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GENOMIC AGONISM AND PHENOTYPIC ANTAGONISM BETWEEN ESTROGEN AND PROGESTERONE RECEPTORS INFORM BREAST CANCER PROGNOSIS AND THERAPIES

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Abstract

Estrogen receptor alpha (ER) and progesterone receptor (PR) play critical roles in breast cancer, however the clinical value of PR is controversial and it is unclear how PR modulates estrogen signaling. This study reports that PR reprograms estrogen signaling as a genomic agonist and a phenotypic antagonist. In isolation, estrogen and progestin are genomic agonists as they regulate genes in similar directions but with differing intensities of gene expression and with varying functional annotation of the genes induced. Similarly, in isolation, progestin is a weak phenotypic agonist of estrogen action, however, in the presence of both hormones, progestin antagonizes estrogen-regulated processes and it behaves as a phenotypic antagonist of estrogen. This principle of genomic agonism and phenotypic antagonism rationalizes the good prognosis associated with PR-positivity of ER+ tumors. Importantly, when both the hormones are present, progestin dominates estrogen action such that the levels of transcriptomes, cellular processes and receptor recruitment observed with joint activation of ER and PR correlate with those observed with PR alone, but not ER alone. Despite the correlation, the transcriptomes on dual treatments are optimally different from individual treatments such that the ingenuity analyses predicted antagonism of oncogenic processes and indicated major tumor suppressive functions of concerted but not individual activity of these receptors. PR remodels nucleosomes to noncompetitively redirect ER genomic binding to distal enhancers and sites that link PR and ER/PR complexes. BRCA1 binding motifs were highly enriched at the binding sites of ER/PR complexes, thus implicating a potential role for BRCA1 in ER/PR crosstalk. Additionally, PR isoform-specific reprogramming of ER chromatin binding highlighted pro- and anti-tumorigenic effects of PR in breast cancer. The two PR isoforms PRA and PRB differentially remodel ER binding. PRA/PRB heterodimers expand ER chromatin binding events fourfold, while PRA primarily decreases ER binding by 75% and PRB redistributes ER binding.

PR is frequently lost in ER+ milieu due to hypermethylation and copy number loss of its gene locus and loss of PR independently modulates the prognostic value of ER. The well-known prognostic value of ER depends on the presence and activity of PR. While combination therapy with PR agonist and tamoxifen is reported to result in cytostatic inhibition of tumor growth, this study reports that joint therapies using PR antagonist lead to cytotoxic tumor regression. PR potentiates the value of ER since synergy between various PR-antagonists such as CDB4124, CDB4453 or EC313 and tamoxifen results in a remarkable regression of ER+/PR+ T47D xenografts, while individual therapies inhibit tumor growth without regression. In conclusion, the present study provides mechanistic explanations for how PR contributes to the clinical value of ER and it strongly implicates the use of PR both as a prognostic/predictive biomarker and as a therapeutic target in ER+/PR+ breast cancers.

Preface: Definition of Genomic Agonism and Phenotypic Antagonism

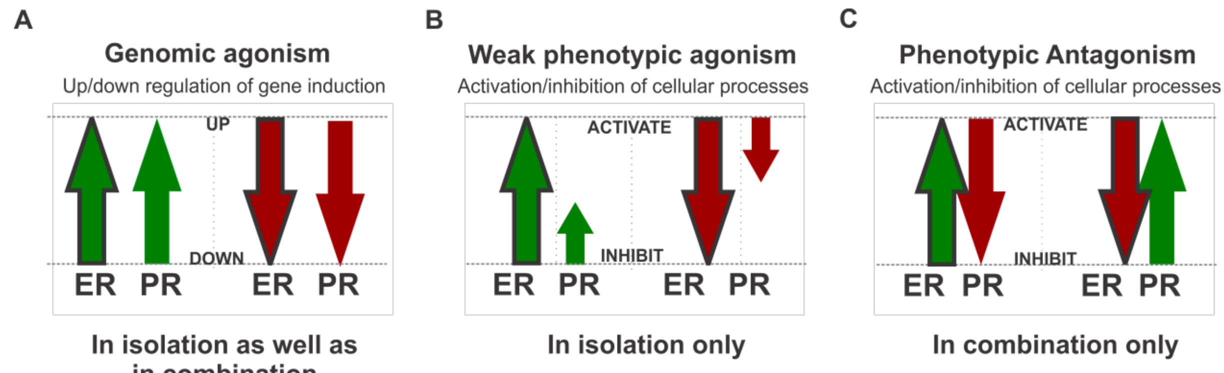


Figure I: Definition of genomic agonism and phenotypic antagonism. (A) Genomic Agonism: In isolation as well as in combination, activated ER and PR regulate expression of majority of genes in similar directions. Compared to vehicle treated cells, genes up (or down) regulated by ER are similarly up (or down) regulated by PR. **(B) Activity of ER and PR in isolation:** In isolation, progestin is a weak phenotypic agonist of estrogen action. Individually, estrogen and progestin activate most of the oncogenic pathways in similar directions but progestin lacks the degree of activation induced by estrogen. **(C) Phenotypic Antagonism:** When both ER and PR are active, PR opposes ER-regulated cellular processes and phenotypes. For example, while ER would activate a cellular pathway, presence of PR would inhibit that cellular pathway with a net outcome of opposing ER-regulated phenotypes.

The classic theory in biology is that transcription of genetic information (coding as well as non-coding parts of the genome) results in transcripts, which either directly or after translation to proteins, impacts cellular processes and phenotypes. In nuclear receptor biology, it is often assumed that positive or negative reinforcement of gene expression could translate to similar positive or negative reinforcement of the phenotype (*1*). However, it is well known that many intermediate processes, such as protein post-translation modifications, epigenetics, histone modifications, non-coding RNAs, activation of other signaling pathways, levels of transcripts and multiple other non-genomic pathways could contribute to the final biological phenotype (*2*–*4*). The intersection of these myriad processes, oftentimes in a combinatorial fashion, has the possibility of creating non-linear relationship between gene expression and phenotypes. For

example, the activity of progesterone receptor is known to be context dependent and it changes based on concentrations, presence of other hormones and other non-genomic signaling pathways (*5*). Similarly, growth factor signaling is known to significantly affect estrogen receptor's chromatin binding and biology (*6*, *7*). Hence, in addition to studying the genomic actions (regulation of gene expression) of a steroid receptor, it is equally important to understand the net biological consequence of activating that steroid receptor.

This study reports that estrogen and progestin have different biological consequences when considered in isolation versus when both the hormones are present together. In isolation, estrogen and progestin are genomic agonists as they regulate genes in similar directions but with differing intensities of gene expression and varying functional annotation of the genes induced **(Fig. IA)**. Similarly, in isolation, progestin is a weak phenotypic agonist of estrogen action because estrogen and progestin alone activate most of the oncogenic pathways in similar directions but progestin lacks the degree of activation induced by estrogen **(Fig. IB)**. However, in the presence of both hormones, progestin opposes estrogen-regulated cellular processes and progestin behaves as a phenotypic antagonist of estrogen with a net outcome of opposing ER-regulated phenotypes **(Fig. IC)**. This principle of genomic agonism and phenotypic antagonism helps to understand the good prognosis associated with PR-positivity of ER+ tumors.

Introduction

Estrogen and progesterone are key ovarian hormones that are essential for the maintenance of normal breast tissue and are critical in the regulation of its cancers (*2*, *3*, *8*). These hormones orchestrate unique gene expression programs by binding and activating estrogen receptor alpha (ER) (*2*) and progesterone receptor (PR) (*9*, *10*). Both ER and PR are members of nuclear receptor family that contain amine- and carboxy-terminal transactivation domains and DNA binding domains. In concert with other signaling pathways, activated ER and PR bind to their respective hormone response elements (AGGTCA for ER and AGAACA for PR) and recruit co-regulators to regulate gene expression. ER exists as two subtypes, namely ERα and ERβ, which are coded by genes in the regions 6q24-27 and 14q22–24 respectively. ERα is the better studied of the two ER isoforms and is the main focus of this study (*11*, *12*). PR is expressed as two major isoforms PRA and PRB, which are coded from two different transcription start sites of PR gene in the region 11q22–23. PRB is full length PR and PRA has a truncated amino terminal domain (*13–16*). In normal breast tissue, PRA and PRB are expressed at similar levels and loss of co-ordinate expression of PRA and PRB is an early event in breast cancer (*17–20*).

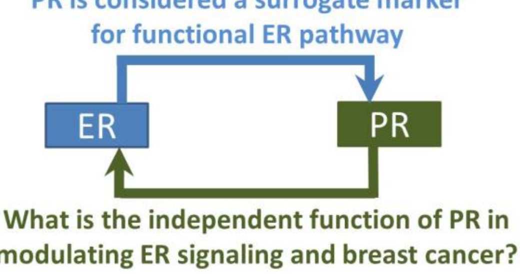


Figure II: Rationale to study ER/PR crosstalk. Although PR is an estrogen-induced gene, it is unclear whether and how PR modulates estrogen signaling and breast cancer.

ER and PR as well as Her2 are commonly used as biomarkers in breast cancer (*21*). PR is an estrogen-induced gene and it is considered a surrogate marker for a functional ER pathway. While it is

widely agreed that ER is an established biomarker in breast cancer, there is a longstanding controversy about the independent function of PR in breast cancer and how PR influences estrogen signaling (*3*) (**Fig II**). Interestingly, despite the controversy over the clinical value of PR, breast tumors are routinely evaluated for ER and PR expression, although tumor ER status is given priority. These clinical decisions largely stem from relatively weak PR staining observed in tumors (*22–25*), routine use of anti-ER but not anti-PR targeting therapies (*3*, *26*), insufficient understanding of the intersection of ER and PR signaling and lack of large enough patient cohorts to conclusively study the clinical value of PR (*21*, *22*, *25*, *27–36*). Mechanistic understanding of ER/PR crosstalk is needed to optimize breast cancer prognostic markers.

Similarly, while ER-targeted therapies are widely used for breast cancer treatments, the clinical efficacy of PR-targeted therapies alone or in combination with ER-targeted therapies is not well appreciated (*10*, *26*). Furthermore, millions of women routinely use estrogen and synthetic progestin formulations as hormone replacement therapies or as contraceptives and it is still unclear why the addition of progestin to these estrogen-only formulations increases breast cancer risk (*37–42*). The major hindrance to the study of PR-targeting therapies is that approved anti-PR agents, such as mifepristone, onapristone and ORG2058, also target GR and/or AR (*43*, *44*). Various in-vitro, mouse models and human xenograft studies have shown additive effects between anti-PR drugs and tamoxifen, however it is difficult to attribute this synergy to PR activity due to major antiglucorticoid activities of these drugs (*26*, *45–47*). Lack of molecular-level details of ER/PR crosstalk hampers the efforts to optimize ER and PR targeting therapies in breast cancer and impedes the development of low-risk hormonal replacement therapies.

Early efforts to understand the interaction between ER and PR were restricted to promoter-proximal regions for a limited number of genes engineered in artificial vector constructs (*48–54*). PR is reported to interact with ER in the cytoplasm and this interaction is critical for activation of c-src/ERK pathways by progestin (*55*, *56*). Another study found that unliganded PRB forms a complex with ER and PELP1 protein to enhance estrogen responsiveness in breast cancer (*57*). Further studies with next-generation technologies are needed since these early works had limited scope due to multiple reasons. For example, earlier techniques could not capture real chromatin dynamics, artificial vectors are not representative of real genomic loci and earlier studies lacked the global understanding of ER/PR crosstalk (*58*, *59*). Moreover, the genomic recruitment of ER/PR complexes and the functional impact of ER/PR complexes on the modulation of estrogen signaling and breast cancer have not been studied. These gaps in knowledge impede the optimization of breast cancer biomarkers, hinder the use of PR-targeted therapies in combination with ER-targeted therapies, and hamper development of low-risk hormonal replacement therapies and contraceptives.

In section I we study the genome-wide intersection of estrogen and progesterone signaling in twelve primary ER+ patient tumors and various PR-positive (multiple T47Ds, ZR75 cells) and PR-deficient (PR-deficient T47D and MCF7 cells) models for breast cancer. We found that estrogen and progestin induced genes expression in similar directions, suggesting genomic agonism between these hormones. While these sex hormones independently regulated genes in similar directions, there are differences in the intensities of gene expression and the functional annotation of the genes induced. Importantly, when both hormones were present, progestin elicited a shift of estrogen action so that the progestin effects were dominant. Pathway analyses revealed that individually, estrogen and progestin regulated oncogenic pathways in similar

directions, suggesting phenotypic agonism between these hormones in isolation. However, in the presence of both the hormones, progestin functionally antagonized estrogen-regulated cellular processes and oncogenic phenotypes in breast cancer, suggesting phenotypic antagonism on dual hormonal treatments. Interestingly, PR reprogrammed ER-regulated levels of transcriptomes to resemble its own and the cellular pathways enriched on joint hormonal treatments correlated with progestin-only treatments. Despite the correlation, the transcriptomes on dual treatments were optimally different from individual treatments such that the ingenuity analyses predicted antagonism of oncogenic processes and indicated major tumor suppressive functions of concerted but not individual activity of these receptors. These findings are particularly significant since estrogen signaling is considered the key pathway in breast cancer and the principle of genomic agonism and phenotypic antagonism might help to understand good prognosis associated with PR positivity of ER+ tumors.

Because genomic binding of ER is critical to its function as a signaling molecule and transcriptional modulator, in section II we investigate how PR modulates ER chromatin binding. PR positivity in human tumors was associated with highly consistent ER binding, suggesting that PR influences ER genomic recruitment. In accord with these results, while activated PRA/PRB heterodimers expanded ER chromatin binding events four-fold, PRA decreased ER binding events by 75% and PRB alone primarily redistributed ER genomic recruitment. These results suggest that PR modulates ER chromatin binding in an isoform-specific manner. Interestingly, PR redirected ER to sites that correlated with genomic binding of PR and complexes of ER and PR. Progestin-mediated redirection of ER binding was significantly diminished in PR-deficient cells, indicating a critical role for PR in remodeling ER binding.

Given that PR clearly modulates ER binding and activity, section III provides the mechanistic insights into how PR orchestrates ER binding with the objective that these details might help to optimize breast cancer prognosis and treatments. Immunoprecipitation of ER pulled down PR suggesting that these receptors interact non-competitively in breast cancer. ER/PR complexes were recruited to the genome and these receptors also interacted via long-distance chromatin looping between their hormone response elements. ER/PR complexes mostly bound to H3K4me1+/H3K27ac1+ enhancers and importantly, to the enhancers with highest H3K4me1+ signal intensity. Additionally, there was a significant overlap between ER binding and DNase hypersensitive regions created by activated PR, suggesting that PR influenced chromatin accessibility for ER binding. In agreement, depletion of either of the known chromatin remodelers FOXA1 or NF1C did not significantly impact ER/PR functional crosstalk and suggested that PR could have pioneering functions in chromatin remodeling for ER binding.

Section IV highlights the prognostic value of PR-mediated reprogramming of estrogen signaling. Although PR is an estrogen-induced gene, an analysis of METABRIC and TCGA cohorts revealed that PR is frequently lost in ER+ tumors due to copy number loss and hypermethylation of the PR gene locus. The independent loss of PR in patient tumors alters estrogen signaling and tumor biology because activated PR is required for ER-regulated genes to differentiate patient survival. In support of these observations, ER staining was not able to differentiate survival outcomes within patients with PR-negative tumors, whereas tumor ER status significantly correlated with positive survival outcomes within the PR-positive patient cohort.

Section V describes that the PR-mediated reprogramming of estrogen signaling is therapeutically relevant and that potent PR modulators, especially in combination with

antiestrogens lead to regression of breast cancer. We study the efficacy of three novel and highly selective PR modulators (SPRMs)/antagonists CDB4124, CDB4453 or EC313 in treating ER+/PR+ T47D xenografts. Although ER is the major therapeutic target in ER+/PR+ breast cancers, all three SPRMs effectively inhibited estrogen-driven growth of ER+/PR+ T47D xenografts. While individual drug treatments with tamoxifen or individual SPRMs inhibited tumor growth, their combined treatment resulted in cytotoxic regression of tumor volume. Importantly, although combination therapy with PR agonist and tamoxifen is reported to result in cytostatic inhibition of tumor growth, this study reports that joint therapies using PR antagonist lead to cytotoxic tumor regression. Currently studies are ongoing to compare how agonist or antagonist activated PR reprograms estrogen signaling and response to ER-targeting breast cancer therapies.

In summary, our research describes extensive overlap between estrogen and progesterone signaling, and strongly implicates the use of PR both as a prognostic/predictive biomarker and as a therapeutic target in ER+/PR+ breast cancers.

I. Genomic agonism and phenotypic antagonism between ER and PR in breast cancer

Background

Ovarian hormones estrogen and progesterone orchestrate menstrual cycles, these hormones are essential for reproduction and they are critical in regulation of hormone responsive cancers in women (*3*). The relationship between these two hormonal receptors is temporal in pre-menopausal women; the rise in circulating estrogen at ovulation stimulates ER-mediated upregulation of PR in reproductive tissues, rendering them responsive to circulating progesterone secreted by the corpus luteum. After menopause, when the ovaries cease production of estrogen and progesterone, reproductive tissues such as the breast are mainly exposed to locally produced hormones, particularly estrogen synthesized by breast adipose tissue (*3*).

Estrogen signaling is the first hormone-driven oncogenic pathway identified in breast cancer and about eighty percent of all breast tumors are positive for ER (*2*, *60*). ER+ breast cancers respond reasonably well to ER-targeting therapies and antiestrogens are the current mainstay of hormonal therapies in breast cancer. However, not all patients with ER+ tumors respond equally well to antiestrogens and a subset of patients that do initially respond, eventually develop resistant tumors that are still positive for ER (*2*, *61*). A classical theory was proposed that the ER+ tumors that do not respond to antiestrogens could be independent of estrogen signaling. Many downstream targets of estrogen signaling, such as TFF1, PDzK1 and PR, were investigated as a surrogate for functional ER pathway and response of ER+ tumors to tamoxifen. Among these surrogates, PR is a widely used biomarker for functional estrogen signaling, mainly because during a menstrual cycle, a progesterone wave is downstream of estrogen surge and PR is a classical estrogen-induced gene (*27–32*, *34*). Additionally, compared

to other surrogates, the presence of PR was found to have greater predictive power for patient survival and response to antiestrogens.

For several decades, while estrogen signaling has been the primary focus of breast cancer research, evidence emerged that PR signaling might also be a key oncogenic signaling pathway in breast cancer. For example, activation of PR expands stem cells and drug-resistant populations, PR can stimulate the proliferation of mammary epithelial cells and PR selects for receptor-negative cells (*62–65*). Importantly, accumulating clinical data have suggested that PR is more than a passenger of ER activity and PR could be a driver of ER activity. The large ATAC trial demonstrated that ER+ patients with PR- but not PR+ tumors responded to estrogen deprivation, thus demonstrating that PR is not required for functional estrogen signaling (*66*, *67*). Also the addition of PR ligand medroxy progesterone acetate to estrogen-only hormone replacement therapies increases breast cancer risk suggesting the PR is an active player in breast cancer (*37–42*). Lastly, multiple clinical trials produced controversial results about the independent prognostic value of PR. However, a few of them suggested that PR can have independent clinical value over ER (*27–32*, *34*). Although these data suggested that critical crosstalk occurs between ER and PR, the mechanistic details of these interactions are not well described and these gaps in knowledge impede the optimization of breast cancer biomarkers and therapies.

Model systems: Twelve primary ER+ tumors, T47D-derived cells, ZR75 and MCF7 cells

Estradiol (E2) and the synthetic progestin R5020 (promegestone) are commonly used for ER and PR ligands for cell and animal studies (*2*, *68*). At 10nM concentrations, estradiol and R5020 have high specificity for ER and PR respectively (*68*, *69*). In females ovarian

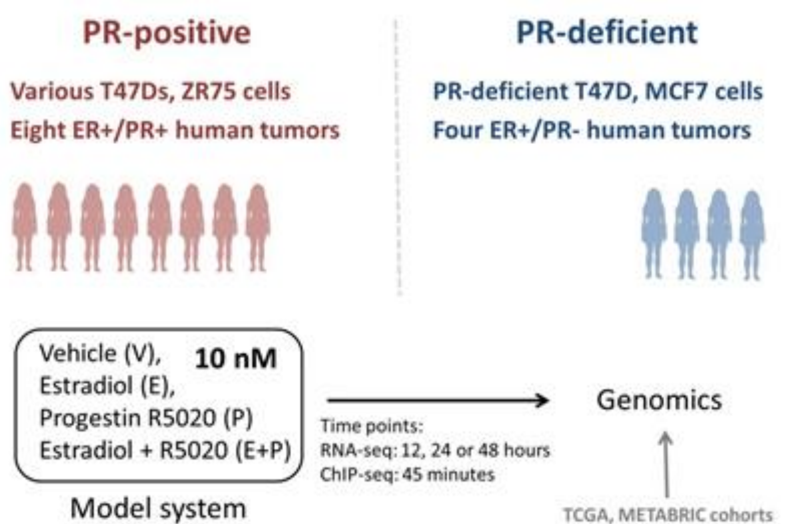


Figure 1.1: Study design and model systems used to investigate ER/PR crosstalk. ER/PR crosstalk was studied in multiple ER+/PR-positive (eight patient tumors, various T47Ds and ZR75 cells) and ER+/PR-deficient (four patient tumors, T47D and MCF7 cells) models. Each of the model system was treated with vehicle (V), estradiol (E), progestin (R5020) or both the hormones together. RNA-seq was performed 12, 24 or 48 hours and ChIP-seq was performed 45 minutes post treatments. Genomics was integrated with TCGA and METABRIC cohorts for clinical analyses.

hormones estrogen and progesterone orchestrate monthly menstrual cycles, which are vital for maintenance of female physiology and reproductive functions. Both estradiol and progesterone are present in a women's body and breast cancers grow in an environment with both the hormones in pre-menopausal women (*3*). In post-menopausal women ovaries cease production of these hormones, however there is local production of estrogen and progesterone and these hormonal signaling pathways could drive growth of ER+/PR+ cancers in post- menopausal women..

Hence, we studied ER/PR functional crosstalk in various model systems treated with vehicle, estrogen (estradiol), progestin (R5020) or concomitantly with both the hormones (**Fig. 1.1**). Solid tumors like those of the breast, are notoriously heterogeneous and the very poor rate (about 5%) of translation from basic science to clinical application in the solid tumor field is often attributed to study models that do not recapitulate this complexity (*70*). For this reason we studied the functional relationship between ER and PR in sliced sections of eight ER+/PR+ and

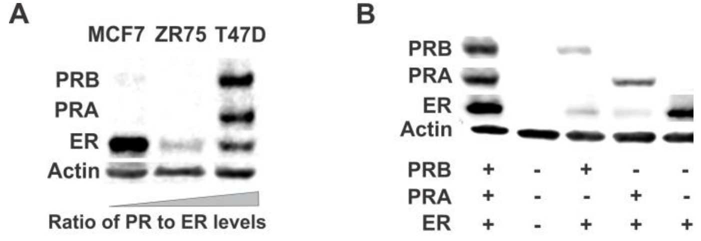


Figure 1.2: ER and PR protein levels in various cell models used in the study. (A) ER and PR levels in T47D, ZR75 and MCF7 cells. **(B)** ER and PR levels in T47D cells and its derived sublines that lack either PR or both ER and PR.

four ER+/PR- independent human breast tumors treated *ex-vivo* with different treatments. This novel ex-vivo procedure was initiated within an hour of surgery to retain innate tissue architecture and viability (*71*). Findings from human tumors were further investigated in widely used cell models of ER+/PR+ breast cancers, namely T47D, MCF7 and ZR75 cells (**Fig. 1.2A**) (*72*, *73*). Compared to T47D cells, MCF7 and ZR75 cells express much lower levels of PR protein. However, PR protein expression can be induced on estradiol treatment in all three cell lines (*74–76*). While ER levels in ZR75 and T47D cells are comparable, MCF7 cells express significantly higher levels of ER. The ratio of PR to ER levels is highest in T47D, followed by ZR75 and then MCF7. For this reason we view MCF7 as a PR-deficient breast cancer cell model. These three cell lines together provide a system to study ER/PR interactions with different relative levels of ER and PR. In addition, investigations in three different cell models help to rule out any cell line specific effects on our observations. Among these three cell models, T47D cells were chosen for in-depth research of ER/PR interactions because compared to MCF7 and ZR75 cells, T47D cells have abundant endogenous expression of both ER and PR proteins. In addition, various T47D sublines have been derived that lack either PRA, PRB, both the isoforms of PR or ER along with PR (**Fig. 1.2B**) (*77–79*). These T47D sublines along with the parent T47D cells provide an opportunity to study PR isoform specific ER/PR crosstalk.

