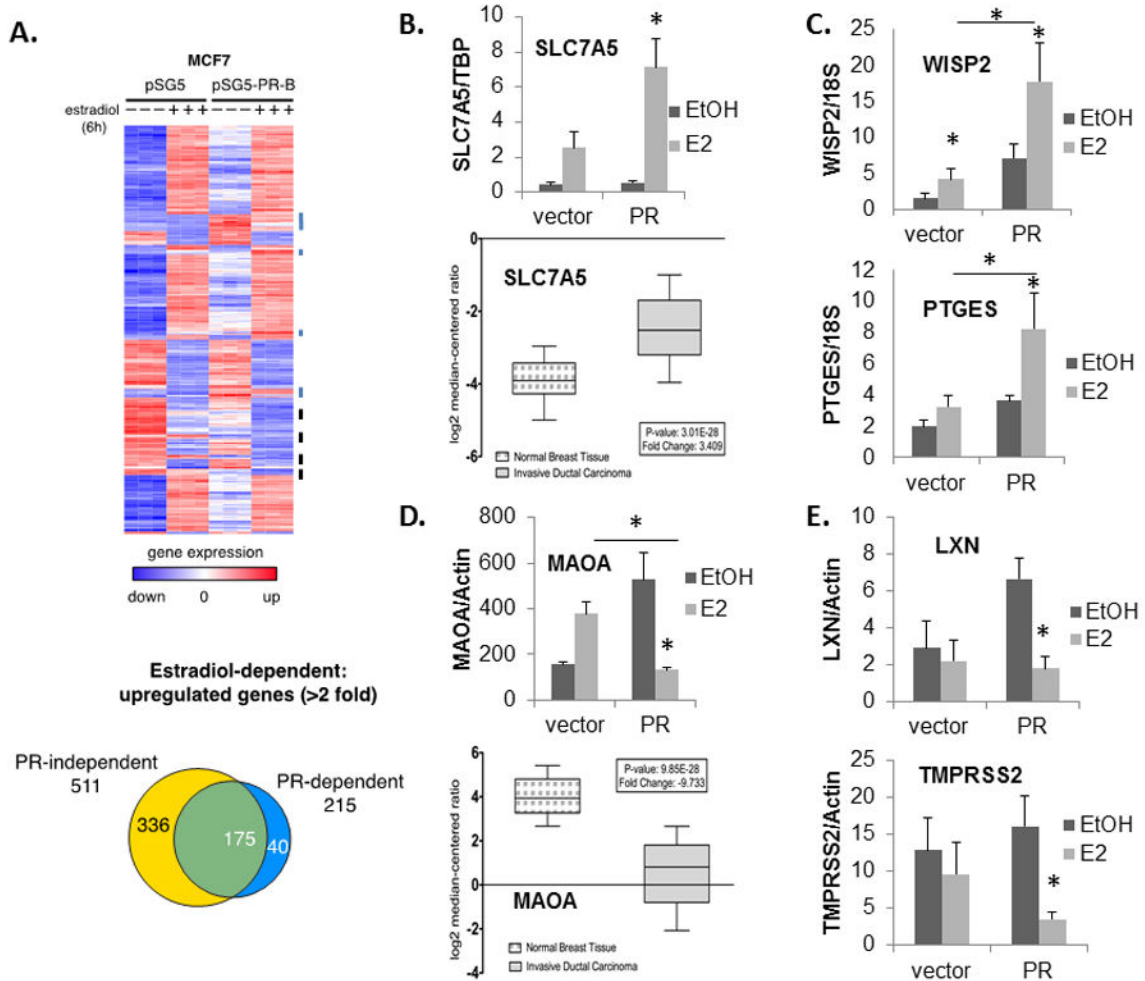
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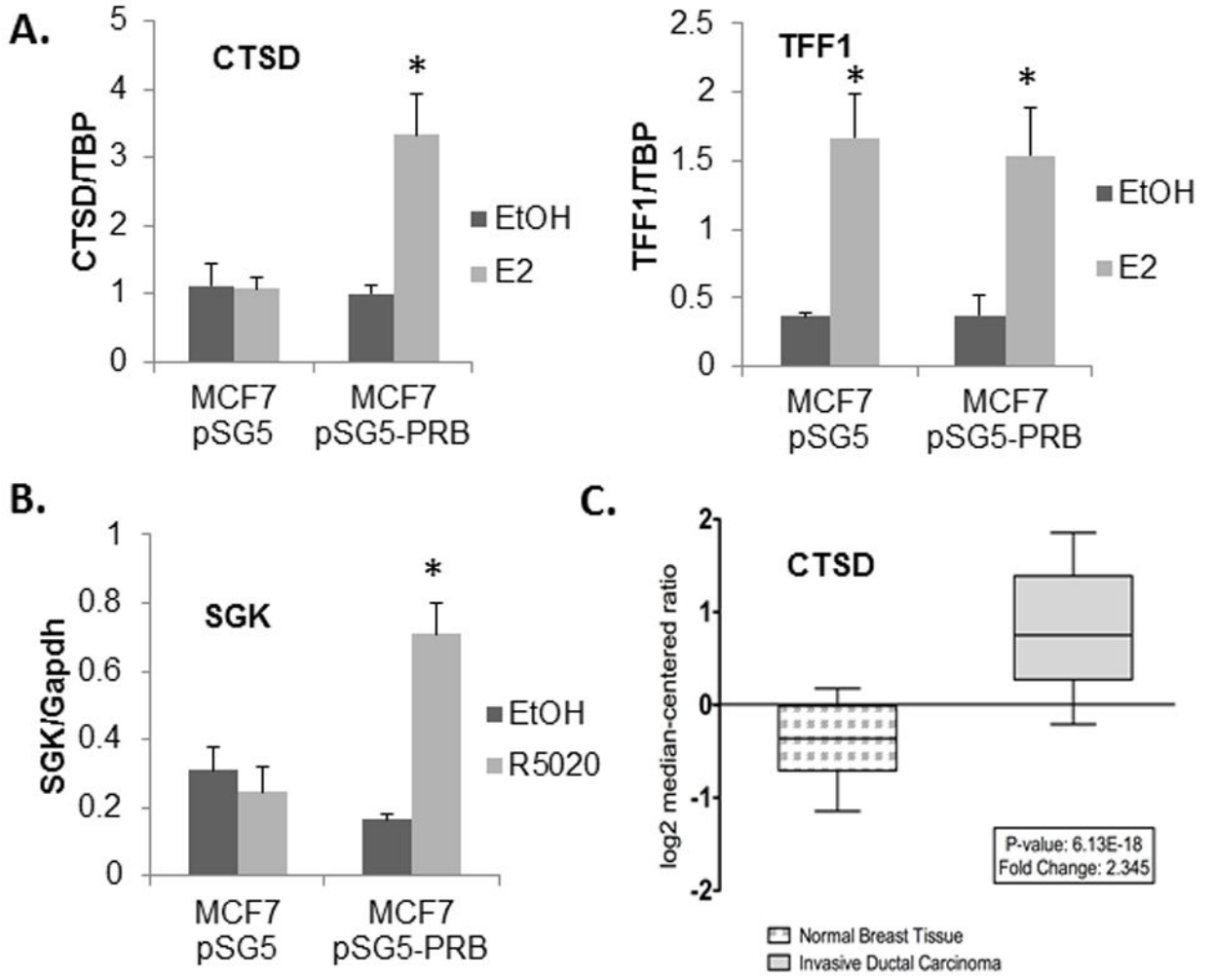
**Figure 1.**