Results

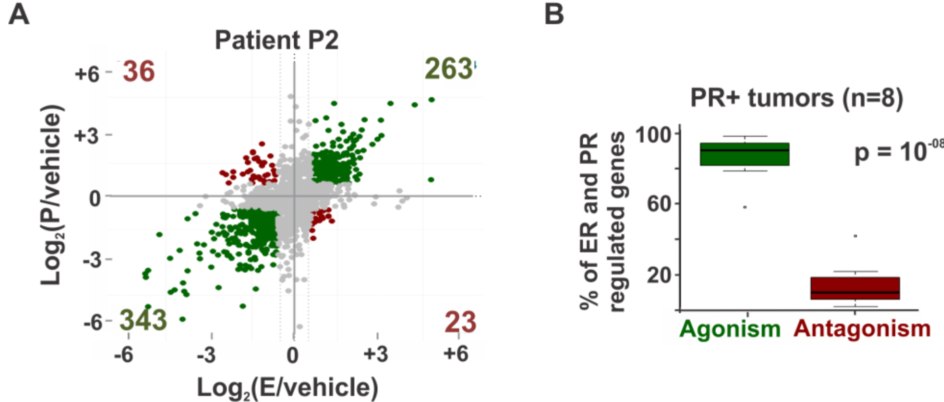


Figure 1.3: ER and PR regulate genes in similar directions and progestin is a genomic agonist of estrogen. **(A)** PR is an agonist of ER-regulated gene expression. Axes denote log fold change of gene expression in representative ER+/PR+ tumors in response to estrogen (E) and progestin R5020 (P) treatment relative to vehicle (V). **(B)** Box plot depicts the percentage of all ER-and PR-regulated genes for which progestin is an agonist or antagonist of E2-regulated gene expression (n = 8 tumors).

Genomic agonism: ER and PR regulate genes in similar directions but with differing intensities of expression and functional annotation of genes induced.

In ER+/PR+ patient tumors, T47D and ZR75 cell models, approximately eighty five percent of genes up- (or down-) regulated by estrogen alone were similarly up- (or down-) regulated by progestin alone, suggesting genomic agonism between these hormones (**Figs. 1.3A, 1.3B and 1.4A - I**). These results were noted in seven out of eight ER+/PR+ tumors (P1 – P3 and P5 - P8) and in all of the ER+/PR+ breast cancer cell models. In T47D cells, similar results were obtained after stringent analyses were done to correlate gene expression with receptor binding to only include genes that have ER and PR binding sites within 100kb of their promoters (**Figs. 1.4I**). While these sex hormones independently regulated genes in similar directions, there were differences in the intensities of gene expression and the functional annotation of the genes induced.

PR reprograms ER-regulated patterns of transcriptomes to correlate with its own

Importantly, when both hormones were present, progestin elicited a shift of estrogen action so that the progestin effects were dominant. In all ER+/PR+ cell models, the levels of transcriptomes on joint activation of ER and PR were similar to the PR-regulated transcriptomes, but were significantly different from those controlled by ER alone (**Figs. 1.5A and 1.6A**). Remarkably, despite the intrinsic heterogeneity between patients and also within slices of the same patient tumor, PR significantly modulated ER-regulated transcriptomes for

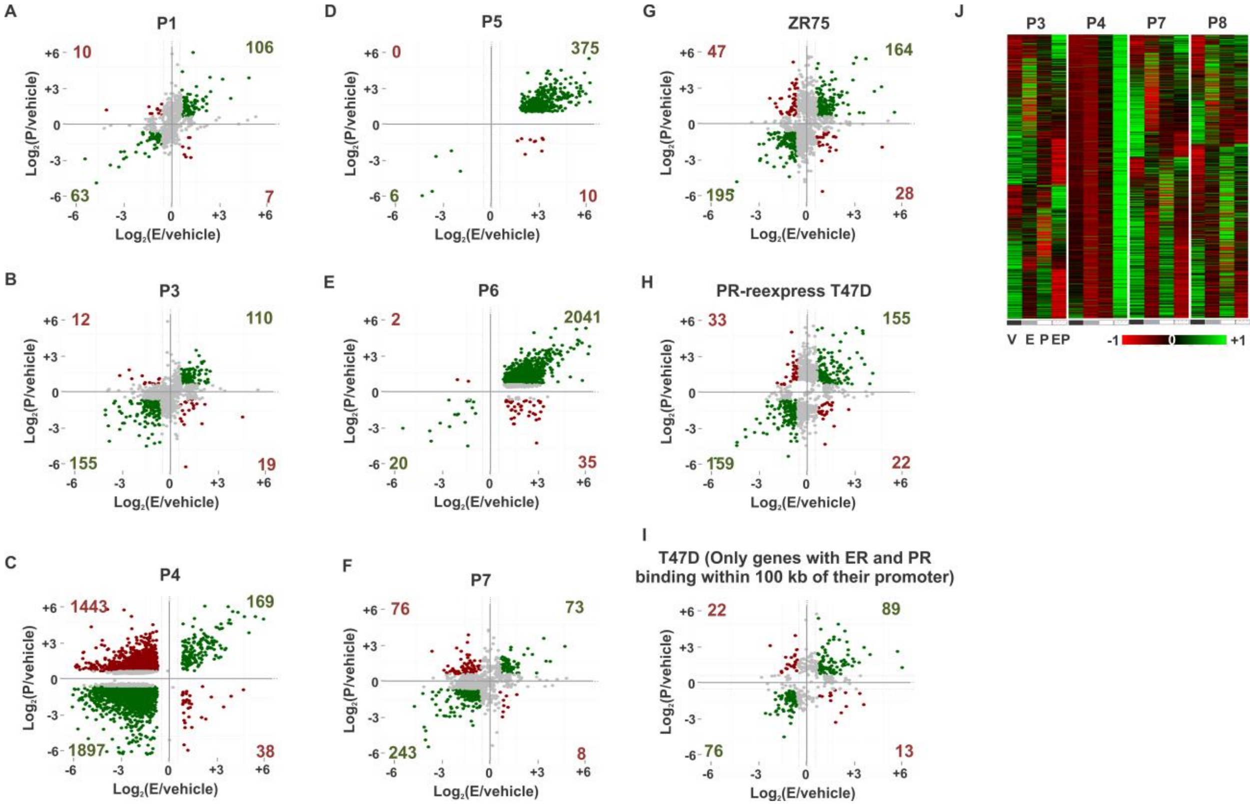


Figure 1.4: Progestin is a genomic agonist of estrogen in ER+/PR+ patient tumors. (A-J) PR is an agonist of ER-regulated gene expression. Axes denote log fold change of gene expression in ER+/PR+ patient tumors **(A - G)** and T47D, ZR75 and PR-rexpress T47D cells **(H - J)** in response to E and P treatment relative to vehicle. **(K)** Expression of E- and P-regulated genes in four ER+/PR+ patient tumors treated *ex-vivo* with vehicle (V), estrogen (E), progestin R5020 (P) or concomitantly with both hormones (E+P). All heatmaps are row-normalized and include union of ER- and PR-regulated genes.

majority of the primary tumors (P1 to P6) (**Figs. 1.4J and 1.5A**). Furthermore, in support of the observations in cell models, PR predominantly reprogrammed ER-regulated levels of transcriptomes to correlate with PR-regulated transcriptomes in a subset of tumor samples (P1, P3 and P4) (**Figs. 1.5A and 1.6A**). Despite the correlation, the transcriptomes on dual treatments were optimally different from individual treatments such that the ingenuity analyses predicted antagonism of oncogenic processes and indicated major tumor suppressive functions of concerted but not individual activity of these receptors (**Fig. 1.7**). These findings are significant since estrogen signaling is considered the key signaling pathway in breast cancer. Since estrogen-regulated gene expression is a known favorable prognostic marker (14), these findings of genomic agonism between ER and PR highlights a longstanding controversy of good prognosis associated with PR-positivity of ER+ tumors.

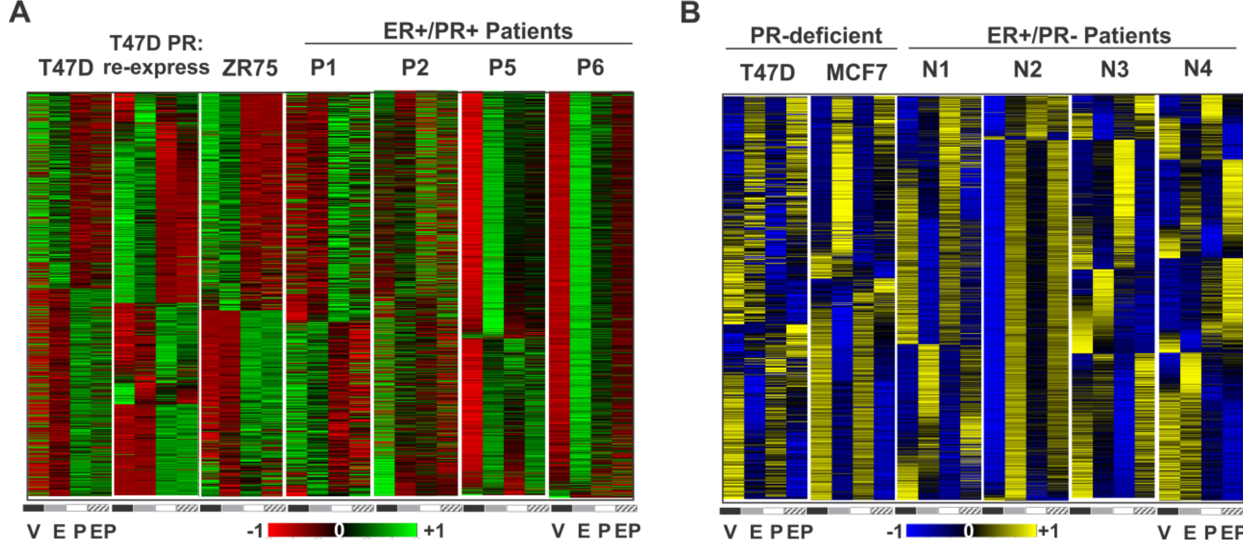


Figure 1.5: Heatmaps - PR reprograms ER-regulated levels of transcriptomes to resemble its own. **(A, B)** Expression of estrogen- and progestin-regulated genes in **(A)** PR+ milieu (four ER+/PR+ human tumors, T47D, ZR75 and PR re-express T47D cells) and **(B)** PR-deficient milieu (four ER+/PR- tumors, PR-deficient T47D and MCF7 cells) treated *ex-vivo* with V, E, P or with both hormones (EP). All heatmaps are row-normalized and include the union of ER- and PR-regulated genes.

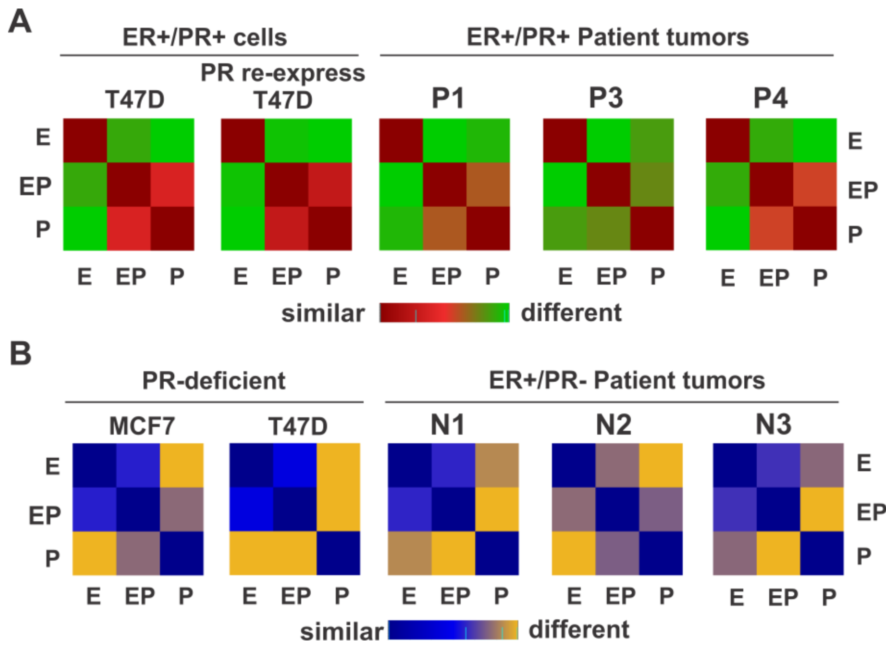


Figure 1.6: Similarity Matrices - PR reprograms ER-regulated levels of transcriptomes to resemble its own.(A - B) Similarity matrices represent correlation between E-, P- and EP-regulated levels transcriptomes in **(A)** ER+/PR+ milieus (three ER+/PR+ human tumors, T47D and PR re-express T47D cells) and **(B)** ER+/PR- milieus (three ER+/PR- tumors, PR-deficient T47D and MCF7 cells).

Phenotypic antagonism: In isolation, PR is a phenotypic agonist but when both the hormones are present, PR antagonizes estrogen-regulated cellular processes

In addition to genomic agonism with ER-regulated transcription, when both the hormones were present, activated PR reprogrammed the ER-regulated transcriptome to be enriched for antagonistic processes (**Fig. 1.7**). While estrogen regulated pro-proliferation and pro-metastatic processes, progestin when combined with estrogen, opposed these cellular pathways, suggesting phenotypic antagonism in the presence of both the hormones (**Figs. 1.7 and 1.8A**). These results further rationalize the favorable prognosis associated with the presence of PR in ER+ tumors. Interestingly, progestin in isolation behaved as a weak phenotypic agonist, thus underscoring the complexities of ER/PR crosstalk. In support of these pathway predictions, progestin in isolation had low proliferation potential, and on dual hormone

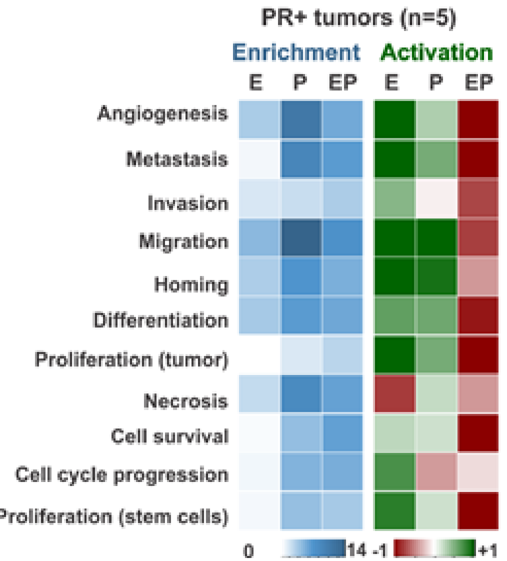


Figure 1.7: Pathway analyses of ER- and PR-regulated transcriptomes. Enrichment (p values) and direction of activation of functional processes by E-, P- and EP-regulated transcriptomes in five human tumors treated for 24 hours.

treatment, progestin abrogated estrogen-induced proliferation in ER+/PR+ tumors and multiple cell models (**Figs. 1.8B and 1.9**). Progestin also abrogated estrogen induced cell migration and invasion such that the final phenotype on joint activation of these receptors was similar to that observed on progestin-only treatment (**Figs. 1.8C and 1.8D**). These results suggest that while progestin is a genomic agonist of ER-regulated gene expression, it reprograms estrogen signaling as a phenotypic antagonist of estrogen-regulated cellular processes in ER+/PR+ breast cancer.

Eighty percent of ER+ breast cancers are also positive for PR (4) and due to the ubiquitous use of antiestrogens in treating PR+ breast cancers (15), it is important to understand whether PR is necessary and sufficient for the functional impact of progestin on estrogen signaling. In contrast to PR-positive tumors, progestin did not significantly modulate ER-regulated levels of transcriptomes in four ER+/PR- tumors, ER+/PR-deficient T47D and MCF7 cells (**Figs. 1.5B and 1.6B**). Stable re-expression of PR in PR-deficient T47D cells rescued these dominant effects of progestin, suggesting that PR is necessary and sufficient to reprogram gene expression controlled by ER (**Figs. 1.5A and 1.6A**). Importantly this modulation of ER-regulated

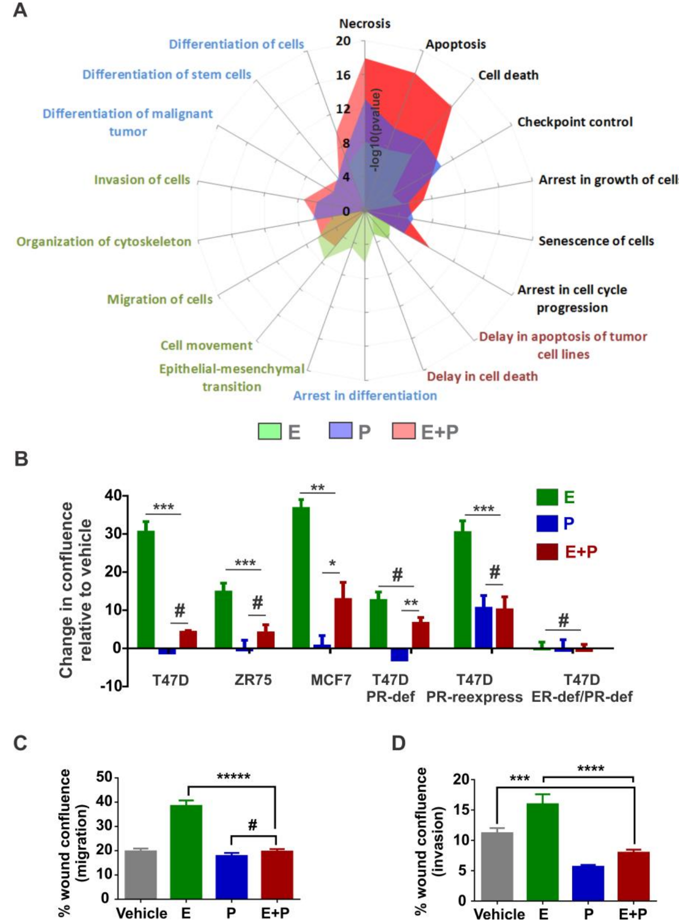


Figure 1.8: Effects of P on E-regulated proliferation and invasion. (A) Pathways enriched in E, P and E+P regulated transcriptomes. Negative log10 of p value is plotted. **(B)** Cell confluence of various ER+/PR+ and ER+/PR-deficient cell models treated with E, P or E+P. **(C)** Migration and **(D)** matrigel invasion of T47D cells in response to treatment with E, P or E+P.

gene expression was observed as early as two hours post progestin treatment, indicating that PR directly affected the transcriptional activities of ER (**Figs. 1.10A and 1.10D**).

In addition to these initial effects of PR on estrogen signaling, PR also reprogrammed ER-regulated transcriptomes following relatively long-term hormonal treatments of 12, 24 or 48 hours (**Figs. 1.5A and 1.6A**). These observed progestin effects were specific to PR since progestin did not induce gene expression in ER-low/PR-deficient T47D cells that are positive for glucocorticoid receptor (**Figs. 1.10B and 1.10C**). All next-generation sequencing results were validated with real time PCR (**Figs. 1.10E – 1.10G**).

In agreement with these observations, the effects of progestin on estrogen-induced cell proliferation were insignificant in ER+/PR- tumors (**Fig. 1.9**) and in PR-deficient T47D cells (**Fig. 1.8B**). However, the effects of progestin were rescued in PR-deficient T47D cells upon

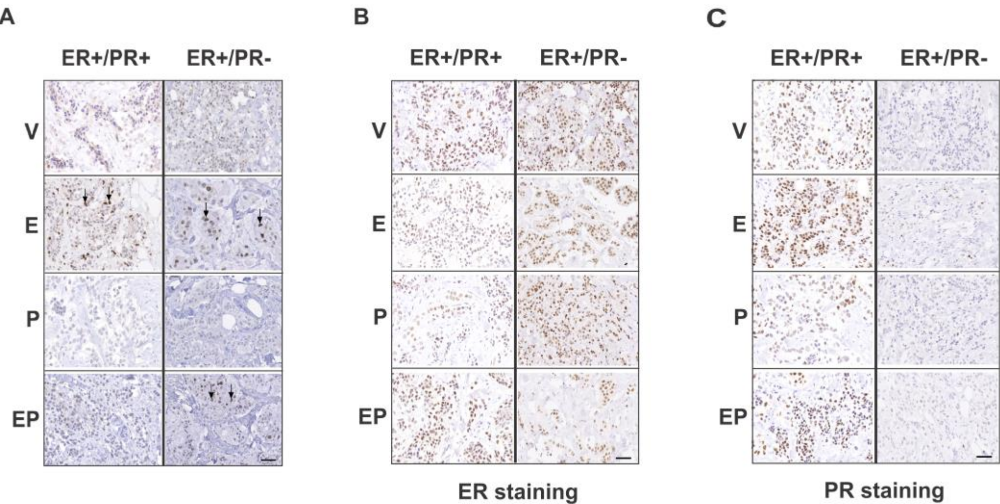


Figure 1.9: Effects of progestin on estrogen-induced proliferation of ER+/PR+ and ER+/PR- human tumors. (A) Changes in proliferation as measured by Ki67 staining of PR+ and PR- patient tumor explants treated ex-vivo with V, E, P or EP. **(B)** Anti-ER and **(C)** anti-PR immunohistochemistry to determine ER and PR levels (scale = 200 um).

stable re-expression of PR (**Fig. 1.8B**). Also, in accord with these results, PR expressed in MCF7 cells under estrogenic conditions was sufficient to abrogate estrogen-induced cell proliferation in response to progestin (**Fig. 1.8B**). These findings illustrate that PR is capable of reprogramming estrogen-induced gene expression, cellular processes and cell proliferation in primary human tumors and various cell models.

Discussion

The functional role of PR in modulating estrogen signaling and breast cancer biology is controversial. To address this question, human tumor tissues (and multiple cell models) were exposed *ex-vivo* (and *in-vitro*) to different combinations of estrogen and progestin. These experiments revealed that in isolation, both of these hormones regulate genes in similar directions indicating genomic agonism between them. Similarly in isolation, progestin is a weak phenotypic agonist, however, when both the hormones are present, progestin antagonizes

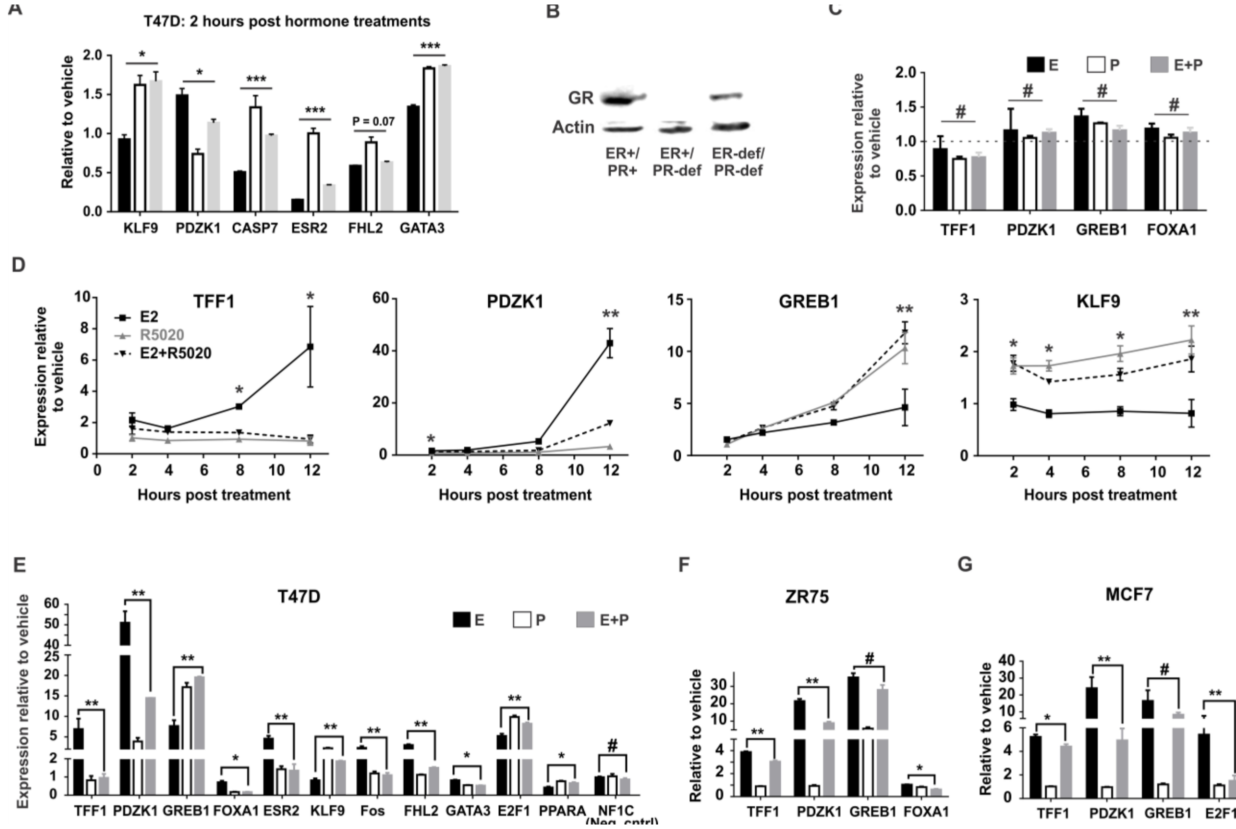


Figure 1.10: Transcriptional effects of PR on ER-regulated gene expression. (A) For primary responders to estrogen treatment, progestin modulates estrogen-induced gene expression in T47D post two hours of hormonal treatment. **(B)** GR levels in ER-low/PR-deficient T47D cells **(C)** Estrogen and progestin do not regulate gene expression in ER-low/PR-deficient/GR+ T47D cells. **(D)** Two, four, eight and twelve hours' time course to study modulation of estrogen-regulated gene expression by progestin in T47D cells. **(E - G)** Progestin modulates estrogen-induced gene expression in **(E)** T47D, **(F)** ZR75 and **(G)** MCF7 cells. Fold change over vehicle treatment is shown.

estrogen-regulated cellular processes and phenotypes. Importantly, on dual hormonal treatments, progestin dominates estrogen action as the gene expression patterns of tissues exposed to both hormones are most similar to progestin alone in a minority of tumor samples and all the PR+ cell models. Ingenuity analyses predicts these gene expression patterns (on dual hormone treatments) to be tumor suppressive. These transcriptomic observations are reflected in functional assays, where progestin abrogates estrogen-mediated increases in cell proliferation, ki67 staining of tumors, cell migration and invasion. Further experimentation in PR-deficient systems and in

models obtained after introduction of PR in PR-deficient systems (PR re-expressed) demonstrates that PR is necessary and sufficient for these progestin actions.

II. PR modulates ER chromatin binding and estrogen signaling in an isoform-specific manner.

Background

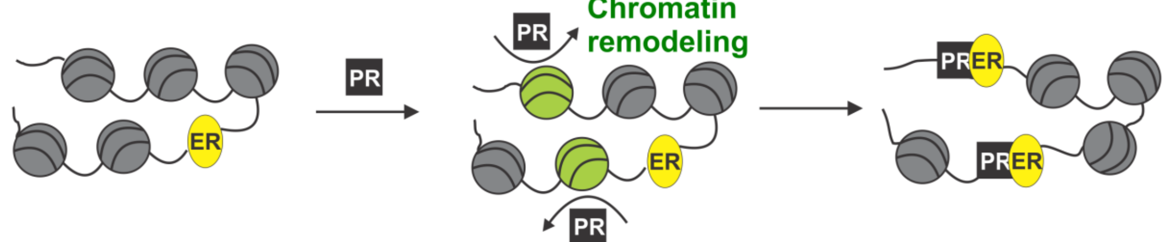


Figure 2.1: PR binds closed chromatin and remodels nucleosomes to regulate gene expression. PR requires nucleosomes for optimal binding and function.

PR binds closed chromatin and remodels nucleosomes to regulate gene expression.

PR belongs to the NR3C subfamily of nuclear receptors, which includes androgen receptor (AR), Glucocorticoid Receptor (GR), Mineralocorticoid Receptor (MR) and Progesterone Receptor (PR) (*80*). Activated GR is reported to modulate chromatin landscape and to reprogram ER chromatin binding in murine cancer cells (*81*, *82*). Similarly, activated AR reprograms ER binding in breast cancer cells (*83*). AR also remodels chromatin landscape in prostate cancer cells (*84*, *85*). Both PR and GR are reported to bind to closed chromatin and repressed chromatin structure favors binding of PR. Upon binding to closed nucleosomes, PR recruits chromatin remodeling machinery, such as SWI/SNF complexes, to open up chromatin and regulate gene expression (**Fig. 2.1**) (*86–92*). In contrast to the chromatin remodeling role of NR3C family of receptors, the chromatin binding aspect of ER has been controversial (*58*, *82*). It is reported that opening of chromatin by pioneer factors facilitates ER binding, while there has been evidence that ER recruitment can also influence binding of pioneer factors and create

chromatin accessibility (*82*, *93*). Nevertheless, chromatin binding of ER and PR is critical to their function as ligand-activated nuclear transcription factors (*59*, *94*). It is not known whether or how PR remodels ER binding and response. Hence, it is important to understand ER and PR crosstalk at the chromatin level and determine what happens to the estrogen-stimulated ER cistrome in the presence and absence of activated PR.

The two isoforms of PR, PRA and PRB, have distinct functional roles in breast cancer

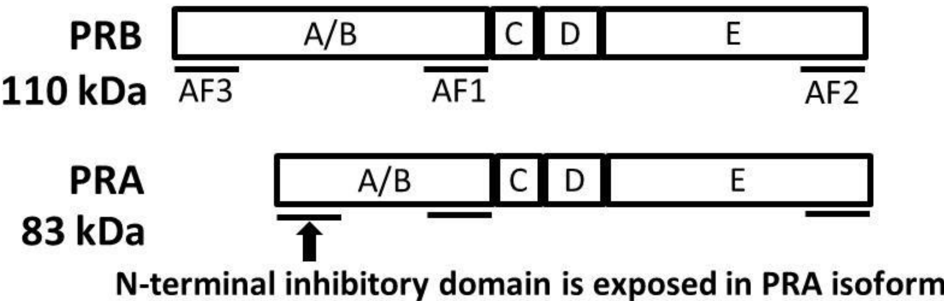


Figure 2.2: Modular structures of two PR isoforms PRA and PRB. PRA and PRB are encoded by two different transcription start sites of the PR gene.

PR exists as two isoforms PRA and PRB, which are coded by two different transcription start sites of the PR gene (**Fig. 2.2**). PRA is the shorter version of the two PR isoforms and it has truncated amino terminal domain. Homodimers as well as heterodimers of PR isoforms are transcriptionally active. Expression of PRA and PRB is tightly controlled and both isoforms are present at similar levels in normal breast tissue. Loss of co-ordinate expression of PRA and PRB is an early event in breast carcinogenesis (*17*). Higher cellular levels of PRA compared to PRB results in aberrant targeting of genes, abnormal nuclear PR foci formation, morphological changes, epithelial to mesenchymal transition and higher cell motility and invasiveness (*13*, *14*, *95*). Furthermore, higher PRA to PRB ratios predict short disease free survival and resistance to tamoxifen therapy (*18*). An earlier study reported that in T47D cells

PRA, PRB and both isoforms regulate 4, 65 and 25 genes respectively (*78*). Differential gene regulation by PRA and PRB in ER+/PR+ breast cancer is still not understood as this study identified a very low number of differentially regulated genes (*78*). The intersection of ER and PR signaling can be anti- or pro-tumorigenic and due to very different biologies of PR homo- and heterodimers, it is important to understand how different PR isoforms differentially reprogram estrogen signaling.

Results

PR positivity is associated with consistent ER binding patterns in human breast tumors

Because genomic binding of ER is critical to its function as a signaling molecule and transcriptional modulator (*59*, *94*), it is important to understand whether PR modulates ER genomic binding. To understand whether PR positivity in human tumors influences ER binding, we performed principal component analyses of ER genomic binding patterns for nine ER+/PR+ and six ER+/PR- patients (*96*). Interestingly, high variance was observed in ER binding among six ER+/PR- patient tumors; whereas ER binding was remarkably consistent among all nine ER+/PR+ patient tumors (**Fig. 2.3**). These results suggest that PR positivity in tumors is associated with highly organized ER binding

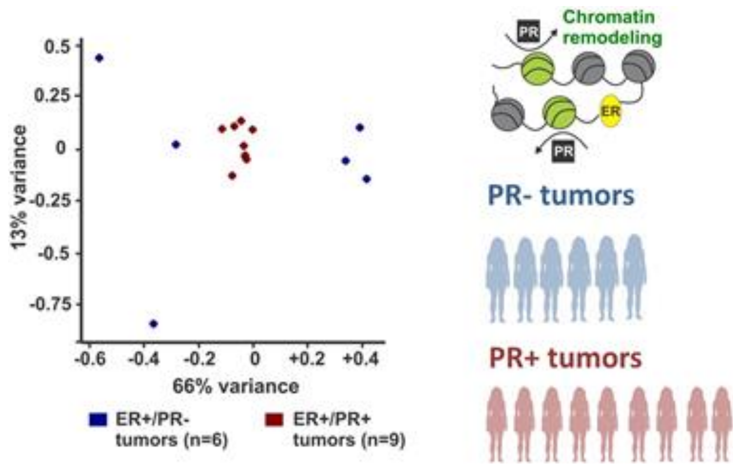


Figure 2.3: ER chromatin binding in PR+ and PR- tumor cohorts. Principal component analysis (PCA) plot displays 79% total variance between ER binding events in nine ER+/PR+ and six ER+/PR- patient tumors.

and indicates that PR could be influencing ER genomic binding.

PR reprograms ER chromatin binding in a PR isoform-specific manner

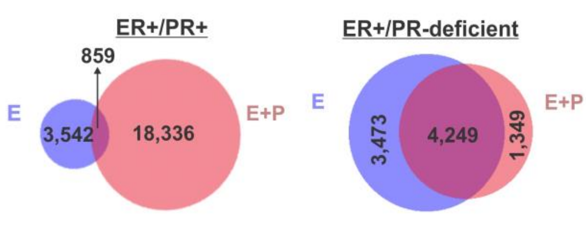


Figure 2.4: Progestin mediated reprogramming of ER binding in PR+ and PR-deficient T47D cells. ER binding sites in PR+ and PR-deficient T47D cells treated with E or with E+P. The overlap represents ER binding that is unchanged in the presence of progestin.

In accord with these results, activated PR increased ER binding events four-fold in ER+/PR+ cells (**Figs. 2.4, 2.5A and 2.5B**), and redirected ER to sites that correlated with genomic binding of PR and complexes of ER and PR (**Fig. 2.7A**). Progestin-mediated expansion and redirection of ER binding was significantly diminished in PR-deficient cells (**Figs. 2.7A and 2.7C**), suggesting a critical role for PR in remodeling ER binding. Interestingly, while activated PRA/PRB heterodimers expanded ER chromatin binding events four-fold (**Fig. 2.5A**), PRA decreased ER binding events by 75% (**Fig. 2.5B**) and PRB primarily redistributed ER genomic recruitment (**Fig. 2.5C**). PR isoform-specific reprogramming of ER chromatin binding highlights the paradoxical pro- and anti-tumorigenic effects of PR in breast cancer. These results necessitate further research on ER/PR crosstalk before use of PR-targeting therapies for breast cancer treatments. ER and PR binding was confirmed by ChIP-PCR (**Figs. 2.6A – 2.6C**). In PR+ but not PR-low T47D, the distribution of ER binding around the ER binding sites obtained after remodeling by PR is below random distribution, suggesting substantial remodeling of ER binding by activated PR (**Figs. 2.7B and 2.7C**).

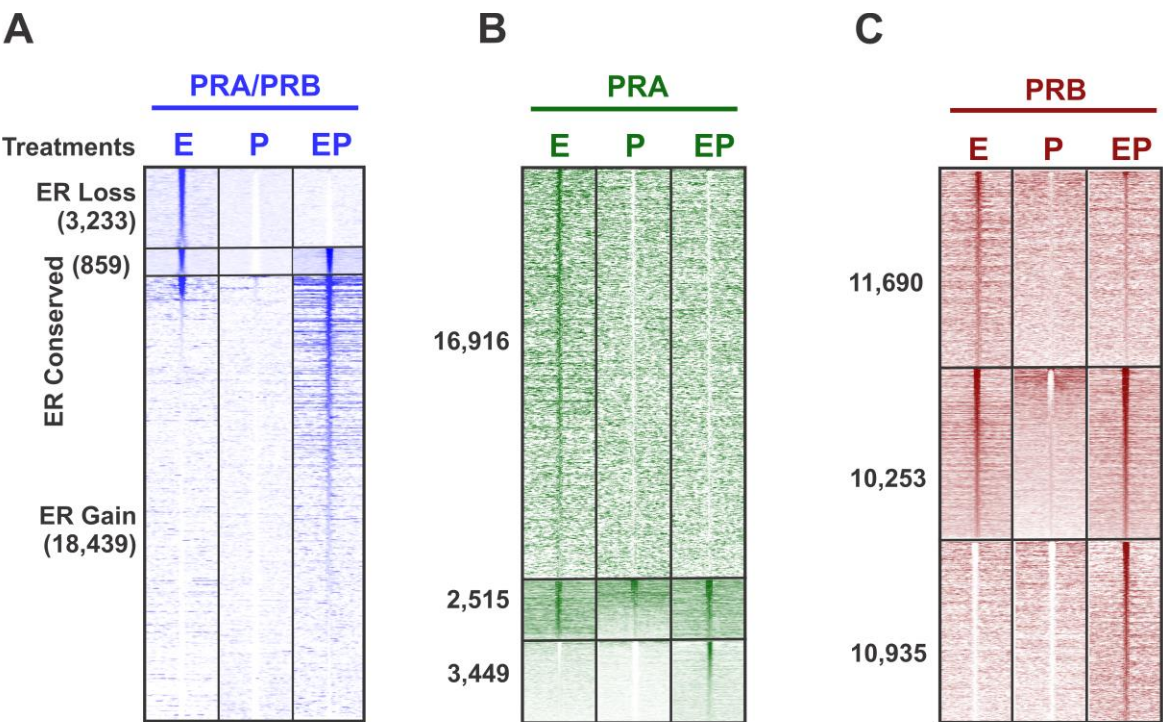


Figure 2.5: PR reprograms ER binding in PR+ and PR-deficient T47D cells. (**A - C**) Heatmaps display signal intensity of sequencing obtained on anti-ER ChIP before and after remodeling by **(A)** PRA/PRB heterodimers and individually by (**B**) PRA and (**C**) PRB homodimers. Genomic window of the union of all ER binding sites observed prior to and after remodeling by PR is displayed. Overlap of at 1 bp was considered to categorize ER binding as lost, conserved or gained due to remodeling by PR.

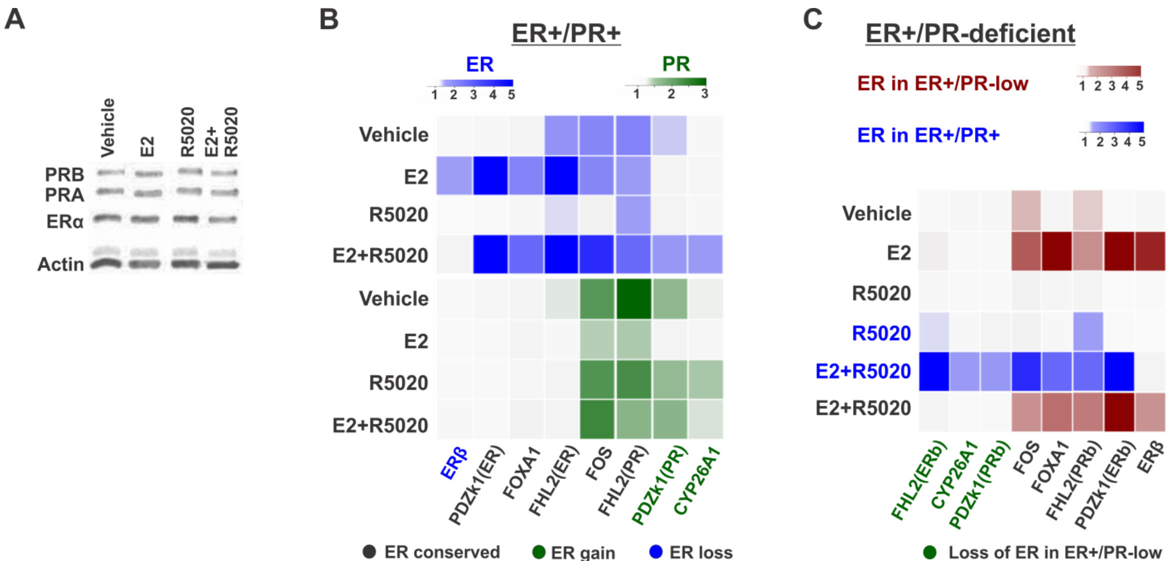


Figure 2.6: RT-PCR validation of progestin mediated reprogramming of ER binding. (**A**) ER and PR levels in T47D cells do not change in response to 45 minutes of treatment with different hormones. **(B)** Heatmaps summarize ChIP-qPCR for ER (blue) and PR (green) binding in T47D cells and ER binding in **(C)** PR-deficient T47D cells (red). ER binding sites that are conserved (black), lost (blue) or gained (green) after reprogramming by PR are shown.

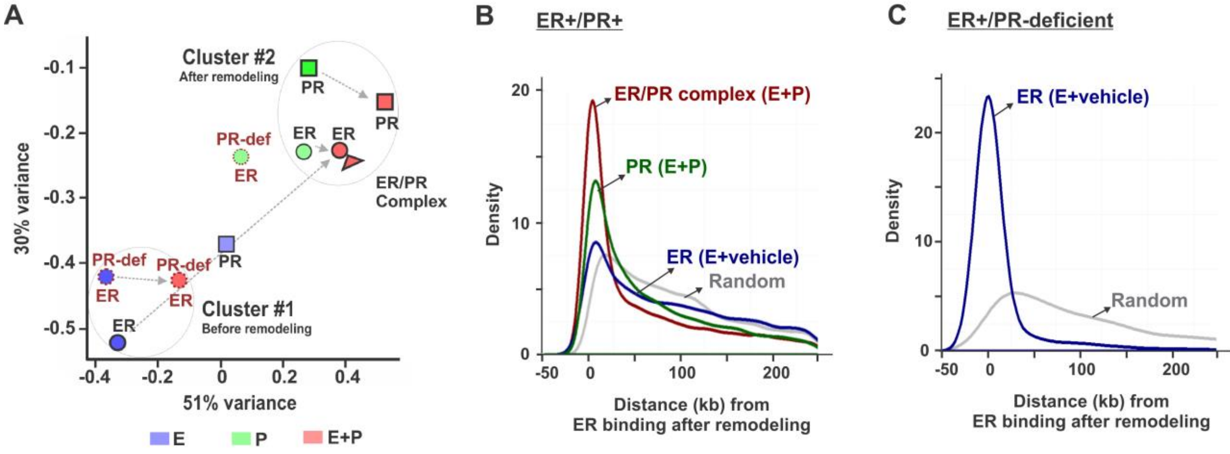


Figure 2.7: PR reprograms ER binding in PR+ and PR-deficient T47D cells (A) PCA plot depicts 81% total variance between binding events for ER, PR and ER/PR complexes observed upon treatment with E, P or E+P. **(B, C)** Distributions of receptor binding around ER bound sites that were observed in **(B)** PR+ and **(C)** PR-deficient T47D cells treated with E+P. Distributions for ER binding observed without progestin and binding for PR, ER/PR complexes are plotted.

In tumors and cell models, ER binding in presence of PR occurred distal to promoters

While ER largely bound near transcriptional start sites in PR-negative tumors and in PR-deficient cells, in PR-positive cells and tumors, ER binding primarily occurred distal to

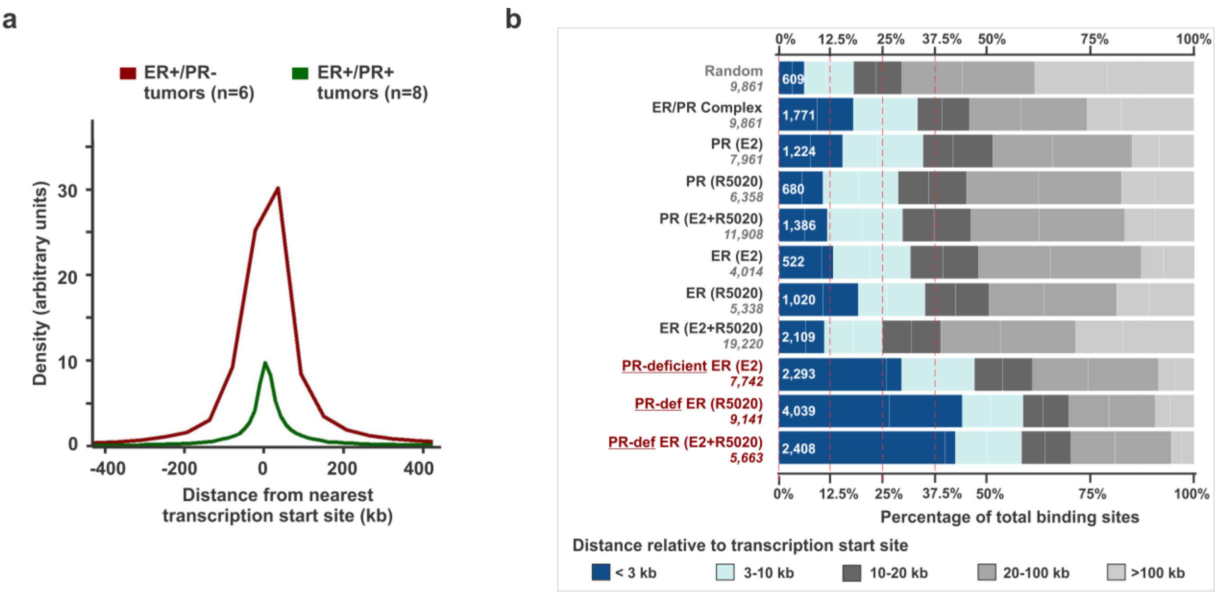


Figure 2.8: ER and PR binding around transcription start sites. (A - B) ER binding sites in **(A)** ER+ human tumors (eight ER+/PR+ and six ER+/PR-) and **(B)** PR+ and PR-deficient T47D cells treated with E or with E+P. One outlier patient tumor in ER+/PR+ group is not plotted.

Motif	Pvalue	Proteins
ER loss		
AGGTCA	10^{-59}	ESR1, -2, PPARG, RORA, RXR:RAR, NR2F1, NR1H2
GGG CA GTGACC	10^{-31}	ESR1, -2, PR, GR, AR, NFIC, PPARG, RORA
ER conserved		
[illegible]	10^{-31}	NFIC, PR, GATA3
[illegible]	10^{-29}	ESR1, -2, PR, GR, AR PPARG, RXR:RAR
ER gain		
CCTCAGCC	0	NFIC, KLF5, EGR1, SP1
CCACCA	10^{-215}	PR, NFIC:TLX1,coREST, Myc:MAX, KLF5
ATGGTCT	10^{-55}	PR, GR, AR, Myc:MAX
CATGTTGG	10^{-37}	PR, BRCA1, NFIC:TLX1, FOXA1, -D3, p53, coREST

Figure 2.9: Motifs enriched at ER and PR binding sites. Transcription factor binding motifs enriched in ER binding sites that are lost, conserved or gained due to reprogramming by PR. The significance of the enriched motif is reported by p value. The adjacent column lists proteins that potentially bind to the identified binding motifs.

promoters (**Figs. 2.8A and 2.8B**). Furthermore, these redirected ER binding sites did not correlate with ER binding sites observed in the absence of progestin (**Fig. 2.7A**). Progestin-mediated expansion and redirection of ER binding was significantly diminished in PR-deficient cells (**Figs. 2.7A and 2.7C**), suggesting a critical role for PR in remodeling ER binding. In accord with these findings, PR and PR-associated coregulators (*97*) were the most highly enriched motifs at redirected ER binding sites (**Fig. 2.9**).