PR-B expression increases breast cancer cell growth in response to estradiol. **(A)** Western blots of PR-B and ER- $\alpha$ . Unmodified MCF7 and MCF7 cells expressing pSG5 or pSG5-PR-B were treated with estradiol (1nM; E2) for 24 h. **(B)** Soft agar colony formation of MCF7 pSG5 or pSG5-PR-B cells grown in ethanol (EtOH), estradiol (E2), or synthetic progestin, R5020 (14 days). **(C)** Soft agar colony formation assay in cells expressing vector or PR-B. Cells were treated with EtOH, estradiol (1nM; E2), IGF1 (5nM), both estradiol and IGF1, or R5020 (10nM) for 21 days. **(D–E)** Soft agar colony formation of MCF7L and BT474 cells grown in ethanol, estradiol, or the anti-progestin onapristone (1 $\mu$ M; Ona) for 7 days ( $\pm$ SEM, \* $p$ <0.05). **(F)** Western blots of PR, ER, and Actin (loading control) from BT474 and MCF7-L cells were treated for 24 hours with estradiol (1nM) and/or onapristone (1 $\mu$ M).

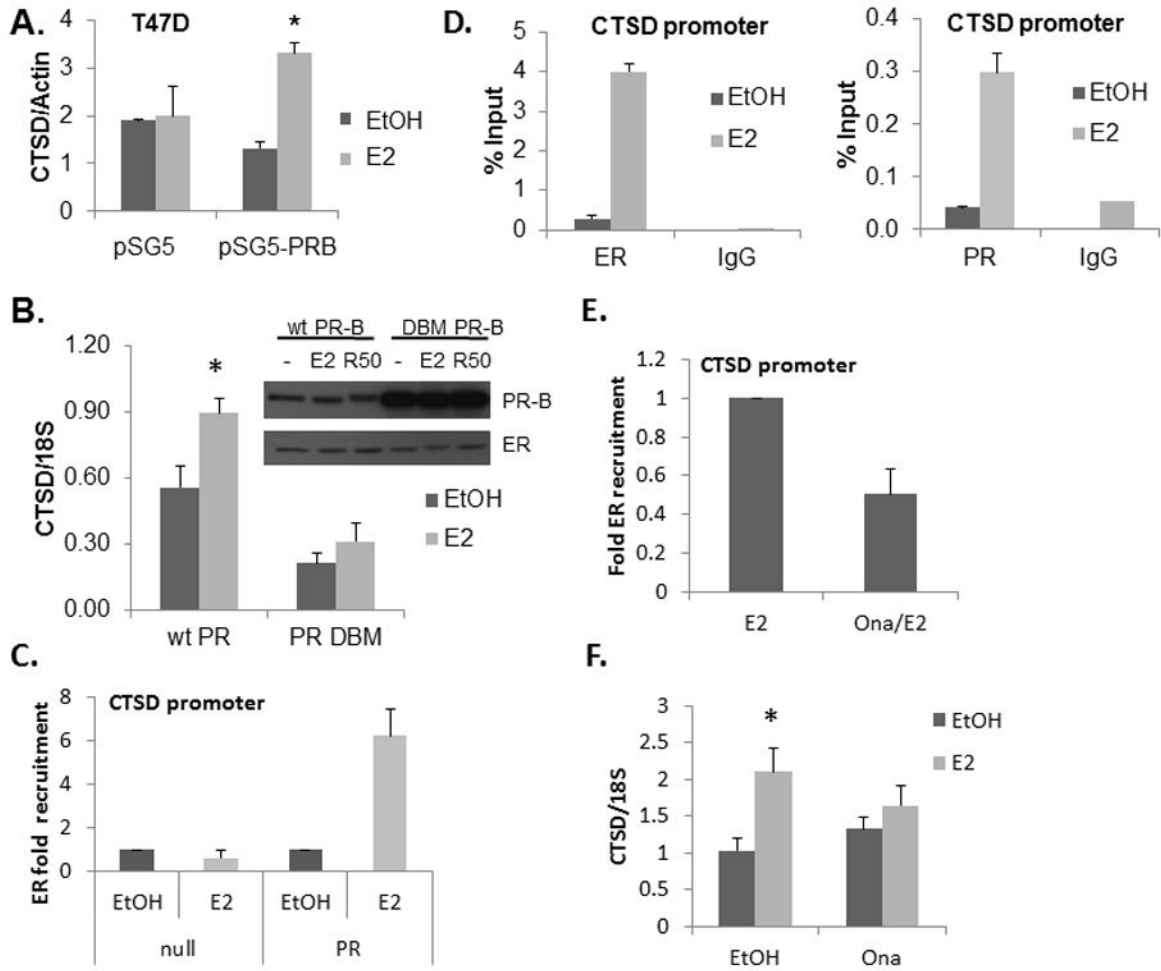


**Figure 2.**

Gene expression profiling identified novel PR-dependent, estradiol-regulated genes. (A) Heat map of normalized gene expression levels in MCF7 cells stably expressing vector or PR-B. Cells were treated with EtOH or estradiol (6h). Blue lines (to the right of the heat map) indicate upregulated genes and black lines (to the right of the heat map) indicate downregulated genes between lanes 2 and 4. The Venn diagram shows the number of genes upregulated in response to estradiol (>2 fold,  $P < 0.001$ ) in MCF7 cells expressing vector only or PR-B. (B, D) *SLC7A5* and *MAOA* mRNA expression. qRT-PCR of *SLC7A5* and *MAOA* in MCF7 cells expressing vector or PR-B and treated with estradiol (6h) (upper panels). Relative *SLC7A5* and *MAOA* mRNA expression in normal breast tissue and invasive breast carcinoma from the TCGA database (lower panels). (C, E) qRT-PCR to examine *WISP2*, *PTGES*, *LXN*, and *TMPRSS2* in MCF7 cells expressing vector or PR-B and treated with estrogen (6h) ( $\pm$ SD or SEM, \* $p < 0.05$ ).