PR reprograms estrogen signaling to be pro or anti-tumorigenic based on relative ratios of PRA and PRB

PRA is the shorter of the two isoforms and PRA has a truncation in amino terminal transactivation domain that possibly exposes an inhibitory domain, through which PRA is hypothesized to be more effective than PRB at recruiting negative coregulators of gene expression (*13*, *51*, *53*, *98*). In agreement with the ability of PRA at reducing ER chromatin binding, compared to PRB, PRA inhibited almost 2.5 times the number of genes and with greater

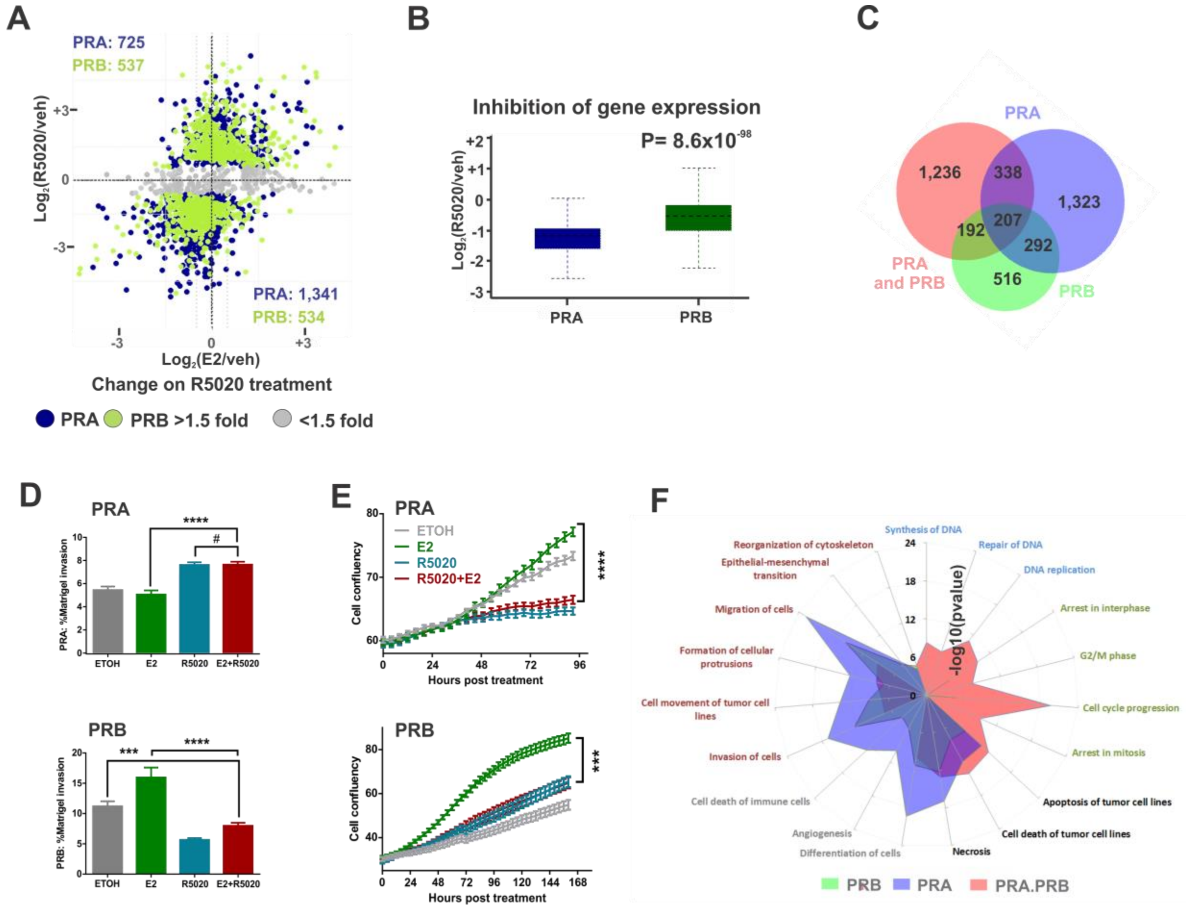


Figure 2.10: Progestin signaling can be anti- or pro-tumorigenic based on relative ratios of PRA and PRB. Both the PR isoforms reprogram estrogen regulated levels of transcriptomes and phenotypes to resemble their own. **(A)** Axes denote log fold change of gene expression in T47D cells in response to estrogen (E) and progestin R5020 (P) treatment relative to vehicle (V). Blue and Green dots represent T47D cells that express either PRA or PRB respectively. **(B)** Magnitude of inhibition of gene expression by PRA or PRB. **(C)** Venn diagrams show overlap of genes that are regulated by PRA/PRB heterodimers, or homodimers of PRA and PRB. **(D)** Matrigel invasion and **(E)** proliferation of ER+ T47D cells expressing only PRA or PRB isoform. The cells are treated with vehicle, E, P or E+P. **(F)** Pathways enriched in E, P and E+P regulated transcriptomes of T47D cells expressing both PRA and PRB or either PRA or PRB alone. Negative log10 of p value is plotted.

magnitude (**Figs. 2.10A and 2.10B**). In conclusion, PRA is more effective than PRB at inhibiting gene expression and ER chromatin binding. PRA and PRB homodimers and heterodimers are transcriptionally active and there is less than 10 percent overlap between the genes regulated by progestin in T47D cells that express similar levels of PRA and PRB, or express either of the two

PR isoforms (**Fig. 2.10C**). It indicates that PRA, PRB and PRA/PRB heterodimers regulate different sets of genes in breast cancer. Pathway analyses revealed that pathways for DNA repair, cell cycle arrest, differentiation and cell death are enriched in T47D cells expressing similar levels of both the PRA and PRB (**Fig. 2.10F**). In contrast, PRA but not PRB preferentially regulated pathways associated with malignancy such as cellular migration, invasion, epithelial to mesenchymal transition, dedifferentiation and maintenance of stem cells (**Fig. 2.10F**). Additionally, activation of PR can be pro- or anti-tumorigenic depending on the relative ratios of PRA and PRB. Each of the PR isoforms modulate estrogen signaling in an isoform-specific manner. For example, activation of PRA increased cellular invasion and decreased cellular proliferation. In contrast, PRB decreased cellular invasion but increased cell proliferation (**Figs. 2.10D and 2.10E**). These findings provide evidence for PR isoform-specific malignant phenotypes and necessitate further research on ER/PR crosstalk before using of PR-targeting therapies for breast cancers.

Discussion

Chromatin binding of ER and PR is important for the genomic actions of these receptors. In agreement to the known chromatin remodeling property of PR, activation of PR reprograms ER chromatin binding and redirects binding of ER to the sites bound by PR and genomic locations enriched for the motifs of PR-associated coregulators. Activated PR primarily shifts ER binding away from the promoters (in cell models and human tumors) and to distal enhancer regions. This PR-mediated remodeling of ER binding is clinically relevant since PR positivity is associated with more consistent ER binding patterns in chromatin in breast tumors. These effects of progestin are much diminished in PR-deficient systems indicating that progestin is acting through PR. Further experimentation revealed that PR remodels ER chromatin binding

in a PR isoform-specific manner and while PRA/PRB heterodimers expand ER binding, PRA shuts ER genomic recruitment and PRB primarily redistributes ER binding. In line with different biologies of PR isoforms, based on the relative ratios of the two PR isoforms, progestin reprogrammed estrogen signaling to be pro or anti-tumorigenic.

III. PR forms complexes with ER and redirects ER binding to enhancers and motifs enriched for PR, ER/PR complexes and BRCA1 protein.

Background

Previous studies on artificial vector constructs suggested that PR might non-competitively interact with ER (*49*, *50*, *52*, *53*). For example, interaction of PR with ER in cytoplasm is reported to be critical for activation of c-src/ERK pathways by progestin (*55*, *56*). A more recent study reported that unliganded PRB is complexed together with PELP1 and ER containing transcription complexes in T47D and MCF7 breast cancer cells (*57*). It is not known whether such interactions occur on endogenous gene loci and what the function of these interactions is genome-wide. Based on these observations we hypothesize that there is genome-wide non-competitive interaction between ER and PR in breast cancer.

Steroid receptors can alter chromatin binding landscape of each other

There is an ongoing controversy about the role of Forkhead protein (FOXA1) as a pioneer factor that facilitates chromatin accessibility for nuclear receptors such as ER, PR, androgen (AR) and glucocorticoid receptors (GR) (*99–101*). For example, FOXA1 is reported to be a key determinant for the recruitment of ER, ER function and estrogen signaling in breast cancer (*59*, *94*, *102*). In contrast, recent evidence from single molecule tracking of live cells suggests that nuclear receptors like ER and GR can also pioneer the recruitment of FOXA1 to the genome (*93*). Interestingly, contrary to the classical theory of so-called pioneer proteins, fast chromatin residence times of 8-10 seconds are observed for FOXA1, suggesting that steroid receptors can alter the response of FOXA1 though highly dynamic and fast DNA interactions (*93*). These results indicate that pioneer proteins may not be absolutely essential to allow

chromatin to remain open and thus permitting access of steroid receptors to binding sites. It is possible that pioneer proteins behave as transient chromatin remodelers and that the initial theory of pioneer proteins (*58*, *59*) was useful but not able to capture the complexity and dynamic nature of steroid receptors interactions with chromatin.

Multiple studies have shown that steroid receptors can alter the binding landscape for each other, thereby mediating crosstalk between different steroid receptors. GR genome binding could be dependent on chromatin accessibility and it is reported that GR uses the existing chromatin state of the cell for gene regulation (*81*, *92*). On the other hand, both GR and PR are known to bind to closed nucleosomes, recruit chromatin remodeling machinery, such as SWI/SNF complexes, to open up chromatin and regulate gene expression (**Fig. 2.1**) (*86–92*). In support, FOXA1 is not absolutely essential for GR chromatin binding and FOXA1 knockdown is known to significantly redistribute GR binding in prostate cancer cells (*103*). Similarly, FOXA1 is not absolutely required for recruitment of AR, i.e. AR genome binding events can be dependent as well as independent of FOXA1. Additionally, FOXA1 plays a dual role in androgen signaling and it may enhance or inhibit androgen responsiveness of AR regulated genes (*85*). One study suggested that FOXA1 might play a similar role with PR as with AR since FOXA1 was not required for progestin response in MCF10A cells that overexpress FOXA1 and PR (*104*). This study found that FOXA1 overexpression in MCF10A cells led to a decrease in the number of progestin responsive genes in these cells (*104*). The function of FOXA1 in progestin responses is still not well understood and MCF10A cells that express PR and FOXA1 are not a breast cancer cell model. Additionally, there are no published data for the effect of FOXA1 knockdown on progestin responses in breast cancer cell models that express endogenous PR and FOXA1, such as T47D, MCF7 or ZR75 cells.

The nuclear factor 1 (NF1) family of transcription factors contains four members NFIA, NFIB, NFIC, NFIX that bind to the consensus DNA sequence TTGGCN5GCCAA(*105*). NF1 factors are also called CAAT binding proteins since they recognize CAAT motifs near promoters of the genes regulated by them. NF1 synergizes with PR and NF1 depletion significantly reduces progestin response (*87*, *88*, *106*). NFI family members interact with FOXA1 and are potent regulators of androgen receptor signaling in prostate cancer cells (*107*). NF1 promotes binding of GR to promoter regions and there is chromatin dependent synergism between NF1 and GR mediated gene expression (*90*, *92*, *108*). In contrast to the research on the function of NF1 factors in gene expression by PR, GR and AR, not much is known about how NF1 regulates ER-mediated gene expression. In summary, upon binding to nucleosomes, PR as well as GR recruits chromatin remodeling machinery, such as SWI/SNF complexes, to open up chromatin and regulate gene expression. The data for PR-mediated chromatin remodeling presented in Section II, strongly suggest that PR could is a remodeler of ER genomic binding.

Results

Global non-competitive interactions between ER and PR

Given that PR clearly modulates ER binding and activity, mechanistic insights into how PR orchestrates ER binding could help exploit ER/PR crosstalk for breast cancer prognosis and treatment. Overexpression of ER in T47D cells did not decrease or reverse PR mediated modulation of ER signaling (**Figs. 3.1A and 3.1B**). Similarly, modest knockdown of PR did not decrease or reverse PR-mediated modulation of ER signaling (**Figs. 3.1C and 3.1D**). These results suggest that PR non-competitively modulates ER signaling and that these receptors either recruit different coregulators or are complexed together at the regulatory regions of genes

coregulated by estrogen and progesterone. ER and PR are known to recruit similar set of co-regulators. Therefore to test the latter hypothesis we performed co-immunoprecipitation and sequential chromatin immunoprecipitation (ReChIP) assays (*9*, *95*, *97*, *109*). ER and PR

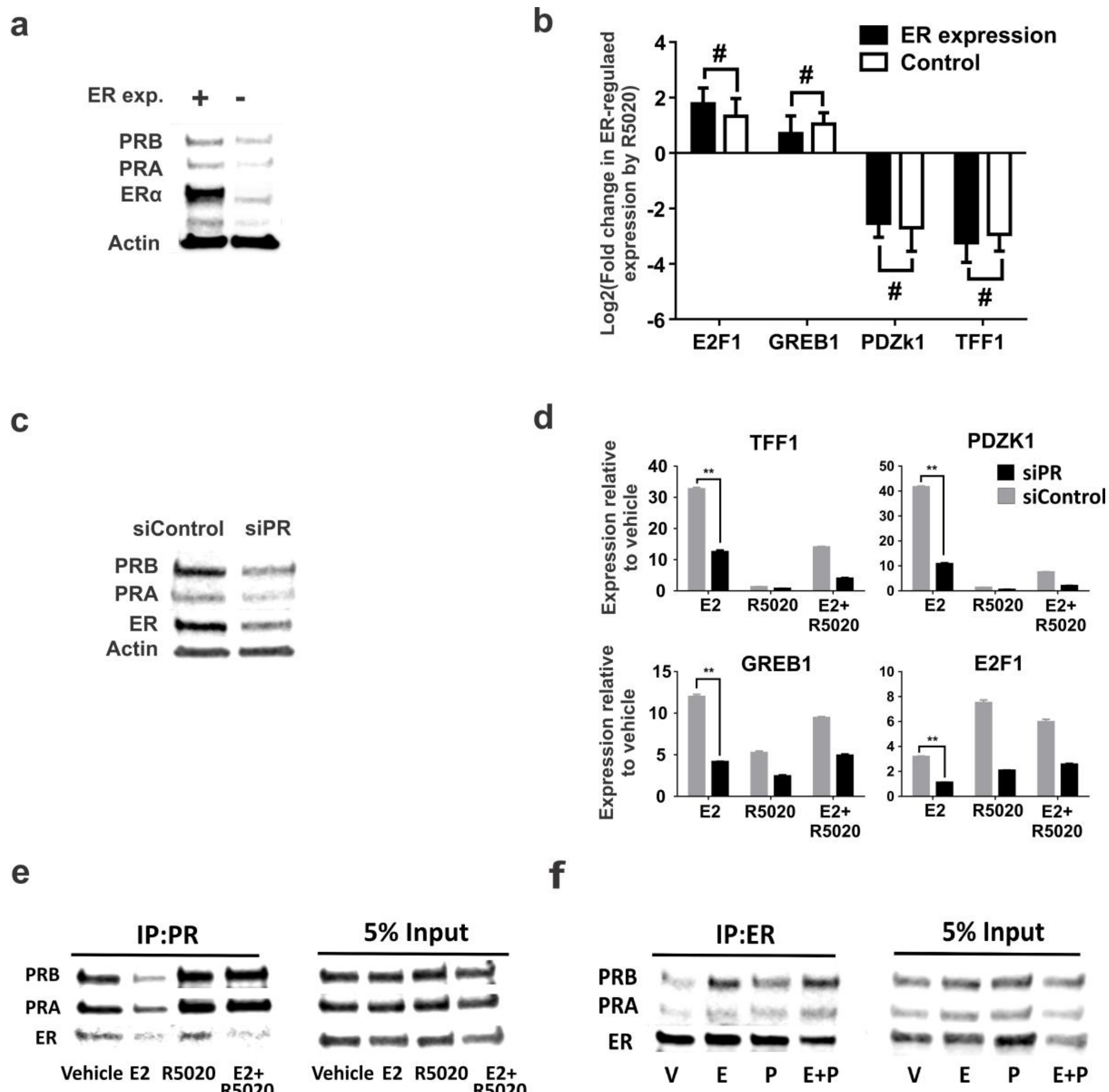


Figure 3.1: Non-competitive interactions between ER and PR. **(A)** Immunoblot showing exogenous expression of ER protein in T47D cells. Expression of empty vector was used as a control. **(B)** Comparison of the effect of ER or control expression on modulation of estrogen-regulated gene expression by progestin. **(C)** Immunoblot of lysate from T47D cells transfected with PR-targeting or non-targeting control siRNA. **(D)** Gene expression inT47D cells treated with E, P or E+P after moderate knockdown of PR. **(E)** Anti-PR immunoprecipitation followed by immunoblotting for both ER and PR in T47D cells treated with different hormones.

immunoprecipitated together after 45 minutes of treatment of T47D cells with estrogen and progestin (**Figs. 3.1E and 3.1F**). Co-immunoprecipitation of ER and PR suggests that these receptors form a complex in these cells. Compared to the vehicle treated T47D cells, significantly higher levels of PR immunoprecipitated with ER in T47D cells co-treated with E2

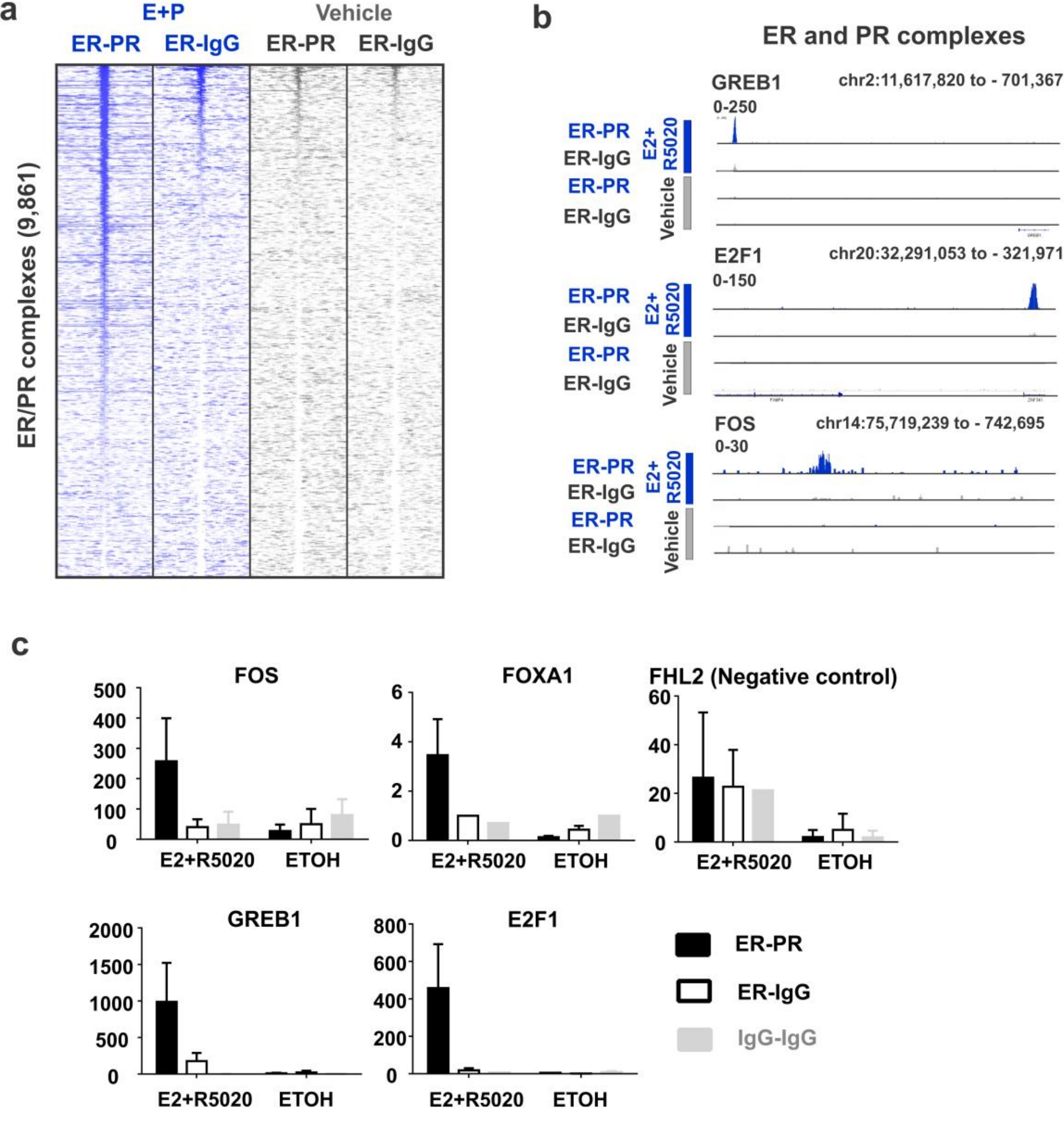


Figure 3.2: Global recruitment of ER/PR complexes to the genome. (**A**) Heatmaps display signal intensity of sequencing obtained on reChIP-seq of anti-ER followed by anti-PR or non-specific IgG control. Genomic window of the binding of ER/PR complexes is displayed. **(B)** Representation of binding of ER/PR complexes near the GREB1, E2F1 and FOS gene loci. **(C)** ReChIP-PCR of anti-ER followed by anti-PR or control anti-IgG ChIP. Fold enrichment with respect to the input is reported.

and R5020, suggesting that simultaneous activation of both ER and PR facilitates the formation of complexes between these receptors. These ER/PR complexes were recruited to the genome upon joint ligand activation, as illustrated by the strong signal obtained from reChIP for ER followed by PR but not when ER was followed by reChIP for non-specific immunoglobulin control (**Figs. 3.2A and 3.2B**). Directed reChIP-PCR specific to FOS, FOXA1, GREB1, E2F1 and FHL2 regulatory regions confirmed the findings from reChIP-seq (**Fig. 3.2C**).

Long-range three-dimensional chromatin looping between ER and PR

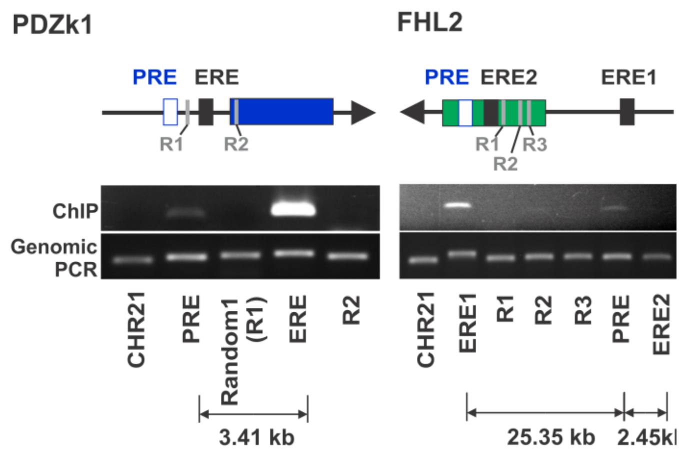


Figure 3.3: Long-range interactions between ER and PR binding sites. Capture of Associated Targets on Chromatin (CATCH) of estrogen response elements at PDZk1 and FHL2 loci pulls down distant progesterone response elements that interact with the pulled down regions. PCR enrichment of the pulled down region, the interacting progesterone response elements and random controls is shown.

In addition to the recruitment of ER/PR complexes, ER and PR also interacted via long-distance chromatin looping between their hormone response elements separated by about 3.5 and 25 kb at the PDZk1 and FHL2 gene loci respectively (**Fig. 3.3**), highlighting that ER/PR complexes can facilitate interactions between distal regulatory regions in the genome. It is important to note that these data are not able to define the nature of the observed ER/PR complexes, which could be direct physical contact or indirect and involve one or more bridging proteins.

PR redirects ER binding to enhancers and sites enriched for BRCA1 binding motifs

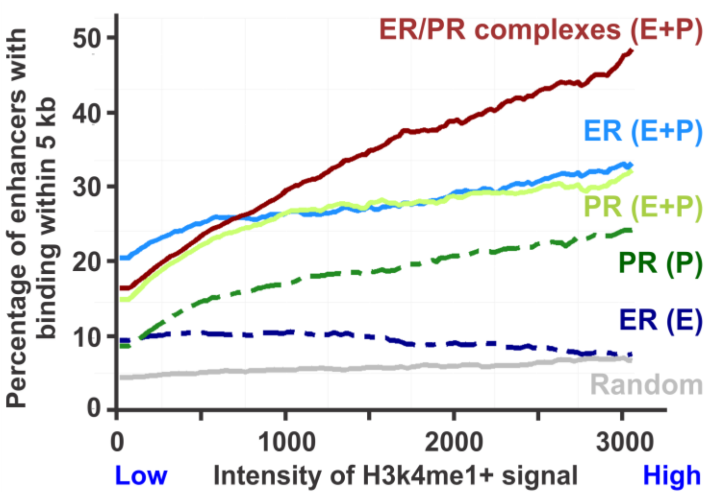


Figure 3.4: PR redirects ER binding to enhancers. Cumulative percentage of H3K4me1+/H3K27ac1+ enhancers that have receptor binding within 5 kb of their center. The enhancers are sorted from left to right in the increasing order of H3K4me1+ signal intensity.

Motif	Pvalue	Proteins
CCTGTA[G/A]T	10^{-165}	BRCA1, ELK4
TAC[A/T]AAAA	10^{-153}	FOXI1, FOXP1, NFI-L3
CAGCCTGG	10^{-61}	PR, ESR1,-2, coREST

Figure 3.5: Motifs enriched at the binding sites of ER/PR complexes. Transcription factor binding motifs enriched in binding sites for ER/PR complexes. The significance of the enriched motif is reported by p value. The adjacent column lists proteins that bind to the identified motifs.

ER/PR complexes mostly bound to H3K4me1+/H3K27ac1+ enhancers and importantly, to the enhancers with highest H3K4me1+ signal intensity (**Fig. 3.4**). Additionally, PR substantially increased ER binding to enhancer regions (**Fig. 3.4**), thus collectively suggesting an active role of these complexes in regulating transcriptional processes. As further evidence for the importance of ER/PR complexes, key proteins in breast carcinogenesis, including BRCA1 (*110*), ER, PR, nuclear factors (*111*) and forkhead proteins (*100*), were the most enriched motifs at the binding sites of these complexes (**Fig. 3.5**). BRCA1/2 carriers are known to have 121% higher levels of serum progesterone (*112*) and PR antagonists have been proposed as cancer prevention therapy in BRCA1-mediated mammary tumorigenesis in mice

(*113*). Despite the clinical evidence of active progesterone signaling in BRCA1-mediated tumorigenesis, it is not mechanistically understood as to how PR might contribute to these cancers. Our finding that ER/PR complexes have a preference for BRCA1 binding motifs suggests a potential role of BRCA1 in ER/PR crosstalk.

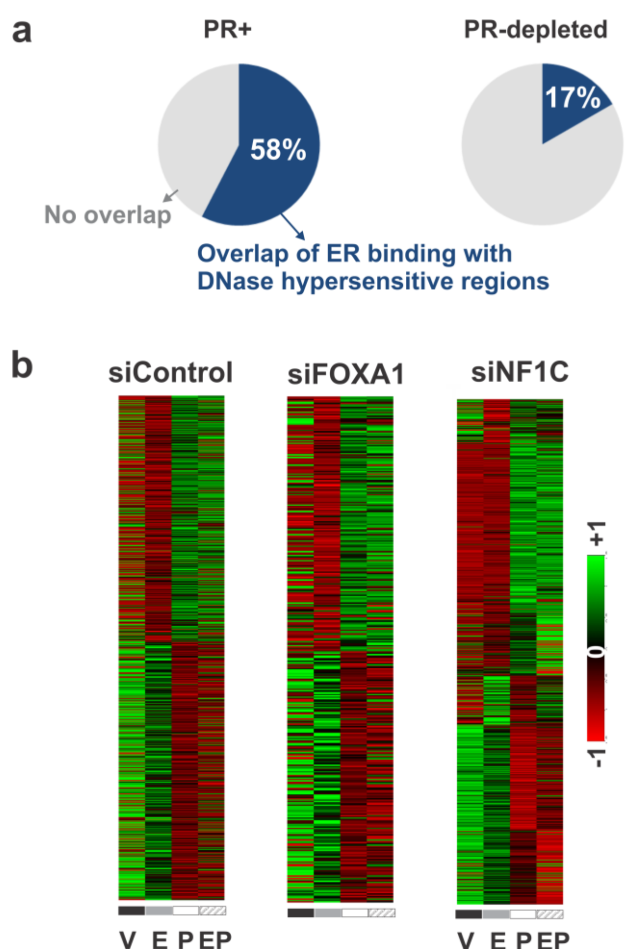


Figure 3.6: Effects of chromatin remodelers FOXA1 and NF1C on ER/PR crosstalk. (A) Percentage overlap of ER binding sites with DNase hypersensitive regions created upon treatment of T47D or PR-depleted T47D cells with progestin. **(B)** Row-normalized heatmaps to depict expression of estrogen- and progestin-regulated genes in T47D cells after siRNA specific depletion of FOXA1, NF1C or a non-specific control. Heatmaps for siFOXA1 specifically include genes that have FOXA1 binding within 100 kb of gene's promoter [26].

While activated PR binds to nucleosomes and remodels chromatin (*87*), ER binding is facilitated by chromatin accessibility that is created upon remodeling of nucleosomes (*58*, *94*). In accord with this view, there was a 58% overlap between ER binding sites and DNase hypersensitive regions created by treatment of T47D cells with progestin. However, depletion of PR reduced this overlap to only 17%, suggesting that PR is required for ER binding to progestin-induced DNase hypersensitive regions (**Fig. 3.6A**).

Effects of chromatin remodelers FOXA1 and NF1C on ER/PR crosstalk

Given that activated PR influenced chromatin accessibility for ER binding, it is not surprising that depletion of either of the known chromatin remodelers FOXA1 (*100*, *114*) or NF1C (*90*, *111*, *115*) (**Figs. 3.7A and 3.7B**) did not significantly impact the effects of PR on ER-regulated gene expression (**Figs. 3.6B and 3.7C**). This finding is interesting because while FOXA1 and NF1C are necessary for both ER and PR regulated gene expression when considered in isolation (**Figs. 3.6D and 3.7E**), they are not required for ER/PR functional crosstalk, even for genes that have FOXA1 binding within 100 kb of their promoters (**Figs. 3.6B and 3.7C**). These results are further supported by recent reports that there is 99% overlap between progesterone-induced ER binding and PR sites, however FOXA1 could be found at only 49% of those sites (*116*). Additionally, under joint hormonal conditions, PR is the only protein that is differentially pulled down with ER (*116*), suggesting a far more important role for PR in regulating estrogen signaling. It is to be determined whether in addition to its genomic actions, PR could be affecting estrogen signaling through non-genomic mechanisms (*86*) or whether there are other chromatin remodelers that might facilitate actions of PR (*117*). Either modulation of ER signaling by PR is not dependent on FOXA1 and NF1C or FOXA1 and NF1C are effective at residual low levels that are left after their knockdown. It is also possible that there is functional redundancy and other coregulators might compensate for the loss of FOXA1 or NF1C. Collectively, these results suggest complex and dynamic interplay between PR and chromatin and indicate that at a subset of sites, PR could bind closed but accessible chromatin (*87*, *118*) and remodel binding of other transcription factors.

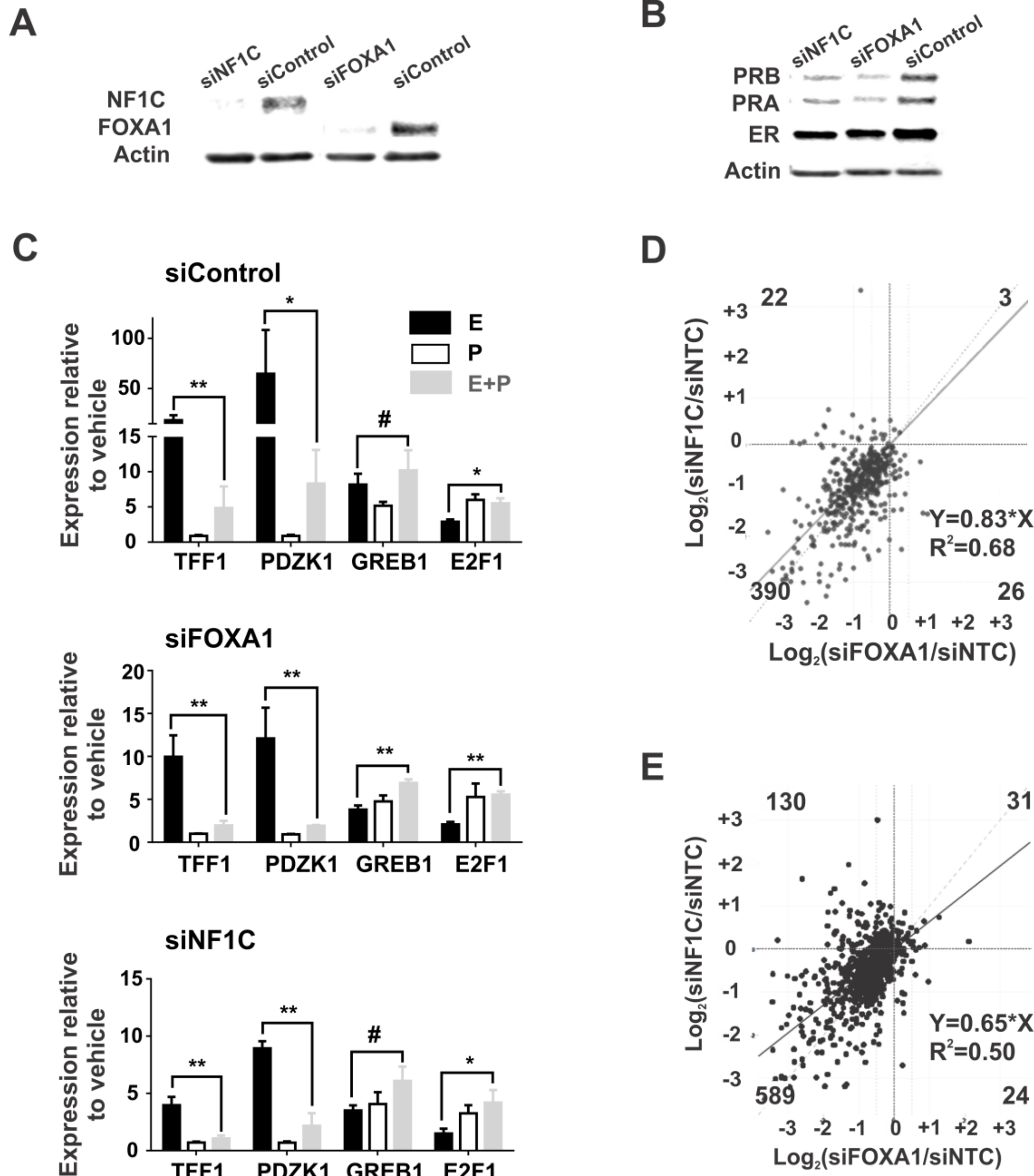


Figure 3.7: Knockdown of FOXA1 and NF1C in T47D cells and its effect on ER- and PR-regulated gene expression. **(A)** Immunoblots of lysate from T47D cells transfected with FOXA1-, NFIC-targeting or non-targeting control siRNA for 48 hours. **(B)** Immunoblots to study the effect of FOXA1 and NFIC knockdown on the protein levels of ER and PR. **(C)** Neither FOXA1 nor NF1C is essential for PR-mediated remodeling of ER-regulated gene expression. Gene expression in T47D cells treated with different hormones after depletion of FOXA1, NF1C or non-target control. Fold change of gene expression on hormone treatment compared to the vehicle is plotted. **(D, E)** FOXA1 and NF1C are essential for ER- and PR-regulated gene expression. Knockdown of FOXA1 and NF1C diminishes **(D)** ER- and **(E)** PR-regulated gene expression. Log fold change in gene expression on siFOXA1 and siNF1C compared to siControl is plotted. Linear regression to compare the effects of FOXA1 and NF1Cknockdown gene expression is plotted.

Knockdown of FOXA1 and NF1C redistributes ER- and PR-regulated gene expression

FOXA1 is not absolutely required for the recruitment of AR and GR and the depletion of FOXA1 significantly redistributes GR and AR binding in prostate cancer (*81*, *85*, *92*, *103*). FOXA1 plays a dual role in androgen and glucocorticoid signaling, where FOXA1 inhibits or enhances different sets of AR- and GR-regulated genes. In support, similar to the chromatin remodeling roles of AR and GR, PR can also remodel nucleosomes to initiate chromatin opening (*81*, *84*, *119*). While chromatin accessibility by FOXA1 facilitates expression of ER- (and PR-) regulated genes, ER (PR) can also regulate expression of a different set of genes in the absence of FOXA1 **(Figs. 3.8A and 3.8B**). These results suggest that similar to the role of FOXA1 in androgen and glucocorticoid signaling, FOXA1 plays a dual role in both estrogen and progestin signaling by facilitating and repressing ER- and PR-mediated gene expression of different sets of genes. These results underscore the complexity and dynamic nature of steroid receptors interactions with chromatin, in comparison to the one's proposed by the classical pioneer factor theory.

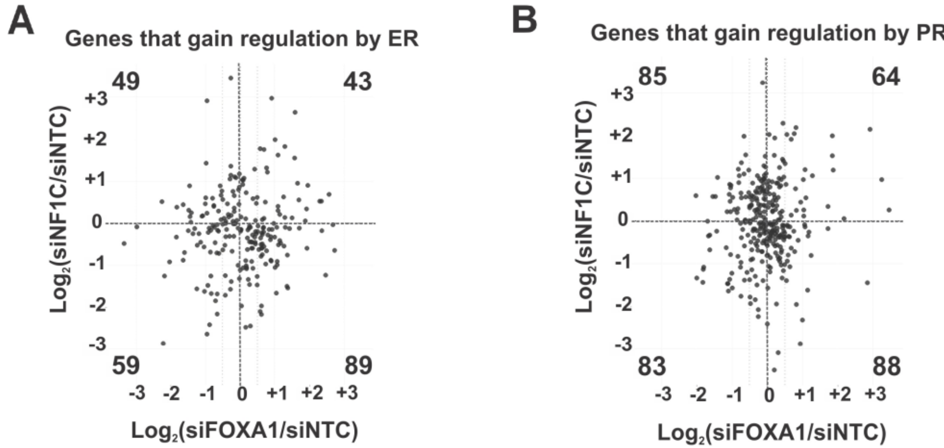


Figure 3.8: Effects of FOXA1 and NF1C on ER- and PR-regulated gene expression. (A - B) Expression of significant number of (A) ER- and (B) PR-regulated genes is de-repressed on the knockdown of either FOXA1 or NFIC. X- and Y-axis denote log2 fold change in gene expression on depletion of FOXA1 or NF1C compared to control knockdown.

Discussion

ER and PR co-immunoprecipitate together in breast cancer cells and changing relative levels of these receptors (by overexpressing ER or moderately depleting PR) did not significantly impact effects of PR on ER-regulated gene expression. These evidences suggest non-competitive nature of ER/PR crosstalk. ER/PR complexes get recruited to the genome and they preferentially bind to enhancer regions and motifs enriched for BRCA1, suggesting a potential role for BRCA1 in ER/PR crosstalk. ER/PR complexes may not always be preassembled since binding sites for these receptors interacted via long-range three-dimensional chromatin looping. In agreement to the chromatin remodeling role of PR, significant overlap of ER binding and DNase hypersensitive regions created by PR demonstrates that PR modulates chromatin accessibility for ER binding. In further support, chromatin remodelers FOXA1 or NFIC are not required for the effects of PR on ER-regulated gene expression.

IV. PR is frequently lost in ER+ tumors due to copy number loss and hypermethylation of PR gene locus. Independent loss of PR alters estrogen signaling and patient survival.

Background

Although for prognostic purposes breast cancer samples are routinely evaluated for ER, PR and Her2 status, there is a long standing controversy about the value PR expression (*27–32*, *34*). Estrogen signaling is considered the key pathway in breast cancer and it is widely agreed that ER is an established biomarker (*120*). The ability of breast cancers to respond to estrogen signaling is a good prognostic marker. PR is a downstream target of ER and PR is believed to be a surrogate marker for functional ER pathways (*28*, *66*). Multiple retrospective and prospective clinical studies show that patients with ER+/PR+ tumors have a better prognosis than patients with ER+/PR- tumors (*27*, *28*, *30–32*). Despite these studies there is no clear consensus whether PR is an independent biomarker or if PR independently influences estrogen signaling and breast cancer. These clinical decisions largely stem from relatively weak PR staining observed in tumors (*22–25*), routine use of anti-ER but not anti-PR targeting therapies (*3*, *26*), insufficient understanding of the intersection of ER and PR signaling and lack of large enough patient

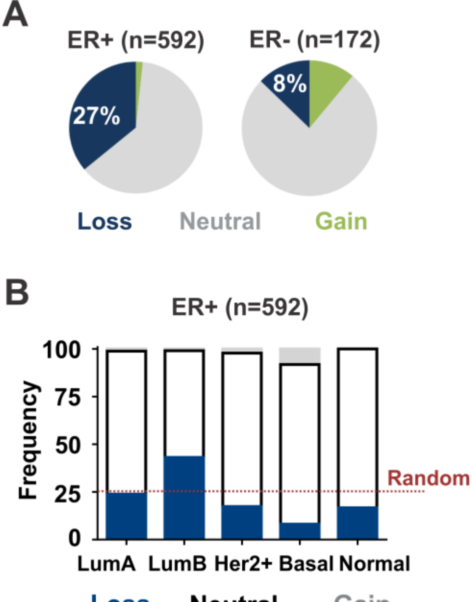


Figure 4.1: In ER+ tumors, PR expression is lost due to copy number loss of PR gene locus. **(A, B)** PR gene locus is frequently lost in ER-positive milieu and luminal-B tumors. Frequency of copy number variation of PR gene locus in TCGA cohort categorized based on **(A)** ER-positivity and **(B)** PAM50 breast tumor subtypes.

cohorts to conclusively determine the clinical value of PR (*21*, *22*, *25*, *27–36*). In addition, the absence of mechanistic details regarding the interaction between ER and PR does not help to settle this controversy. This section describes the possible sources of loss of PR protein in ER+ tumors and investigates how independent loss of PR could alter prognostic value of ER.

Results

PR is lost in ER+ milieu due to copy number loss and hypermethylation of PR locus

In addition to the genomic agonism between estrogen and progesterone, PR-mediated reprogramming of estrogen signaling further highlights the prognostic value of PR as a breast cancer biomarker. Activated PR reprograms the ER-regulated transcriptome to enrich for tumor-suppressive processes (**Figs. 1.7 and 1.8A**) and progestin inhibits estrogen-induced

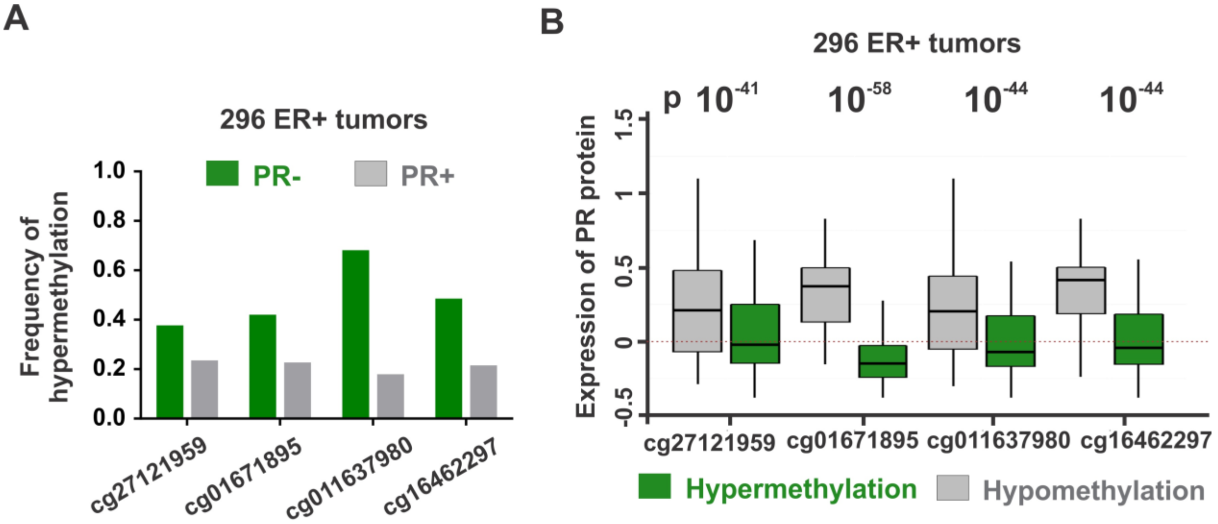


Figure 4.2: In ER+ tumors, PR expression is lost due to hypermethylation of PR gene locus. (A) Frequency of hypermethylation of PR locus in ER+ TCGA tumors categorized based on PR status. **(B)** Hypermethylation of PR gene locus correlates with loss of PR expression in ER+ TCGA tumors, measured using reverse phase protein arrays.

increases in cell proliferation, cell migration and invasion (**Figs. 1.8B – 1.8D**). The breast cancer

relevance of PR is further bolstered by the finding that PR-regulated genes are enriched in gene signatures for estrogen response, breast cancer subtype classifiers, therapy resistance and metastasis (**Fig. 4.4B**).

PR expression is induced by estrogen and the presence of PR in tumors is considered a surrogate for functional estrogen signaling (*14*, *15*, *121*). However in the ATAC trial, ER+/PR- but not ER+/PR+ tumors had a better response to estrogen depletion by aromatase inhibitors compared to anti-ER tamoxifen treatment (*66*, *67*), suggesting that ER+/PR- tumors are dependent on estrogen signaling and that PR might be lost due to other independent mechanisms (*28*, *60*, *122*, *123*). An analysis of METABRIC (*36*) and TCGA (*21*) cohorts revealed that PR is frequently lost in ER+ tumors (**Fig. 4.1A**) due to copy number loss (**Figs. 4.1B and 4.4A**) and hypermethylation of the PR gene locus (**Fig. 4.2A**). In further support of the loss of PR protein expression, hypemethylation of the PR gene locus was associated with loss of protein expression (**Fig. 4.2B**). Within luminal breast cancers, the PR gene locus was lost at a higher frequency in aggressive luminal B cancers subtypes (**Figs. 4.1B and 4.4A**), suggesting an association between loss of PR gene locus and aggressiveness of luminal cancers.

Independent loss of PR modulates estrogen signaling and patient survival

The independent loss of PR in patient tumors modulates estrogen signaling and tumor biology since activated PR was required for ER-regulated genes to differentiate patient survival (**Fig. 4.3A**). In further support of the clinical value of PR, PR-regulated gene profiles had independent prognostic value (**Fig. 4.4D**) and the PR-positivity of tumors positively correlated with favorable clinicopathological parameters (**Figs. 4.4C**). Consistent with the importance of PR for estrogen signaling, tumor ER staining was not able to differentiate survival

outcomes within patients with PR-negative tumors (**Fig. 4.3B**), while tumor ER status significantly correlated with positive survival outcomes within the PR-positive patient cohort (**Fig. 4.3B**). Collectively, these results suggest that PR is a major contributor to the clinical value of ER.