**Figure 3.** PR-B expression is required for estradiol induction of *CTSD*. (A–B) qRT-PCR of *CTSD*, *TFF1*, and *SGK* in pSG5 or pSG5-PR-B MCF7 cells treated (6h) with the indicated hormone (ie, estradiol or R5020) ( $\pm$ SD, \* $p < 0.05$ ). (C) TCGA database analysis of relative *CTSD* mRNA expression in normal breast tissue and invasive breast carcinoma.



**Figure 4.**

PR is required for ER occupancy on the *CTSD* promoter. (A) qRT-PCR of *CTSD* in pSG5 or pSG5-PR-B T47D cells treated with ethanol or estradiol (24h). (B) qRT-PCR of *CTSD* mRNA in T47D cells stably expressing pSG5-PR-B or pSG5-DBM PR-B and treated with estradiol (24h). (Inset) Western blot of PR-B and ER in T47D cells stably expressing PR-B or DBM PR-B. ( $\pm$ SEM, \* $p < 0.05$ ). (C) pSG5-PR-B MCF7 cells treated with vehicle or estradiol (1h) were subjected to ChIP assays to examine ER recruitment to estradiol responsive regions in the *CTSD* distal promoter. Chromatin associated with ER immunocomplexes was subjected to qPCR, normalized to inputs, and expressed as estradiol-induced fold change over ethanol treatment. IgG was used as a negative control. Fold change values from two independent experimental repeats are presented. (D) MCF7 pSG5-PR-B cells were treated with ethanol or estradiol (40min). ChIP assays were performed using ER (right) or PR (left) antibodies compared to IgG negative controls. qPCR values normalized to input controls are shown, data is representative of 4–8 independent experiments. (E) ChIP assays to examine ER recruitment to the *CTSD* distal promoter were performed in MCF7L cells treated with ethanol or estradiol. Estradiol-induced ER recruitment was normalized to inputs and is expressed as fold blockade by onapristone. Values represent the average of three independent experimental repeats. (F) MCF7L cells