Discussion

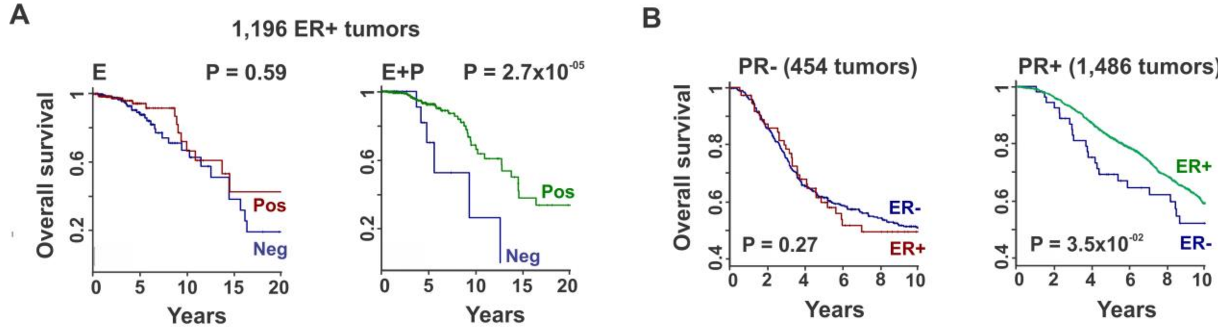
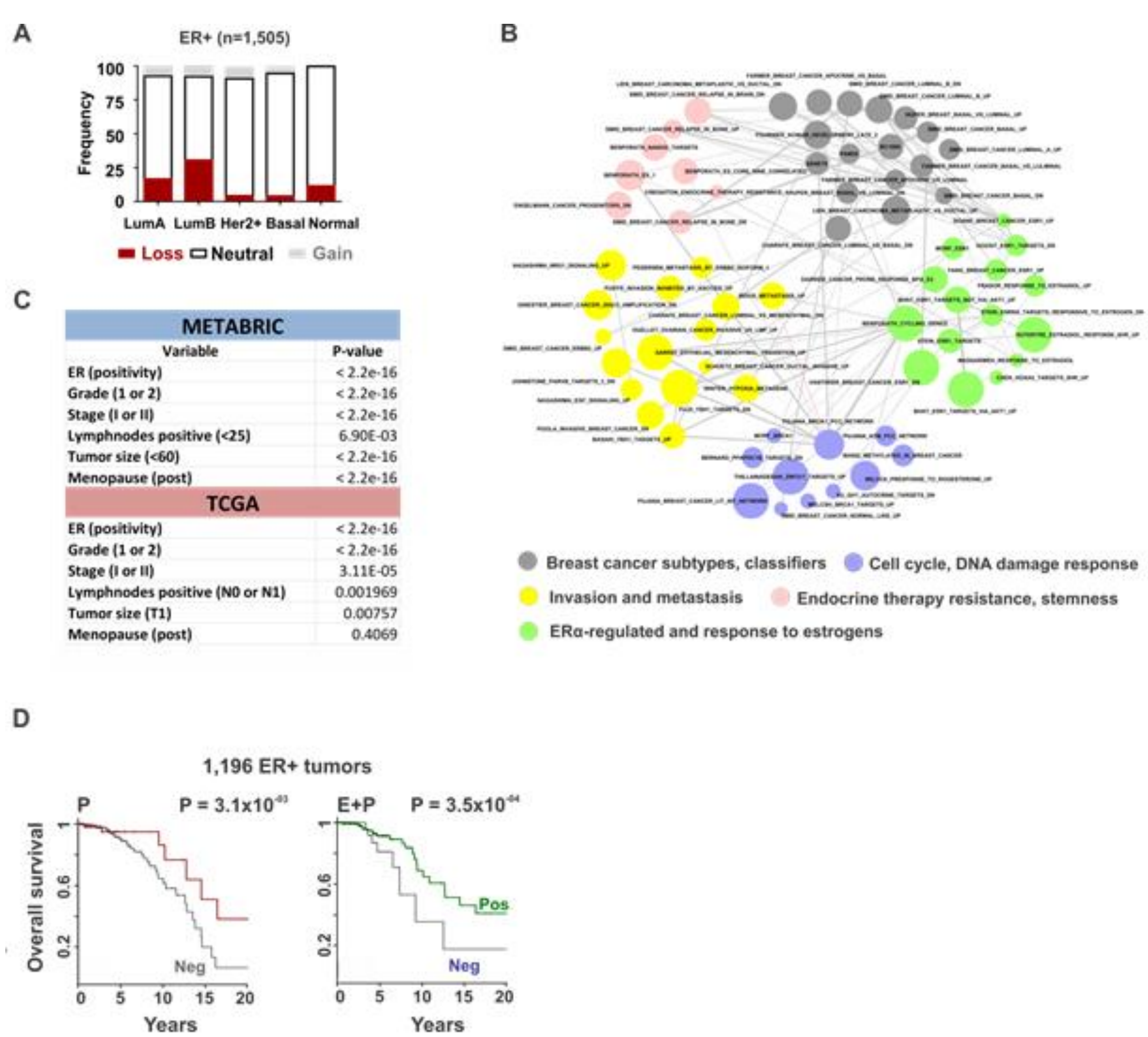


Figure 4.3: Prognostic value of ER depends on activity and presence of PR. (A) Overall survival in TCGA cohort classified by positive or negative correlation to estrogen-regulated signature scores. Curves are presented for before (red) and after (green) progestin-mediated reprogramming of estrogen signaling. **(B)** Overall survival as determined by the differential tumor staining for ER in PR-negative (red) and PR-positive (green) patient cohorts from METABRIC.

PR is an estrogen-induced gene. In clinical decision making PR is not given much importance (relative to ER) because PR is considered a surrogate for functional estrogen signaling. PR is clinically relevant since PR can be independently lost in ER+ tumors due to hypermethylation and/or copy number loss. Loss of PR alters estrogen signaling because well-known prognostic value of ER depends on the presence and activity of PR. In further support of PR's clinical value, PR-regulated genes are enriched in gene signatures for estrogen response, breast cancer subtype classifiers, therapy resistance and metastasis. Reprogramming by PR contributes to the prognostic value of ER-regulated genes and presence of PR in tumors significantly improves the value of ER in differentiating patient survival. Association between

PR-positivity of tumors and favorable clinicopathological parameters further bolsters the clinical significance of obtaining PR and ER values for breast cancer samples.



METABRIC	
Variable	P-value
ER (positivity)	< 2.2e-16
Grade (1 or 2)	< 2.2e-16
Stage (I or II)	< 2.2e-16
Lymphnodes positive (<25)	6.90E-03
Tumor size (<60)	< 2.2e-16
Menopause (post)	< 2.2e-16
TCGA	
ER (positivity)	< 2.2e-16
Grade (1 or 2)	< 2.2e-16
Stage (I or II)	3.11E-05
Lymphnodes positive (N0 or N1)	0.001969
Tumor size (T1)	0.00757
Menopause (post)	0.4069

Figure 4.4: PR tumor staining is clinically relevant and it is associated with favorable clinical outcomes. **(A)** Frequency of copy number variation of PR locus in METABRIC categorized based on PAM50 breast tumor subtypes. **(B)** Network of functional modules enriched in PR-regulated genes. Each node represents a breast cancer signature annotated with its MsigDB identifier. The node size is inversely proportional to the bonferroni adjusted p-value and the edge width correlate with the overlap size of the enrichment between the functional modules. The enriched gene signatures are curated in five categories. **(C)** Associations between positive tumor PR staining and favorable clinicopathological variables in METABRIC and TCGA cohorts. P values are determined by chi-square test. **(D)** Overall survival in TCGA cohort classified by positive or negative correlation to PR signature scores. Curves are presented for progestin scores in the absence (red) and presence (green) of estrogen.

V. Synergy between antiestrogen tamoxifen and the selective PR modulators (SPRMs) CDB4453, CDB4124 and EC313 results in rapid regression of T47D xenografts. Function of PR agonists and antagonists as breast cancer therapies.

Background

Both estrogen and progesterone signaling are critical for hormone responsive cancers in women. Although ER-targeted therapies are routinely used in breast cancer treatments, there is a lack of understanding for the efficacy of anti-PR therapies alone or in combination with anti-ER therapies. The major hindrance to the study of anti-PR therapies is the lack of approved therapeutic PR modulators that are highly specific for PR (*26*, *46*, *124*). The majority of anti-PR modulators such as mifepristone, onapristone and ORG2058 also target glucocorticoid (GR) and/or androgen receptors (*26*, *45*, *46*). Research in breast cancer cell lines, mouse models and xenograft studies have shown additive effects between tamoxifen and mifepristone. However it is unclear whether this observed effect is due to PR activity because mifepristone is a major anti-GR therapeutic (*125*, *126*). Moreover, clinical trials to study efficacy of joint treatments of breast cancer with tamoxifen and mifepristone have been inconclusive and

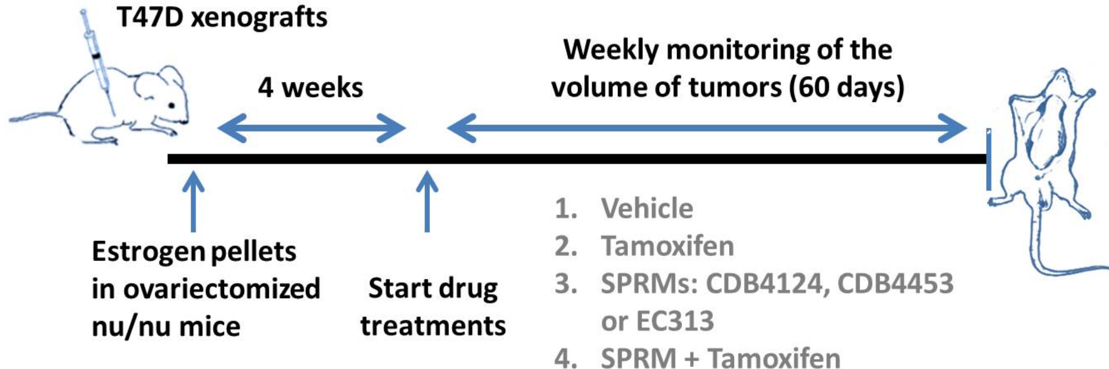


Figure 5.1: Experimental outline: Setup used for the preclinical studies with various PR antagonists (CDB4124, CDB4453 or EC313), alone and in combination with tamoxifen.

have resulted in glucocorticoid signaling related side effects in patients (*127*). Highly-selective PR modulators (SPRMs) might be a viable alternative alone or in combination with antiestrogens for breast cancer treatments. For this reason we investigated SPRMs CDB4453 and CDB4124 from Repros therapeutics and EC313 from Evestra Inc (*43*, *44*, *128*, *129*). Compared to mifepristone, CDB4453 is about 100 times more specific for PR than GR (*43*, *44*). Similarly, CDB4124 is shown to inhibit PR mediated growth in T47D breast cancer cell lines and xenografts (*130*, *131*).

An unsolved paradox in breast cancer is how activated PR can be pro- or anti-tumorigenic in different contexts or how both PR agonists and antagonists can have growth inhibitory effects on PR-positive breast cancer cell models (*8*, *132*, *133*). Both PR agonists (high dose megace (*134–137*) and medroxyprogesterone acetate (*138–143*)) as well as PR antagonists (mifepristone (*127*, *144*), onapristone (*145*), lonaprisan (*146*)) (*26*, *133*, *147*) have been studied as treatments for ER+/PR+ breast cancers with limited success. In addition, multiple clinical trials have studied the efficacy of PR-targeting therapies in breast cancers that have relapsed on antiestrogen therapies (tamoxifen, aromatase inhibitors) and are still positive for PR (*26*, *137*). While PR agonists inhibit estrogen-induced growth in ER+/PR+ breast cancer models, agonist activated PR can be overall tumorigenic by expanding stem cell pool and increasing drug resistant cell population therapy resistance pool (*148*). The complexity of targeting PR for breast cancer treatments is further highlighted by the pro- and anti-proliferative effects of PR ligands in a dose-dependent and PR isoform-specific manner (*19*, *149*). Interestingly, various preclinical studies have reported additive effects of combined antiestrogen and antiprogestin treatments (*45*, *133*), though these findings have not yet been successfully tested in clinical trials (*133*). Our study highlights the observed synergy between tamoxifen and three different SPRMs CDB4124,

CDB4453 or EC313 in treating T47D xenografts (**Fig. 5.1**). A recent independent study (*116*) reported relatively mild effects of combining tamoxifen with progesterone. The molecular details of how agonist or antagonist signaling intersects with tamoxifen signaling are still unclear and they are the focus of ongoing studies.

Results

Joint therapies with tamoxifen and any of the SRPMs (CDB4124, CDB4453 or EC313) results in rapid tumor regression

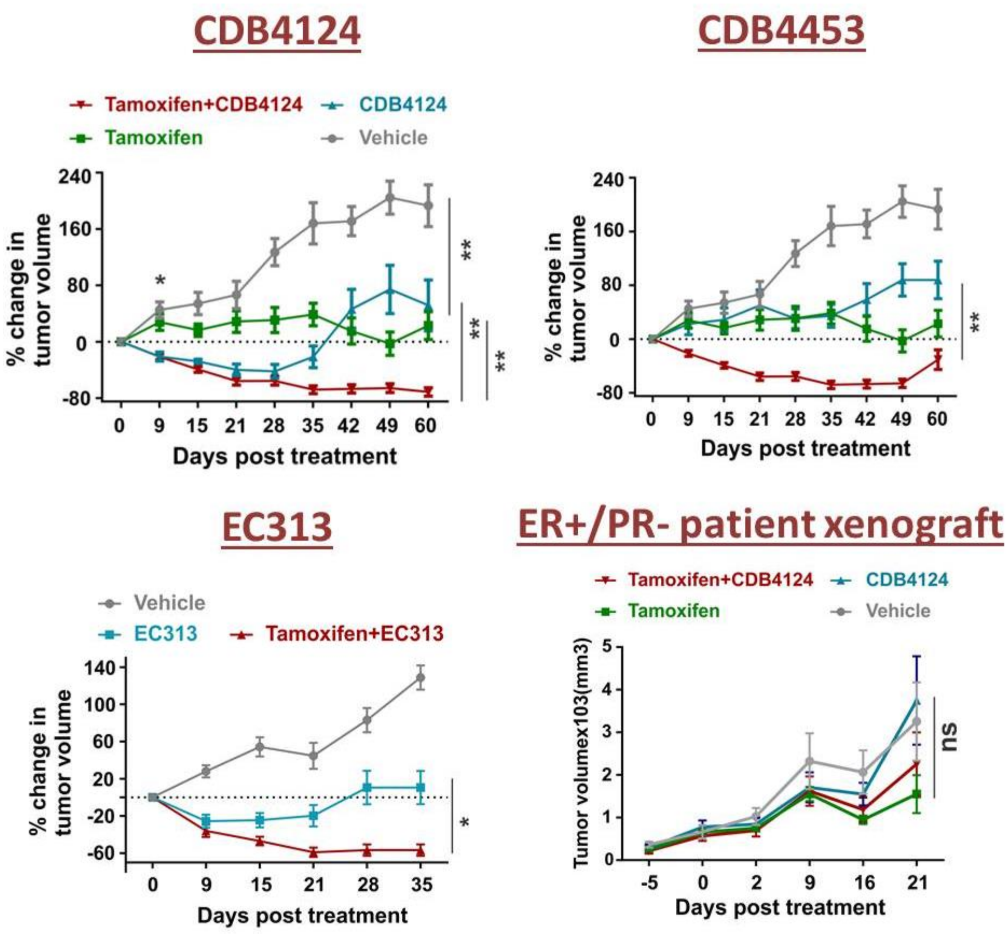


Figure 5.2: Synergy between PR antagonists (CDB4124, CDB4453 or EC313) and tamoxifen results in regression of T47D xenografts. T47D xenografts were grown in ovariectomized nude mice containing estrogen silastic implants and were treated with placebo, tamoxifen, PR antagonist or tamoxifen plus PR antagonist. Three different PR antagonists were studied. Average tumor volume at the start of therapies was 125 mm^3 and percentage change in tumor volume is shown (n = at least 12).

Although ER is the major therapeutic target in ER+/PR+ breast cancers (*33*), SPRMs CDB4124, CDB4453 or EC313 (*43*) effectively inhibited the estrogen-driven growth of ER+/PR+ T47D xenografts (**Fig. 5.2**). These findings highlight the therapeutic value of ER/PR crosstalk in treating ER+/PR+ breast cancers. Importantly, while individual drug treatments inhibited tumor growth, their combined treatment resulted in synergistic regression of tumor volume (**Figs. 5.2 and 5.3A**). The effectiveness of joint treatment was further highlighted by the speed of tumor regression, which occurred in just nine days of combination therapy whereas individual drug treatments required significantly longer achieving tumor growth inhibition, without any net regression at the end of the study. Importantly, tamoxifen/CDB4124 combination therapy maintained the observed 70% tumor regression throughout the course of the study while tumors treated with CDB4124 alone developed resistance after four weeks of therapy. This observed synergy was not seen *in-vitro* in PR-deficient T47D cells and *in-vivo* in ER+/PR- patient derived xenograft model, suggesting that PR is required for these synergistic effects (**Figs. 5.2 and 5.3B**). While combination therapy with two other SPRMs (CDB4453 or EC313) also led to

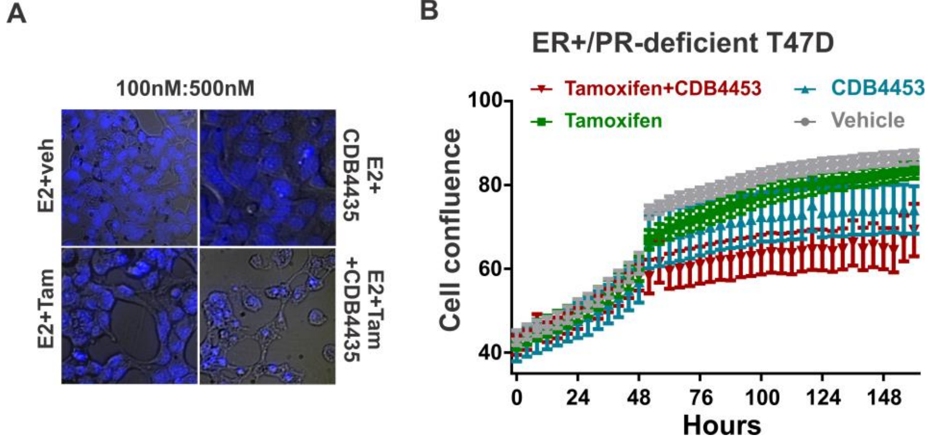


Figure 5.3: Synergy between PR antagonists and tamoxifen T47D cell model system. (A) Overlap of fluorescent DAPI staining on phase contrast images of T47D cells treated with different drugs for 5 days. **(B)** Cell confluence of ER+/PR-deficient T47D grown in estrogenic media (10 nM estradiol) containing vehicle, 4-OH tamoxifen, CDB4453 or CDB4453 plus 4-OH tamoxifen. In-vivo results were highly reproducible, and in contrast, in-vitro results had high day-to-day variability.

tumor regression, these drugs were less effective than CDB4124 in inhibiting growth of xenografts **(Fig. 5.2)**. These results suggest that different SPRMs can have different anti-tumor activity and underscores the need to identify/engineer SPRMs with effective therapeutic value. Collectively, these preclinical findings with three different SPRMs indicate that PR-mediated reprogramming of estrogen signaling is therapeutically relevant and that potent SPRMs, especially in combination with antiestrogens, could be effective therapies in ER+/PR+ breast cancers.

Joint therapies with tamoxifen and any of the SRPMs (CDB4124, CDB4453 or EC313

Whereas our study highlights synergy between tamoxifen and three different PR antagonists CDB4124, CDB4453 or EC313 in treating T47D xenografts (**Fig. 5.2**), an

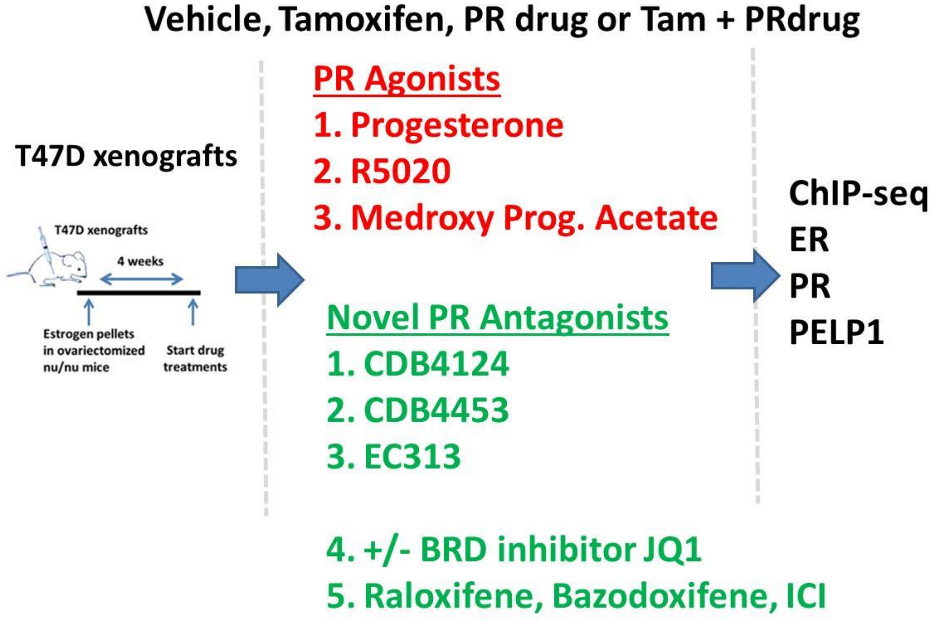


Figure 5.4: Experimental outline used to study how three different PR agonists (natural progesterone, synthetic progestin R5020 or medroxy progesterone acetate) or three different PR antagonists (CDB4124, CDB4453 or EC313) reprogram tamoxifen signaling. A subset of mice that were treated with EC313 plus tamoxifen was also treated with bromo-domain inhibitor JQ1. Additionally, another subset of mice was treated with EC313 plus three different estrogen modulators, raloxifen, bazodoxifene or fulvestrant.

independent study (*116*) reported mild additive effects of combining tamoxifen with progesterone. Side-by-side comparison of how PR agonists and antagonists reprogram tamoxifen signaling would help to optimize use of PR-targeting therapies in treatments for breast cancer. For this purpose, we are studying how three PR agonists (natural progesterone, synthetic progestin R5020 and medroxy progesterone acetate) and four highly-selective PR antagonists (CDB4124, CDB4453, EC313 and EC317) reprogram tamoxifen signaling in ER+/PR+ T47D xenografts **(Fig. 5.4)**.

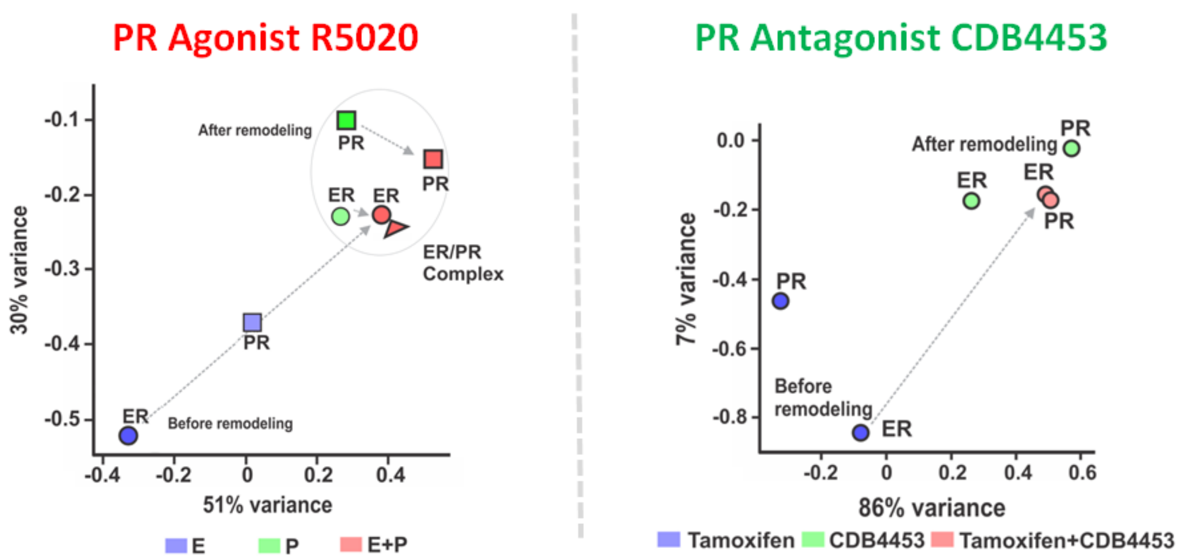


Figure 5.5: Agonist as well as antagonist activated PR redirects ER binding to binding loci for PR and ER/PR complexes. **(A)** PCA plots to depict variance between binding events for ER, PR and ER/PR complexes observed upon treatment with E, PR drug (agonist R5020 or antagonist CDB4453) or E+PR drug. Experiments were R5020 were done in-vitro and experiments with CDB4453 were done in-vivo.

Similar to the PR agonist R5020, the PR antagonist CDB4453 reprograms ER chromatin binding to correlate with the binding of PR **(Fig. 5.5)**. Ongoing studies will address whether PR differentially reprograms estrogen signaling in an isoform-, dose- and ligand-

selective manner. These studies should resolve some of the mechanistic complexities of PR signaling and serve as a guide to more effective PR-targeting therapies in breast cancer.

Discussion

PR-targeted therapies have been studied with limited success in various cell lines, mouse models and clinical trials (*26*, *150*). Majority of approved PR modulators have cross reactivity with other nuclear receptors, thus limiting their efficacy (*43*). Importantly, there is a lack of knowledge about combined use of ER and PR-targeting therapies in clinic (*26*). ER/PR crosstalk is therapeutically relevant since combination therapies with tamoxifen and any of the three SPRMs (CDB4124, CDB4453 or EC313) result in tumor regression, while individual therapies inhibit tumor growth. Importantly, SPRM alone inhibits estrogen-mediated growth of T47D xenografts suggesting that targeting of PR alone can have some anti-tumor effect, although tumors rapidly develop resistance to individual treatment with any of the SPRMs. SPRMs act through PR because these anti-tumor effects of individual or combination therapies with SPRM are not observed *in-vitro* in PR-deficient systems and *in-vivo* in ER+/PR- patient-derived xenograft model. Out of all the three SPRMs tested, CDB4124 shows maximum efficacy indicating that it is important to identify/engineer effective SPRMs for breast cancer treatments. In contrast to the findings of cytotoxic tumor regression upon joint therapies with tamoxifen and SPRM, combination therapy with PR agonist progesterone and tamoxifen results in cytostatic inhibition of tumor growth (*116*). It is interesting that agonist- and antagonist-activated PR potentiates responses to tamoxifen, although only antagonist drives tumor regression. Further research is needed to optimize PR-targeting therapies in clinic since agonist-activated PR is known to enrich stem cell and receptor-negative populations, expand drug resistant clones and contribute to tumor relapse (*65*, *148*). These results indicate that PR-mediated reprogramming of

estrogen signaling is therapeutically relevant and further analyses of tumor markers for patient selection would help to translate PR-targeting therapies, especially in combination with antiestrogens, as treatments for ER+/PR+ breast cancers.

Discussion

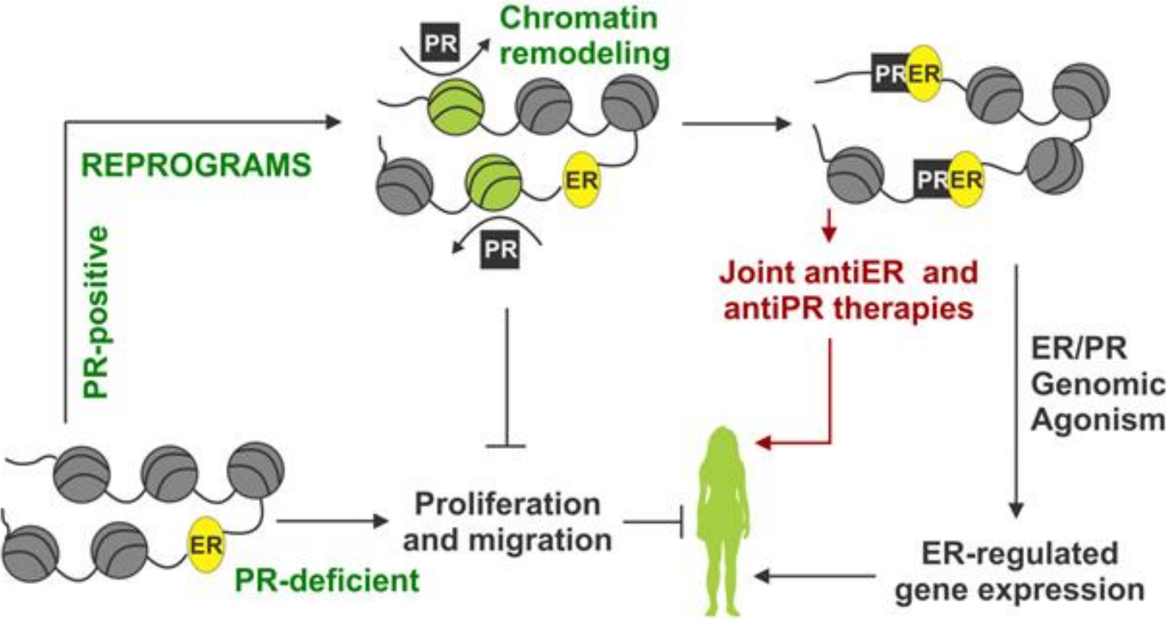


Figure 6.1: Proposed model for ER/PR crosstalk: PR remodels chromatin to redirect ER binding and reprogram estrogen signaling in a PR isoform-specific manner. PR is a genomic agonist and a phenotypic antagonist of estrogen signaling.

Estrogen and progesterone are key ovarian hormones that orchestrate menstrual cycles, are essential for reproduction and are critical in the regulation of breast cancers (*2*, *151*, *152*). During the menstrual cycle, estrogen levels are high pre-ovulation while progestin levels are high post-ovulation. In pregnancy, progestin levels increase over tenfold compared to progestin's normal plasma levels and they return to baseline post-partum. These two hormones regulate different sets of physiological processes in the two halves of menstrual cycles as well as in pregnancy (*3*, *152*). Our findings of extensive overlap between estrogen and progesterone signaling indicate that these two hormones have been co-evolving to balance gene expression and physiologic phenotypes. Furthermore, the dominance of progestin over estrogen signaling suggests functions for progestin to reprogram estrogen effects post-ovulation during menstrual

cycles and also as a hormone to maintain pregnancy. This feedback between ER and PR controls vital cellular processes and its dysregulation may lead to cancer.

In isolation, progestin and estrogen regulate genes in similar directions while there are differences in the intensities of gene expression and the functional annotation of the genes induced, suggesting that these hormones are genomic agonists. Similarly, in isolation, both of these hormones activate oncogenic pathways in similar directions although pathway activation scores with progestin alone were lower than those observed with estrogen-only treatments. These results indicate that when considered in isolation, PR is a weak phenotypic agonist of estrogen-mediated phenotypes. Importantly, when both the hormones are present, progestin dominates estrogen action such that the levels of transcriptomes, cellular processes and receptor recruitment observed with joint activation of ER and PR correlate with those observed with PR alone, but not ER alone. Despite the correlation, the transcriptomes on dual treatments are optimally different from individual treatments such that the ingenuity analyses predicted antagonism of oncogenic processes and indicated major tumor suppressive functions of concerted but not individual activity of these receptors. Not surprisingly, when both the hormones are present, PR becomes a phenotypic antagonist and opposes estrogen-mediated phenotypes. The consequences of genomic agonism and phenotypic antagonism are reflected in the observation that the well-known prognostic value of ER in breast cancer depends on the presence and activity of PR.

Early investigations into the interaction between ER and PR were restricted to promoter-proximal regions for a limited number of genes engineered in artificial vector constructs (*48–54*). However, ER commonly binds to enhancer regions distal to promoters (*58*, *59*) and we report that binding of PR and ER/PR complexes also occurs primarily at the distal enhancers. Additionally, earlier studies lack a genomic focus and they do not capture real

chromatin dynamics because they were performed on engineered constructs. PR binds to nucleosomes and recruits chromatin remodeling complexes to initiate the opening of chromatin by displacement of histones H1 and H2A/H2B dimers. PR-mediated chromatin remodeling reprograms the regulation of gene expression (*87*, *89*). Our results showing significant PR-mediated reprogramming of ER binding and activity suggest that nucleosome remodeling by PR might facilitate reprogramming of estrogen signaling. Chromatin remodelers FOXA1 and NF1C play dual roles in both estrogen and progestin signaling by facilitating and repressing ER- and PR-mediated gene expression of different sets of genes. Importantly, depletion of either of these factors did not significantly impact the effects of PR on ER-regulated gene expression, suggesting PR itself can have pioneering functions to open up chromatin for ER genomic binding.

Earlier studies have reported that PR interacts with ER in the cytoplasm and that this interaction is critical for activation of c-src/ERK pathways by progestin (*55*, *56*). Another study found that unliganded PRB forms a complex with ER and PELP1 protein to enhance estrogen responsiveness in breast cancer (*57*). The genomic recruitment of ER/PR complexes and the functional impact of ER/PR complexes on the modulation of estrogen signaling and breast cancer are not well studied. We found that PR forms complexes with ER to reprogram ER binding to enhancers and regulatory loci enriched for BRCA1, PR, ER and nuclear factors. BRCA1 has been widely implicated in breast cancer risk, genesis and progression (*110*) and BRCA1/2 carriers are known to have 121% higher levels of serum progesterone level (*112*). Additionally, PR antagonists have been proposed as cancer prevention therapy in BRCA1-mediated mammary tumorigenesis in mice (*113*). Despite the clinical evidence for active progesterone signaling in BRCA1-mediated tumorigenesis, it is not mechanistically understood

how PR might contribute to these cancers. Our finding of preferential recruitment of ER/PR complexes to BRCA1 and ELK4 binding loci indicates a potential role of BRCA1 in ER/PR crosstalk.

Interestingly, despite the controversy over the clinical value of PR, breast tumors are routinely evaluated for ER and PR expression, although tumor ER status is given priority. These clinical decisions largely stem from relatively weak PR staining observed in tumors (*22–25*), routine use of anti-ER but not anti-PR targeting therapies (*3*, *26*), insufficient understanding of the intersection of ER and PR signaling and lack of large enough patient cohorts to conclusively study the clinical value of PR (*21*, *22*, *25*, *27–36*). We found that PR-regulated genes are extensively enriched in breast cancer signatures and that they can diagnostically differentiate breast tumor subtypes and survival outcomes. Furthermore, we observed that PR reprograms binding of ER to genes highly expressed in luminal but minimally expressed in more aggressive basal-like cancers. Although PR is an estrogen-induced gene, PR can be independently lost in ER+ tumors due to hypermethylation and copy number loss of the PR gene locus. The loss of PR alters estrogen signaling and breast cancer responses because activated PR significantly contributes to the prognostic value of ER-regulated genes. In support, PR tumor status significantly correlates with favorable clinical indices such as positive ER status, smaller tumor size, fewer numbers of positive nodes and lower tumor stage.

The major hindrance to the study of anti-PR therapies in breast cancer treatment is that approved anti-PR drugs, such as mifepristone, onapristone and ORG2058, also target GR and/or AR (*43*, *44*). Various in-vitro, mouse models and human xenograft studies have shown additive effects between anti-PR drugs and tamoxifen, however it is difficult to attribute this synergy to PR activity due to major antiglucorticoid activities of these drugs (*26*, *45–47*).

Clinical trials to study the therapeutic efficacy of joint tamoxifen and mifepristone treatments have been inconclusive and have resulted in glucocorticoid signaling related side effects in patients. A few new-generation anti-PR drugs such as lonaprisan have low antiglucocorticoid activities and other PR-specific drugs, like CDB4124, CDB4453, EC313 and EC317 are under development (*26*, *43*, *44*). We report that synergy between the PR-modulators CDB4124, CDB4453 or EC313 and tamoxifen results in rapid regression in T47D human xenografts, while individual treatments inhibit tumor growth without net regression at the end of the study. Importantly, SPRM alone inhibits estrogen-mediated growth of T47D xenografts suggesting that targeting of PR alone can have some anti-tumor effect, although tumors rapidly develop resistance to individual treatment with any of the SPRMs. These xenograft findings underscore the therapeutic relevance of ER/PR crosstalk and provide a strong rationale to explore joint anti-ER and anti-PR targeting therapies in ER+/PR+ breast cancer treatments. An independent study (*116*) reported cytostatic effects of combining tamoxifen with progesterone. The molecular details of how agonist or antagonist signaling intersects with tamoxifen signaling are still unclear and are the focus of ongoing studies. It is interesting that agonist- and antagonist-activated PR potentiates responses to tamoxifen, although only antagonist drives tumor regression. Further research is needed to optimize PR-targeting therapies in clinic since agonist-activated PR is known to enrich stem cell and receptor-negative populations, expand drug resistant clones and contribute to tumor relapse (*65*, *148*). Further analyses of tumor markers for patient selection would help to translate PR-targeting therapies, especially in combination with antiestrogens, as treatments for ER+/PR+ breast cancers.

In summary, extensive crosstalk occurs between estrogen and progesterone signaling. PR reprograms estrogen signaling as a genomic agonist and a phenotypic antagonist.

These findings provide mechanistic explanations for how PR contributes to the clinical value of ER and it strongly implicates the use of PR both as a prognostic/predictive biomarker and as a therapeutic target in ER+/PR+ breast cancers.

Future Directions

Many exciting avenues of investigations emerge from this study. It would be interesting to study the molecular mechanism that decouples genomic agonism between ER and PR to the observed phenotypic antagonism in the presence of both hormones. The genomics data can be mined to come up with prognostic gene signatures with better predictive powers than existing tools such as OncoType Dx. The mechanism behind dominant reprogramming of estrogen signaling by PR is not well understood and it would be interesting to compare shorter versus longer term reprogramming of estrogen signaling by PR. It is possible that in long-term exposures to progestins, PR moderately reprograms estrogen signaling. This study does not illuminate whether ER and PR are interacting through direct or indirect physical contacts. It would be worthwhile to understand the nature of ER/PR interactions and to investigate cofactor proteins that bring these proteins in close contacts. This knowledge of accompanying adapter proteins would also be useful in generating new therapies of breast cancer.

The chromatin remodeling role of PR and the role of so-called pioneer proteins need further investigations. It is to be seen whether the observed chromatin remodeling feature of PR is a general property that is shared by other nuclear receptors or is this primarily confined to PR or other members of the NR3C family of nuclear receptors. If other members of NR3C family reprogram estrogen signaling (in addition to PR, GR is already known to reprogram estrogen signaling), it would be interesting to study similarities and differences in the reprogramming of estrogen signaling by the other three members of this family. Follow-up on the findings as to why depletion of FOXA1 or NFIC did not significantly impact effects of PR on ER-regulated gene expression would further illuminate finer details of ER/PR crosstalk. It is

possible that PR is affecting ER activity through non-genomic pathways and further studies in this direction will be helpful.

Further research is needed to optimally exploit PR in breast cancer prognosis and treatments. There is a need to identify/engineer more effective SPRMs for breast cancer therapies. It is interesting that agonist- and antagonist-activated PR potentiates responses to tamoxifen, although only antagonist drives tumor regression. Research is needed to optimize PR-targeting therapies in clinic since agonist-activated PR is known to enrich stem cell and receptor-negative populations, expand drug resistant clones and contribute to tumor relapse (*65*, *148*). Further analyses of tumor markers for patient selection are needed to translate PR-targeting therapies, especially in combination with antiestrogens, as treatments for ER+/PR+ breast cancers.

Conclusions

These studies in human tumor tissues (and multiple cell models) exposed *ex-vivo* (and *in-vitro*) to different combinations of estrogen and progestin revealed that in isolation, both of these hormones regulate genes in similar directions indicating genomic agonism between them. Similarly in isolation, progestin is a weak phenotypic agonist, however, when both the hormones are present, progestin antagonizes estrogen-regulated cellular processes and phenotypes. Importantly, on dual hormonal treatments, progestin dominates estrogen action as the gene expression patterns of tissues exposed to both hormones correlated to progestin alone in a minority of tumor samples and all the PR+ cell models. Despite the observed correlation, the transcriptomes on dual treatments are optimally different from individual treatments such that the ingenuity analyses predicted antagonism of oncogenic processes and indicated major tumor suppressive functions of concerted but not individual activity of these receptors. PR is necessary and sufficient for these progestin actions and these genomic observations are reflected in the functional assays of cell proliferation, migration and invasion.

PR positivity of human tumors is associated with more consistent ER binding patterns in chromatin in breast tumors. PR remodels nucleosomes to noncompetitively redirect ER genomic binding to distal enhancers, BRCA1 binding motifs and sites that link PR and ER/PR complexes. The binding pattern of ER in the presence of PR occurred distal to promoters in both human tumors and cell models. PR reprograms ER chromatin binding in an isoform-specific manner. Activated PRA/PRB heterodimers expands ER binding events fourfold, while PRA reduces ER binding by 75% and PRB primarily redistributes ER binding.

ER/PR crosstalk is likely non-competitive in nature because ER and PR co-immunoprecipitate together in breast cancer cells and changing relative levels of these receptors (by overexpressing ER or moderately depleting PR) did not significantly impact effects of PR on ER-regulated gene expression. ER/PR complexes are recruited to the genome and they preferentially bind to enhancer regions and motifs enriched for BRCA1, suggesting a potential role for BRCA1 in ER/PR crosstalk. Interactions between ER and PR can facilitate interactions between distal regulatory elements and the ER/PR complexes may not always be preassembled since binding sites for these receptors interacted via long-range looping. Chromatin remodelers FOXA1 or NFIC are not required for the effects of PR on ER-regulated gene expression. There is significant overlap of ER binding and DNase hypersensitive regions created by PR demonstrating that PR remodels chromatin to modulate chromatin accessibility for ER binding.

The presence and activity of PR is a strong indicator of prognostic value of ER. The observed loss of PR protein expression in a subset of ER+/PR+ breast cancers, due to hypermethylation or loss of the PR gene locus, and associated loss of ER prognostic value, further highlights the role of PR as an essential ER modulator. In agreement, tumor PR status and PR-mediated reprogramming significantly contributes to the ability of ER and ER-regulated genes to differentiate patient survival outcomes respectively.

Therapeutically, synergy between PR-modulators/antagonists CDB4124, CDB4453 or EC313 and tamoxifen results in rapid regression of T47D human tumor xenografts, while individual treatments inhibit tumor growth without net regression at the end of the study. Among all the three SPRMs investigated, CDB4124 had highest efficacy indicating that there is a need to identify SPRM with maximum clinical benefit. These preclinical findings underscore the therapeutic relevance of ER/PR crosstalk. While combination therapy with PR agonist and

tamoxifen is reported to result in cytostatic inhibition of tumor growth, this study reports that joint therapies using PR antagonist lead to cytotoxic tumor regression. Studies are ongoing as to how agonist- and antagonist-activated PR reprograms estrogen signaling.

In conclusion, the principle of genomic agonism and phenotypic antagonism provides mechanistic explanations for the clinical value of PR as a prognostic biomarker and as a therapeutic target. On dual hormonal treatments, progestin dominates estrogen action such that the levels of transcriptomes, cellular processes and receptor recruitment observed with joint activation of ER and PR correlate with those observed with PR alone, but not ER alone. This study strongly implicates using PR both as a prognostic/predictive biomarker and as a therapeutic target in ER+/PR+ breast cancers.

Acknowledgements for the resources and facilities used

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Patient tumor explants

Twelve tumor samples and all relevant clinical information were obtained from women undergoing breast surgeries at Burnside Private Hospital, Adelaide (approval numbers: H-065-2005; H-169-2011) and University of Texas Southwestern medical center (approval numbers: STU 032011-187). Eight tumors are ER+/PR+ (P1 to P8) and four tumors are ER+/PR- (N1 to N4). The collected tissue samples were processed within an hour of surgeries and they were cultured via three-dimensional *ex-vivo* explant methodologies as described before (*71*). Briefly, sliced pieces of tumors were incubated on gelatin sponges for 36 hours in regular RPMI media. Representative pieces of tumors were in parallel fixed in 4% formalin and subsequently immunohistochemistry for ER and PR protein was performed to assess the status of tumors for these receptors. In parallel, apportioned tumors were treated with vehicle, estradiol (E2), R5020, or the combination of estradiol and R5020 for 24 or 48 hours prior to being subjected to ki67 immunostaining and RNA extraction. Due to limited tumor size, R5020 treated explant was not available for tumor N5 and both vehicle and R5020 treated explants were not available for tumor P8. See section on RNA-seq data analysis for details. Pieces of treated ER+/PR+ and ER+/PR- tumor samples were immunostained for proliferation marker ki67 and the slides were scanned at 40X magnification using NanoZoomer microscopy system.

To study the transcriptome changes in response to hormone treatments, sequencing libraries were made from the RNA extracted from each of the treated tissue samples and next generation sequencing was performed. Due to limited tumor size, R5020 treated explant was not available for tumor N1. See the section on RNA-seq data analyses for further details.

Xenograft experiments

Nude mice (J:nu) were obtained from Jackson Labs at an age of 4-6 weeks old. All mice were ovariectomized females with an average weight of 20 grams. Nude mice were anesthetized with isoflourane and an incision was made on the back of the neck. A silastic implant containing 17-β-estradiol was inserted under the skin and several sutures were applied. Circulating estradiol determinations were made by the University of Chicago clinical laboratory by obtaining blood from mice with implants by cardiac puncture. The 5 mg 17-β-estradiol silastic implants were made as follows: a 1.4 cm portion of silastic tubing (Dow Corning 0.078 in x 0.125 in OD Catalog no. 508-009) was filled with 5 mg 17-β-estradiol (Sigma E2758-1G) and 10mg cellulose (Sigmacell Cellulose Type 20, 20um S3504-500G) and sealed with aquarium glue.

Sufficient numbers of T47D cells were cultured in-vitro and at the day of cell injections, the cells were harvested and suspended in PBS. 10 million T47D cells were injected in mammary fat pad along with biodegradable matrigel. Two tumors per mice were grown (left and right hand side mammary fat pad). Approximately after a month of initial cell injections, when the tumors reached 120 mm^3, the mice were implanted with 25 mg and 90 days release pellets forCDB4453, CDB-4124, placebo, Tamoxifen, or CDB-4124/CDB4453 in combination with Tamoxifen (Innovative Research of America). EC313 was administered as five i.p injections (10 mg/kg/day) per week. Xenograft tumor size was measured weeklyand percentage change in tumor volume since the start of therapy is reported. Sixty daysdays after implantation, tumors were excised, weighed, and fixed or stored in liquid nitrogen for subsequent analysis.

Cell culture

Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. T47D cells and derived sublines were provided by Dr. Kathryn Horwitz(*77*). Briefly, ER+/PR-low T47D cells were derived from parent ER+/PR+ T47D cells through flow cytometry and PRA or PRB was stably re-expressed in ER+/PR-low T47D cells to create ER+/PRA+ or ER+/PRB+ T47D cells. Media used to grow ER+/PRA+ and ER+/PRB+ T47D was supplemented with 200 µg/ml of geniticin (life technologies, #10131-027) for selection; media to grow ER-low/PR-low/GR+ T47D cells was supplemented with 50 µg/ml gentamycin sulfate (mediatech, #30-005-CR). Prior to experiments, cells were cultured for 48 hours in phenol red free RPMI 1640 supplemented with charcoal-stripped FBS and 1% penicillin streptomycin (steroid-deprived media). Estradiol (Sigma #E8875-250MG) and R5020 (PerkinElmer #NLP004005MG) dissolved in ethanol (vehicle) were used at a final concentration of 10 nM for all experiments.

Cell migration (scratch wound) assays

T47D cells were grown in 96 well ImageLock plates (Essen Bioscience #4379). After the cells reached approximately 90% confluence, they were deprived of steroids for 48 hours. Thereafter, scratch wounds were made using 96 pin WoundMaker (Essen Bioscience #4493) and washed twice with phosphate buffered saline (PBS). Cells were then treated and the confluence of the wound was analyzed over time using an integrated cell migration analysis module (Essen Bioscience #9600-0012). Wound confluence is expressed as the percentage of the wound area occupied by cells and is plotted 48 hours after drug treatments.

Cell invasion (matrigel invasion) assays

Matrigel (BD biosciences #356231) was dissolved 1:40 in steroid-deprived RPMI 1640 and 50 µl was aliquoted to the bottom of each well of a 96 well ImageLock (Essen Bioscience #4379) plate. Thereafter, the plate was incubated at 37°C for 30 minutes to allow the matrigel to solidify, and excess media was removed. Cells were then plated on the top of the matrigel layer and allowed to grow for 48-72 hours until they reached approximately 100% confluence. Subsequently, scratch wounds were made using a 96-pin WoundMaker (Essen Bioscience#4493) and washed with PBS. Matrigel was then dissolved in steroid-deprived RPMI 1640 containing hormone treatment, and another 50 µl layer of matrigel was applied above the cells. After complete solidification, 200 µl of steroid-deprived RPMI 1640 containing hormone treatment was added to the wells. Confluence of the matrigel invasion was analyzed over time using integrated cell migration analysis module (Essen Bioscience #9600-0012). Matrigel invasion is expressed as the percentage of the matrigel-filled wound area that is occupied by cells. Matrigel invasion represents 48 hours post-treatment.

Confluence and proliferation studies

Cells were plated in a 96 well plate. After reaching approximately 30% confluence, cells were deprived of steroids for 48 hours and then treated as indicated. The cell confluence was measured over time using the Essen Bioscience Incucyte. Confluence is defined as the percentage of area covered by cells. To count cell number, T47D cells nuclei were labeled green with Cell Player NucLight Green (Essen Bioscience) and counted via the Incucyte.

Transfection

siRNA: Cells were cultured to approximately 50-60% confluence, the media was changed to OptiMEM and cells were transfected with 100 nM of each siRNA using lipofectamine 2000. SiGENOME RNA pools for siPGR (M-003433-01), siNF1C (M-008362-00), siFOXA1 (M-010319-01) and non-targeting control (D-001206-13) were obtained from Dharmacon. After 24 hours the media was replaced with steroid-deprived RPMI 1640 for 48 hours and cells were incubated with indicated treatment for 12 hours and collected for analysis.

ER overexpression: Cells were cultured to approximately 50-60% confluence and then transfected with either pcDNA3.1 plasmid containing full-length ESR1 or a control pcDNA3.1 vector using Fugene HD. After 24 hours the media was changed to steroid-deprived RPMI 1640 for 48 hours, and the cells were treated with ethanol, estradiol, R5020 or estradiol plus R5020 for 12 hours before analysis.

Fluorescent microscopy

T47D cells were grown in 96-well plates until approximately 30% confluent. Then the cells were cultured in steroid-deprived RPMI 1640 for 48 hours before being treated for 5 days with vehicle, 4-OH tamoxifen, CDB4453 or jointly with 4-OH tamoxifen plus CDB4453 in the presence of 10 nM estradiol. Final concentration of 100 nM and 500 nM was used for 4-OH tamoxifen and CDB4453 respectively. After 5 days, the cells were washed twice with ice-cold PBS, fixed with 4% paraformaldehyde for 10 minutes and then stained with DAPI (1 ug/ml, Life Technologies, USA) for 10 minutes. Cell images were taken using an Axiovert 100tv (Zeiss, USA).