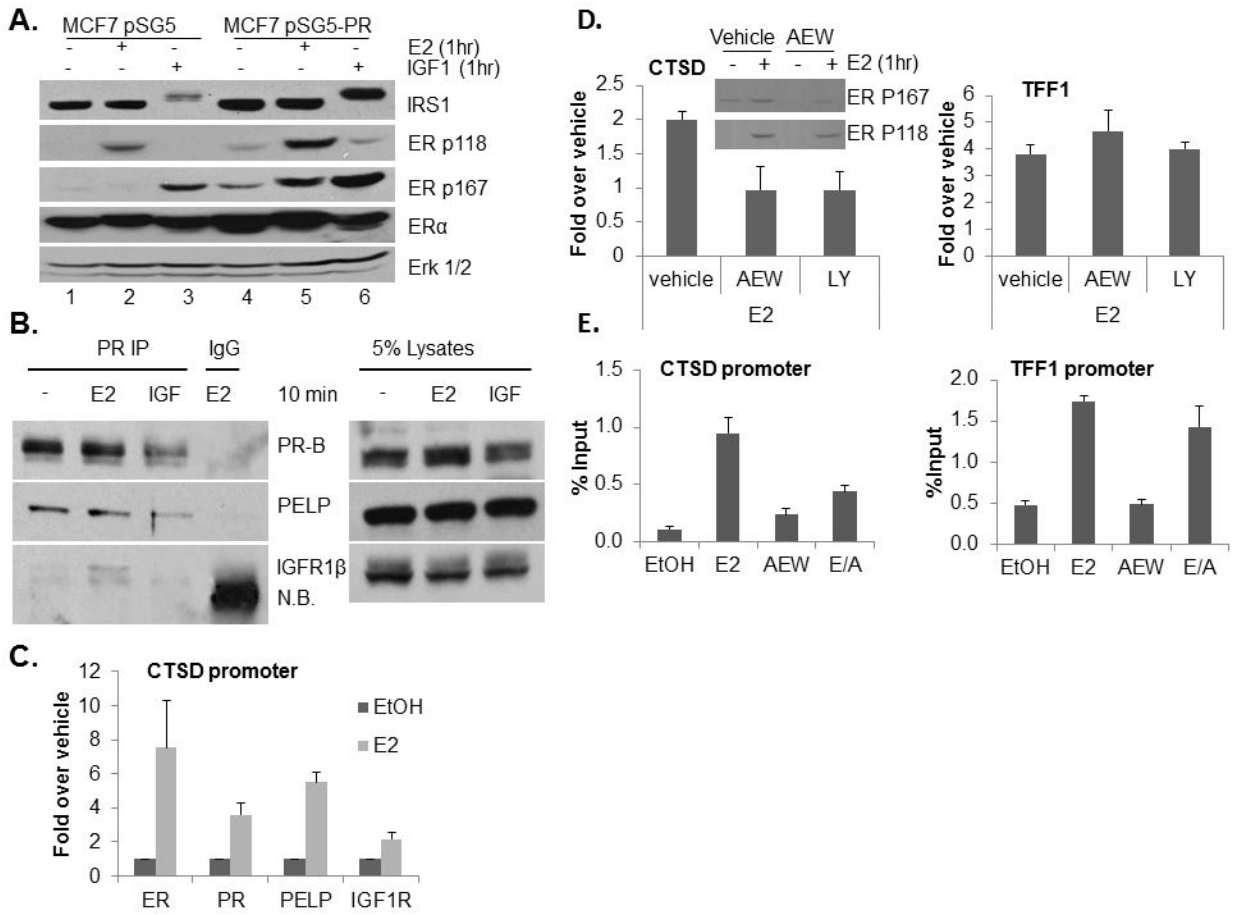
were treated with estradiol, onapristone, or both (24h). *CTSD* mRNA expression was evaluated using qRT-PCR and normalized to a housekeeper gene ( $\pm$ SEM, \* $p < 0.05$ ).

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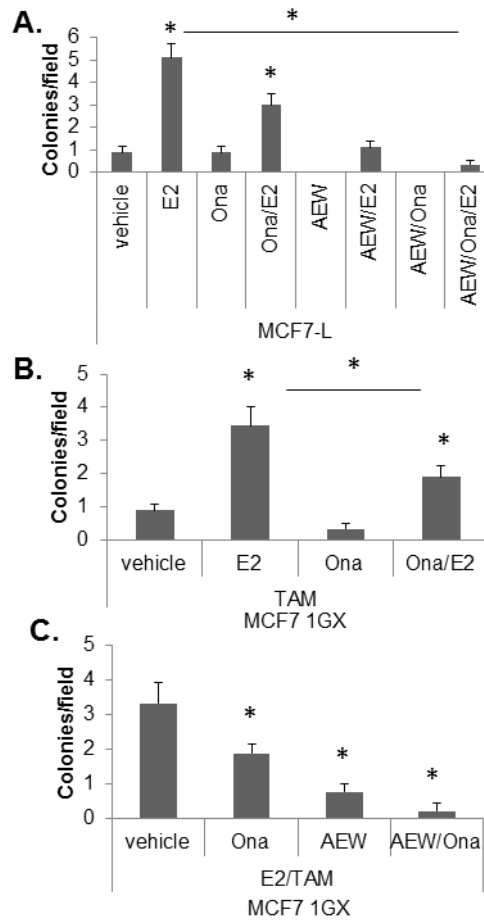
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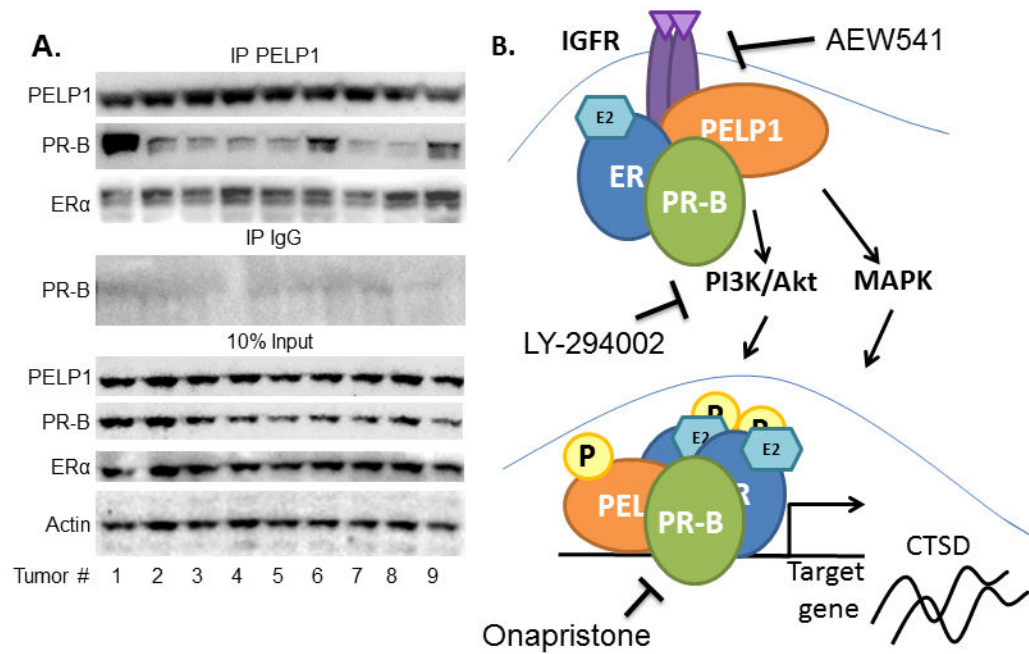
**Figure 5.**