Protein expression

Cells were grown to approximately 60-70% confluence and lysed with standard RIPA buffer. The resulting total cell lysate was run on SDS-PAGE gel, transferred on to nitrocellulose membrane and immunoblotted using antibodies for the proteins of interest. Antibodies used for immunoblotting are anti-ER (HC20 from Santa Cruz), anti-PR KD68 (in-house developed), anti-actin (A-2228 from Sigma), anti-FOXA1 (Ab5089 from Abcam) and anti-NF1C (gift from Dr. Naoko Tanese, NYU). Protein expression was normalized to actin loading control.

Co-immunoprecipitation

T47D cells were plated in 10cm dishes and serum starved for 72 hours before treatment. Cells at approximately 70-80% confluence were then treated as indicated. Cells were washed twice with 5 mL ice-cold PBS, harvested via scraping and pelleted at 4°C. Cells were suspended in 300µl lysis buffer (0.1% CHAPS, 40 mM HEPES, pH 7.5, 120 mMNaCl, 1mM EDTA, 10mM Na Pyrophosphate and 10mM β-glycerophosphate; supplemented with PICS (1:100), 50mMNaF, 0.5nM $NaOV_4$) and incubated on ice for 15 minutes. The cells were lysed via three cycles of snap freeze in liquid nitrogen followed by thaw on ice. Protein concentration was measured and 2 mg of total protein was used for CoIP. Five percent, by volume, of the sample was saved as input. Samples were incubated overnight at 4^oC with 0.8 µg anti-ER (Santa Cruz Biotechnology HC-20) and immunoprecipitated with 30 µl of magnetic protein G beads (Life Technologies #10004D) for 1 hour. After washing three times with lysis buffer, samples were eluted in 20 µl of standard 2X Laemmli buffer and loaded on the gel alongside input samples. The gel was then immunoblotted for ER and PR.

Capture of Associated Targets on Chromatin (CATCH)

T47D cells were grown in regular RPMI media. When the cells reached 80% confluence they were fixed with 1% formaldehyde for 10 minutes, followed by quenching with 250 mM Tris pH 8.0. The fixed cells were harvested, lysed in lysis buffer and sonicated in diagenode biorupter (30 sec high/30 sec low) for 15 minutes. The sheared chromatin was pre-cleared of any endogenous biotin by incubating them with streptavidin beads for 25 min. Subsequently, the pre-cleared chromatin was obtained and the beads were discarded. 20 µl of the pre-cleared chromatin was used as input. Then to pull down the genomic region of interest, the pre-cleared chromatin was incubated with specific biotinylated oligos in a controlled thermal reaction. Biotinylated oligos specific to the genomic regions of interest were designed and ordered from IDT. Unhybridized oligos were removed and the mixture was incubated with streptavidin beads to immunoprecipitate chromatin annealed to the biotinylated oligos. Immunoprecipitated protein-DNA complexes were washed with wash buffer and the washed complexes were eluted from streptavidin beads with elution buffer. Protein-DNA complexes were de-crosslinked to obtain DNA fragments attached to the protein by incubation at 65°C for 6 hours. Finally, the DNA fragments were purified using the phenol:chloroform:isoamyl alcohol extraction method. The interaction between genomic regions was assessed by the PCR enrichment of that genomic region in the final pulled down DNA fragments. PCR was performed with primers designed for regions suspected to be interacting with the targeted pull-down. PCR of the pulled down region was used as a positive control. Primers flanking a disparate genomic region (on chromosome 21), were used a negative control. PCR with control primers flanking regions up- and/or downstream of these interacting regions were used as experimental controls. The primer sequences used for chromosome capture are provided in **Table 2**. The products of the

PCR enrichment were sequenced to confirm the identity of purified regions and other genomic regions that interact with it.

Chromatin immunoprecipitation (ChIP) and ChIP-sequencing

Cells were grown in steroid-deprived RPMI for 48 hours to approximately 80% confluence, before being treated for 45 minutes with ethanol, estradiol, R5020 or estradiol plus R5020. Cells were then fixed with 1% formaldehyde for 10 minutes and the crosslinking was quenched with 0.125 M glycine for 5 minutes. Fixed cells were suspended in ChIP lysis buffer (1 ml 1M Tris pH 8.0; 200 µl 5M NaCl; 1 ml 0.5M EDTA; 1 ml NP-40; 1 g SDS, 0.5 g deoxycholate) and sheared in the Diagenode Biorupter for 20 minutes (30 second cycles). 100 µl of sheared chromatin was removed as input control. A 1:10 dilution of sheared chromatin in ChIP dilution buffer (1.7 ml 1M Tris pH 8.0; 3.3 ml 5M NaCl; 5 ml 10% NP-40; 200 µl 10% SDS; to 100 ml with H_2O), 4 µg antibody and 30 µl magnetic DynaBeads were incubated in a rotator at 4^oC overnight. Chromatin was immunoprecipitated overnight using anti-ER (Santa Cruz Biotechnology HC-20), anti-PR (KD68) or serum IgG. To ChIP for histone enhancer marks, anti-H3K4me1+ (Abcam, ab 8895) and anti-H3K27ac1+ (Abcam, ab 4729) antibodies were used. Next, the immunoprecipitated chromatin was washed with ChIP wash buffer I (2 ml 1M Tris pH 8.0; 3 ml 5M NaCl; 400 µl 0.5M EDTA; 10 ml 10% NP-40; 1 ml 10% SDS; to 100 ml with H_2O), ChIP wash buffer II (2 ml 1M Tris pH 8.0; 10 ml 5M NaCl; 400 µl 0.5M EDTA; 10 ml 10% NP-40; 1 ml 10% SDS; to 100 ml with H_2O), ChIP wash buffer III (1 ml 1M Tris pH 8.0; 5 ml of 5M LiCl; 200 µl 0.5M EDTA; 10 ml 10% NP-40; 10 ml 10% deoxycholate; to 100 ml with H_2O) and TE (pH 8.0). Elution was performed twice from beads by incubating them with 100 µl ChIP-elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65^oC for 15 minutes each. The eluted protein-DNA complexes were de-crosslinked overnight at 65^oC in 200 µM NaCl. After

de-crosslinking, the mixture was treated with proteinase K for 45 minutes followed by incubation with RNAse A for 30 minutes. Finally, DNA fragments were purified using Qiagen PCR purification kit and reconstituted in 50 µl nuclear-free water. Real time PCR was performed using SYBR green (Table S1). For ChIP-seq library preparations, libraries were prepared using KapaBiosystems LTP library preparation kit (#KK8232) according to the manufacturer's protocol, starting with 15 µl of DNA. For the PCR enrichment step of the library preparation protocol, twelve PCR cycles were performed.

ChIP followed by ChIP (ReChIP) and ReChIP-sequencing

T47D cells were treated for 45 minutes with ethanol or estradiol plus R5020. ChIP was then performed using anti-ER (Santa Cruz Biotechnology HC-20) as described above, however, chromatin was eluted in ReChIP elution buffer (1X TE, 2% SDS, 15mM DTT supplemented with protease inhibitors). The eluted anti-ER ChIP sample was diluted 1:20 in ChIP dilution buffer supplemented with 50 µg BSA and protease inhibitors. The secondary ChIP (ReChIP) was performed using anti-PR (KD68) and anti-IgG. Primer sequences used for reChIP-qRT-PCR are provided in Table S1. For reChIP-seq library preparations, the final reChIPed DNA fragments were reconstituted in 30 µl nuclease-free water. 15 µl of DNA was used to prepare ChIP-seq libraries using Kapa Biosystems LTP library preparation kit (#KK8232) according to the manufacturer's protocol. For the PCR enrichment step of the library preparation protocol, twelve PCR cycles were performed.

RNA expression and RNA-sequencing

Cells were grown to approximately 60-70% confluence and then treated as indicated for 2, 4, 8 or 12 hours. Fresh patient tumors were treated *ex-vivo* for either 24 or 48

hours. Total RNA was extracted using QiagenRNAeasy kit, RNA was converted to cDNA, and transcript levels were analyzed in the cDNA by real time PCR using Taqman probes. Transcript specific oligonucleotides labeled with Taqman probes were procured from IDT and Taqman reagents were purchased from applied biosystems. For RNA-seq library preparations, poly-A tailed mRNA was purified from 250ng total RNA using NEBnext Poly(A) mRNA magnetic isolation module (NEB #E7490). The sequencing libraries were prepared using NEBnext RNA-seq library preparation kit (NEB #E7530). For the PCR enrichment step of the library preparation protocol, twelve PCR cycles were performed.

DNase I hypersensitive sites sequencing (DNase-seq) and data analysis

The DNase-seq data was obtained from GSE-41617 (http://public-docs.crg.es/mbeato/dsoronellas/DNAseq/). Briefly, T47D-MTLV cells were treated with R5020 or vehicle for sixty minutes and DNase-sequencing was performed. Additionally, PR was knocked down and a similar DNase-sequencing experiment was performed in PR-depleted T47D-MTLV cells. Publicly available BIGWIG files (DNase-seq reads attached to HG19 genome) were obtained and vehicle treatment was subtracted from the progestin treatment. Subsequently, overlap was calculated between DNase hypersensitive regions and all the ER bindings observed in T47D. An overlap window of 20kb was used for the overlap analyses.

RNA-seq data analysis

About 2.7 billion 50-bp single-end RNA-sequencing reads were generated using Illumina Hiseq system. The sequencing reads were groomed and aligned to the HG19 human genome build using Tophat software. The assembled reads were then constituted in transcripts using the cufflinks package. The cufflinks output of control and experimental samples were

merged using cuffmerge and differential gene expression was estimated by analyzing the merged output using the cuffdiff package. Transcripts that had greater than two-fold expression change between the control and experimental drug treatments were selected and used for downstream analysis, such as to compare differential gene expression between experimental conditions, functional pathway analysis and patient tumor analyses. Genes that were differentially expressed on estradiol, R5020 or combined estradiol and R5020 treatments are plotted in row-normalized heatmaps. Tumor N1 lacks RNA-seq data for R5020 treatment. For this tumor all the available sequencing reads from the corresponding treatment and PR-positivity tumor groups were combined and randomly down sampled to make up for the respective data.

Similarity matrices: HTseq package was used to calculate count matrices by overlapping the exonic BED files from UCSC with the expression BAM files that are obtained after aligning RNA-seq reads to HG19 genome build. Subsequently, the count matrices were imported in DESeq2 package and similarity matrices were computed using sample-to-sample distance function.

ChIP-seq and reChIP-seq analysis

About 1.4 billion 50-bp single-end DNA-sequencing reads were generated using Illumina Hiseq system. The sequencing reads were groomed and aligned to the HG19 build of the human genome using Bowtie 2.0 software. ChIP-peaks for different treatment conditions were called using MACS14 with the respective input files used as background. ChIP peaks with enrichment greater than or equal to 15-fold, with respect to input, were selected for each of the treatment conditions. Subsequently, control ChIP-peaks obtained after vehicle treatment were subtracted from the ChIP-peak data for each of the experimental conditions. For reChIP

experiments, both the reChIP peaks obtained after vehicle treatment and also reChIP peaks obtained with control IgG were used as controls. Hence, both of the reChIP controls were subtracted from the reChIP-signal obtained on joint estradiol plus R5020 treatment. ChIP-peaks and reChIP-peaks obtained after subtraction of control peaks were used for downstream analysis such as to compare differential receptor binding between different experimental conditions, ingenuity pathway analysis and patient tumor analyses. These finalChIP-peak and reChIP-datasets were shuffled 1000 times across HG19 genome to generate the corresponding random datasets that were used to create random null distributions for analysis. Differential binding in ChIP-seq was analyzed using the DiffBind R-package. Receptor binding heatmaps for ChIP were plotted 5 kb up- and down-stream of the ChIP peak using the Repitools R-package. An overlap of at least 1 bp was considered to determine whether two different ChIP peaks are overlapping. Investigations of ChIP-peak overlaps are performed using Genomic Ranges, IRanges and Genomic Alignment R-packages. Annotation of ChIP-peaks was performed using ChIPseeker and ChIPpeakAnno R-packages.

Enrichment analysis of protein binding motifs in ChIP regions

Two hundred and thirteen position weight matrices for transcription factor binding were obtained from the JASPAR and TRANSFAC databases. Binding sites for ER or PR or ER/PR complexes were extended by 100 bp on both 5’ and 3’ ends and their corresponding repeat masked FASTA sequences were obtained from UCSC genome browser. The FASTA sequences were scanned for matches with the position weight matrices using the MEME-ChIP suite. The significance of the enriched binding motif is reported using p-value, which is an estimate of the expected number of motifs with the given log likelihood ratio (or higher), width and site count in a similarly sized set of random sequences. Finally, target transcription factors

corresponding to the enriched binding motif were obtained using TOMTOM from the MEME suite.

Functional pathway analysis

Ingenuity analyses were used to identify cellular processes that are enriched in the transcriptomes observed in ER+/PR+ explants in response to 24 hours of treatment with various hormones. Prior to running Ingenuity analyses, the gene expression was normalized to vehicle treatment. A separate Ingenuity analysis was done with the transcriptome data from T47D and ZR75 cells. The appropriate p-values were calculated using right-tailed Fisher exact test and are subjected to Benjamini-Hochberg correction for multiple testing. The corrected p-values measure the likelihood of association between the genes of interest and the functional pathway that can be due to random chance. Subsequently, differential regulation of functional pathways between treatments was performed using the comparison tool of IPA and visualized on a radar chart by plotting the negative logarithm of the p-value.

Analysis of functional module enrichment in PR-regulated genes

A total of 1,412 cancer-relevant gene signatures were downloaded from all the Homo sapien datasets available in MSigDB v4.0. Target PR-regulated gene set consisted of genes that are differentially regulated on progestin treatment by at least two-fold compared to vehicle control. Transcriptome data from T47D, MCF7 and ZR75 cells was used to perform functional module enrichment analysis. The target PR-regulated gene set was arranged in a descending order (maximally up-regulated genes at the top and maximally down regulated genes at the bottom) and tested for enrichment in each of the human cancer-relevant gene signatures obtained from MSigDB. The obtained p-values were subjected to Bonferroni correction and then

observed enrichment scores were normalized to the enrichment scores obtained for all the dataset permutations. The network for the gene signatures enriched in PR-regulated genes was visualized in Cytoscape v2.8 (table S4). Enrichment results with FDR less than 57 percent were used to make the network. In the network, each node represents a breast cancer signature annotated with its MsigDB identifier. The node size is inversely proportional to the Bonferroni adjusted p-value and the edge width correlates with the overlap size of the enrichment between the functional modules.

Hypermethylation, copy number analysis and PR protein expression analyses

Normalized DNA methylation data for 872 patients was obtained from TCGA's JHU_USC 450k methylation array. Phospho- or total protein expression by reverse phase protein array (replicate-base normalization) was obtained from TCGA's MD Anderson database for 747 patients. Subsequently, ER-positive tumors that have complete data for methylation as well as protein expression were retained for downstream analyses. These ER+ tumors were categorized based on their PR status and frequency for the methylation of PR-annotated probes (cg27121959, cg01671895, cg011637980 and cg16462297) was determined in these subgroups. To assess the relation between methylation status and PR expression, hyper- and hypomethylated tumor groups were defined as the tumors in top and bottom quartile of methylation values. Subsequently, welch two-sided t-test was performed between PR protein expression in the hyper- and hypomethylated subgroups.

Normalized copy number variant (CNV) datasets (germline deleted) were obtained from TCGA database for 1099 patients. Similarly normalized CNV data was obtained from METABRIC for 1,992 patients. Corresponding clinical information was also obtained from

these databases. The tumors were categorized either based on their ER-receptor status or according to PAM50 (*153*) breast cancer classifier (luminal A, luminal B, basal-like, normal and Her2+). The patient tumors were called into PR gene loss, neutral or gain groups based on the copy number alterations of the PR gene locus on chromosome 11. The proportions of tumors in a category with loss, unaltered or gained PR gene locus determined the corresponding frequency of PR copy number alterations in that category. The category labels were permuted to calculate the random frequency levels.

Analysis of ER- and PR-binding and expression as predictors of clinical outcomes

The signature scores were calculated as a spearman correlation between receptor-regulated gene expression and expression of receptor-target genes in a tumor sample. Estrogen-regulated gene expression in T47D cells before and after reprogramming by progestin was used to calculate ER signature scores in the absence or presence of activated PR. Similarly, progestin-regulated gene expression in T47D cells with or without estrogen was used to calculate PR signature scores in the absence or presence of activated ER. Genes with at least one receptor binding site within 100 kb of the gene's transcription start site was identified as receptor-target genes (*1*).Survival analysis was done using the two ER-signature scores to understand how activated PR affects the prognostic value of ER-regulated genes. Similarly, analysis using the two PR-signature scores was done to study the influence of activated ER on the prognostic value of PR-regulated genes. For each of these four independent analyses, ER+ tumors from TCGA cohort (n = 1,196) were divided in two categories based on their positive or negative correlation with ER- or PR-signature scores. Subsequently, for these two tumor categories, Kaplan-Meier survival curves for overall patient survival were plotted. P values were calculated using log-rank test. The analysis was performed using the Survival package in R.

Analysis of prognostic value of a tumor ER and PR immunohistochemical staining

The immunohistochemical status of ER and PR (and other clinical data) were obtained from METABRIC and TCGA patient cohorts. Tumors were divided into sub-groups based on their positivity for ER and PR status. Kaplan-Meier survival curves for overall patient survival were plotted. P values were calculated using log-rank test. The analysis was performed using the Survival package in R. Associations between tumor PR status and other clinical and pathologic variables were studied using chi-square contingency test in R. \

Appendix

Table 1: Clinical information of patient tumors				
Tumor ID	**Treatment time**	**Age**	**Receptor Status**	**Diagnosis**
P1	24 hrs	61	ER+ PR+ HER2+	Invasive carcinoma of micropapillary type.
P2	24 hrs	64	ER+ PR+ HER2-	Invasive carcinoma of no special type.
P3	24 hrs	52	ER+ PR+ HER2-	IDC
P4	48 hrs	53	ER+ (90%) PR+ (75%) Her2-	IDC
P5	48 hrs	55	ER+ (98%) PR+ (57%) Her2-	IDC
P6	48 hrs	63	ER+ (100%) PR+ (95%) Her2-	IDC
P7	24 hrs	78	ER+ PR+ HER2+/-	Infiltrating ductal carcinoma (IDC)
P8	24 hrs	39	ER+ PR+ HER2+/-	IDC with focal invasive micropapillary carcinoma.
N1	24 hrs	47	ER+ PR- HER2-	IDC
N2	48 hrs	54	ER+ PR-Her2-	IDC
N3	48 hrs	74	ER+ PR-Her2-	IDC
N4	24 hrs	48	ER+ PR- HER2-	Invasive carcinoma of no special type.

Table 2A: PCR primers for ChIP-PCR		
Gene near binding site	**Forward primer**	**Reverse primer**
E2F1	GGTGAGATGGGAGTCTGAGG	GGTGCCTGCAAAGTAGGTTC
FOXA1	TGAGGACTGCTTGGTCACAG	CACCAGCCTTTCCAACCTAA
FOS	CCACTCTGGGCAGATCAGTT	CTGGCTAGATCAGGCTTTGG
GATA3	GCCAAAACCTACCAGCAAAT	TCAGCACCATGCACAAGAAT
TFF1 - binding1	CAGGTCTCGGTTCTCTTTGC	GGGCTATACCACTTGCCAGA
KLF9 - binding1	GGCTTGGAAACCAGAATGTC	CAGTGCTGGGACCAAGAAAG
GREB1	GCTCGCTTATGTGGCTTAGG	CGCTGTGCAAAGAACAAAGG
FHL2 - PR	CGCATTCTCTCAGGACAACC	TCCACTCTACACCAGCCTGA
FHL2 – ER	AGACCGCCCTATGTTCGTTA	CAAGGTTTGAGTGGGAGGAA
PDZK1 – PR	GACCCCTCATCTGGGAAAAG	GCCTGAGGATCCTTGGAGAG
PDZK1 – ER	TGAGGAAGCTGCTCAATGTC	CCACACTGGAAGAGCCATTT
ESR1 - Enhancer	TGTAGGCTAGTTTTGTTTAACGATTTTT	GGTGATGGGAGAATTGCTTAGAA
ESR2	CTCCGTGGAGCACATAATCC	TGGCTAACCTCCTGATGCTC
CYP26A1	CAGCCTCCCCTGGAATGTA	GCACCATGTAAGCTGGAGAA
FKBP5	AACACCCTGTTCTGAATGTGG	GCATGGTTTAGGGGTTCTTG
Control R18s	GAGTGTTCAAAGCAGGTCCAA	CCTCTAGCGGTGCAATACAAA

Table 2B: PCR primers for reChIP-PCR			
Gene	**Forward primer**	**Reverse primer**	**Chromosome location**
E2F1	GGACTGTATGCCTCGTGCTA	CTTGGGTCCCTAAGCTCTGA	chr20:32,291,053-321,971
FOXA1	GGTTTCCGAGGAAGGGATTA	CCCGGGACCTAAAAGTCAA	chr14:38,048,757-074,325
FOS	CAGAGAGATGTTGGCTCAGG	CCGATTCTGGAACAGCTTCT	chr14:75,719,239-742,695
GREB1	CCTATGCAGTTTTGCTGCTG	GCCTACCACAAGGTCAGCTC	chr2:11,617,820-701,367

Table 3: PCR Primers for CATCH chromosome capture				
Oligo Name	**Sequence 5' to 3'**	**Product Size**	**Internal Oligo**	**bp DNA Target**
CHR21F	GGCTTGTTTGAGAGAGCGAG	100	(chromosome 21)	n/a
CHR21R	TTAACATTGCCCCTTGTGCC			
PDZK1 EREF	gggattgcGATGAACTCAGG	123	/5BioTinTEG/TGAAAGA TATAGAGGAGGCCAGGAG	158
PDZK1 ERER	ACCAGCTTATCTTCCTCCACC			
PDZK1 PREF	CAGAGTACACAGTCGCCTCT	111	/5BioTinTEG/AGAGTGG AAAGGGAGACTGTCATAA	159
PDZK1 PRER	GCTCCAGTGGTTTTCTCTCC			
PDZK1 R1F	TGCTGGCTTAATGTTGCACA	111	(chromosome 1 near PRE)	n/a
PDZK1 R1R	TGTTCTTGCAGCACTTGTGT			
PDZK1 R2F	tggactcaagcattcctccc	118	(chromosome 1 near ERE)	n/a
PDZK1 R2R	AGCAATCTGGTCAGGAAGCT			
FHL2 EREF	GTCTAGCCCACCAGCCTC	129	/5BioTinTEG/CTAGAAGCC CTGCCTTTCTTTGG	175
FHL2 ERER	GGGTCTGAGCTGTACAAATGC			
FHL2 PREF	CTGAGAGAAACGTTGCGGAG	108	/5BioTinTEG/CCAGGGAAT GATGCCGAGATAAC	193
FHL2 PRER	CAAAAGACAGAGTGCCTTCCA			
FHL2 R1F	AAACCCACCCTTCTGTCCTC	105	(chromosome 2 between ERE/PRE)	n/a
FHL2 R1R	GCTGGACCCTGAGAATGTGA			
FHL2 R2F	CCCTGATCCACCACTGAAGT	109	(chromosome 2 between ERE/PRE)	n/a
FHL2 R2R	GTGGGCAGAGATCACATTCG			
FHL2 R3F	ACACCAGCTATTCCTGTGGT	110	(chromosome 2 between ERE/PRE)	n/a
FHL2 R3R	CGCAGTGTGAATAAGCAGCA			
FHL2 ERE2F	TGAGAGCCAGACGTTTCAGT	105	(intron 3 of FHL2)	n/a
FHL2 ERE2R	GCTGAGCTTTAGTGGCAAGT			

Table 1: Table presents the clinical information of twelve primary tumors used in this study. All the twelve tumors are positive for ER and eight of those are ER+/PR+ (P1 to P8) and four are ER+/PR- (N1 to N4). RNA-seq was performed on tumors treated *ex-vivo* with vehicle, E, P or E+P for 24 or 48 hours.

Table 2: **(A)** Primers used for directed ChIP-qPCR. **(B)** Primers used for directed reChIP-qPCR.

Table 3: Primers used for targeted chromosome capture (CATCH) experiments

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