PR-B coordinates ER signaling and transcriptional complexes. **(A)** Western blots of pSG5 or pSG5-PR MCF7 cells treated with estradiol or IGF1 (1h). **(B)** MCF7 (PR-null/ER-null subline C4-12) cells transiently transfected with ER and PR-B were starved and treated with estradiol or IGF-1 (10min). PR-containing complexes were isolated using PR-specific antibodies and protein G-agarose beads. Western blots were performed on immunocomplexes and whole cell lysates (N.B.; non-specific band). **(C)** ChIP assays were performed in MCF7 pSG5-PRB cells treated with vehicle or estradiol (40min). *CTSD* distal promoter chromatin from immunocomplexes isolated by using antibodies specific for ER, PR, PELP1, or IGF1R was evaluated with qRT-PCR and normalized to inputs. Values are expressed as estradiol-induced fold change over vehicle. Data represent the average of 3–5 independent experimental repeats. **(D)** MCF7 cells were treated with vehicle, AEW541, or LY-294002 and vehicle or estradiol (24h). Cells were subjected qRT-PCR to assess *CTSD* and *TFF1* mRNA levels normalized to housekeeper genes. Values are expressed as the average estradiol-induced fold change over vehicle. Data represent the average of 3 independent experimental repeats. (Inset) Western blots of MCF7L cells treated with vehicle or estradiol and vehicle or AEW541 (1h). **(E)** ChIP assay of ER recruitment to the *CTSD* distal and *TFF1* promoters in MCF7L cell treated with vehicle, estradiol, AEW541, or both estradiol and AEW541 for 40 min. Values, expressed as a percent of input controls, are representative of 3 independent experiments.



**Figure 6.**

PR antagonist blocks estradiol-stimulated growth and sensitizes breast cancer cells to tamoxifen. **(A)** Soft agar colony formation of MCF7L cells grown in ethanol, estradiol, AEW, or onapristone (7 days). **(B)** Soft agar colony formation of MCF7 1GX cells treated with tamoxifen and either vehicle, estradiol, onapristone, or both (7 days). **(C)** Soft agar colony formation of MCF7 1GX cells treated with tamoxifen and estradiol and either vehicle, onapristone, AEW, or both (7 days) ( $\pm$ SEM, \*  $p < 0.05$ ).



**Figure 7.** PR/ER/PELP1 containing complexes in human breast cancer samples. **(A)** Protein complexes were isolated from nine human tumor samples using PELP1-specific antibodies. Western blots were performed on immunocomplexes and input controls. Actin was used as a loading control. **(B)** PR-B is a component of a transcriptional complex where it coordinates estradiol-induced signaling to alter ER-mediated gene regulation.