Progesterone receptor maintains estrogen receptor gene expression by regulating DNA methylation in hormone-free breast cancer cells

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Abstract

Background: How breast cancers respond to endocrine therapy strongly depends on the expression of the estrogen and progesterone receptors (ER and PR, respectively), with double-negative ER–/PR– breast cancers having worse clinical outcome than ER+/PR+ breast cancers. Although much is known about *ER*α gene (*ESR1*) regulation after hormonal stimulation, how it is regulated in the absence of hormones is not fully understood. We used

ER+/PR+ positive breast cancer cells to investigate the role of PR in *ESR1* gene regulation in the absence of hormones.

Methods: We compared the *ESR1* gene expression and its methylation profile between PR-positive breast cancer cells, PR-depleted cells and PR-rescue cells by RT-qPCR and MeDIP-qPCR, respectively. Moreover, we analyzed the PR binding to *ESR1* locus by chromatin immunoprecipitation method and, to methylated or unmethylated progesterone responsive elements by electrophoresis mobility shift assay.

Results: We show that PR binds to the low-methylated *ESR1* promoter and maintains both gene expression and the DNA methylation profile of the *ESR1* locus in hormone-deprived breast cancer cells. Depletion of PR reduces *ESR1* expression, with a concomitant increase in gene promoter methylation. The high amount of DNA methylation in the *ESR1* promoter of PR-depleted cells persists after the stable re-expression of PR and inhibits PR binding to this genomic region. Consequently, the rescue of PR expression in PR-depleted cells is insufficient to restore *ESR1* expression. Consistent with these data, DNA methylation impedes PR binding to consensus progesterone responsive elements in vitro.

Conclusion: These findings help us understand the complex crosstalk between PR and ER, and suggest that the analysis of DNA methylation of *ESR1* promoter in breast cancer cells can help to design the appropriate targeted therapies for different types of breast cancer patients

Key Words

Progesterone receptor; estrogen receptor; gene expression; DNA methylation, breast cancer

Background

Estrogen and progesterone are main players in the normal development and function of the mammary gland as well as in the progression and outcome of breast cancers [1]. Both hormones act through their cognate receptors (estrogen receptor, ER, and progesterone receptor, PR), which function as signaling triggers and as ligand-activated transcription factors [1]. Of the approximately two-thirds of breast cancers that express the ER protein, about half also express the PR protein and are therefore classified as ER+/PR+ [2]. ER+/PR+ tumors tend to be highly differentiated and responsive to hormone therapies, in contrast to ER-positive tumors lacking PR, which are less likely to respond to endocrine therapy [2]. Approximately one-third of breast cancers lack both ER and PR (ER–/PR–) and generally shows poor histological differentiation with higher growth rates [2]. These cancers rarely respond to hormone therapies and exhibit a poor clinical outcome compared to ER+/PR+ breast cancers [2]. Hence, understanding the molecular mechanism that controls the expression of these hormone receptors in tumor cells is an important area of investigation.

Genetic mutations of the ERα coding gene (*ESR1)*, such as deletions, rearrangements, and point mutations, are not frequent enough to explain the loss of *ESR1* expression in up to one-third of human breast cancers [2]. In contrast, a gain of methylation of the CpG island localized at the *ESR1* promoter frequently occurs and results in reduced or no ERα expression [3-4]. CpG islands are cytosine/guanosine-rich genomic sequences located in the regulatory regions of genes. They are generally unmethylated in normal somatic cells, with the

exception of imprinted genes [5]. In cancer cells, however, a gain of CpG island methylation is associated with transcriptional silencing, either directly or through a change in chromatin conformation [5]. Initially, it was reported that there was no correlation between loss of *ESR1* expression and methylation of the coding region of *ESR1* in breast cancer [6]. However, a clear correlation between the lack of *ESR1* expression and gene promoter methylation has been shown [3-4]. Moreover, breast cancer patients with poor prognosis tend to have higher DNA methylation levels at the *ESR1* promoter [7]. The analysis of *ESR1* promoter methylation status, therefore, can be critical for stratifying breast cancers.

Upon hormone exposure, PR and ER mediate breast cancer cell response and strongly affect cell growth, migration and invasion [1]. Both hormone-activated receptors exhibit enhanced binding to specific DNA sequences called hormone-responsive elements, which are generally located within target gene enhancers or promoters [1, 8]. The DNA-bound receptors orchestrate the assembly of large cofactor-containing protein complexes that can either positively or negatively affect gene transcription [1]. In addition, hormone-activated ER and PR attached to the cell membrane can trigger rapid signaling by interacting with several kinases, which also participate in hormonal gene regulation [1, 9].

In the absence of hormones, unliganded PR and ER also profoundly affect the breast cancer cell biology. Unliganded ER inhibits breast cancer cell growth and is required for the maintenance of the cell epithelial phenotype [10, 11]. The silencing of ER in breast cancer cells, indeed, induces epithelial to mesenchymal transition [11]. Moreover, the presence of this protein plays a key role in the breast cancer sensitivity to the endocrine therapy [12].

Interestingly, as the unliganded ER, also the unliganded PR enhances breast cancer response to estrogen and to selective ER modulator used for the endocrine therapy, including tamoxifen and others antiestrogens [12-14]. However, how the presence of PR affects the breast cancer sensitivity to these external stimuli is not completely understood. Thus, the study of the role of unliganded hormone receptors in breast cancer cells helps to understand how epithelial breast cancer cells maintain cell homeostasis and how they respond to external stimuli including the ER modulators used for the endocrine therapy.

About one-third of breast cancers are resistant to steroid hormones but often retain functional hormone receptors [15] and remain highly sensitive to growth factors including EGFR (epidermal growth factor receptor) [16]. Growth factors stimulate breast cancer cell proliferation by enhancing the transcriptional activity of unliganded PR via the protein kinases signaling pathways [16]. The activated unliganded PR directly regulates gene expression by acting at chromatin level [17], even if the molecular mechanisms put into play are poorly understood. In hormone-free breast cancer cells, phosphorylated and under-sumoylated PR positively regulates the expression of growth promoting genes by recruiting steroid receptor coactivator 1 (SRC1) to gene promoters [17]. In contrast, sumoylated PR down-regulated the expression of the same class of genes by recruiting histone deacetylase 3 (HDAC3) to promoters [17]. On the other hand, unliganded ERα directly regulates the expression of hundred genes including the PR-encoding gene (*PGR*) in hormone-deprived breast cancer cells by modulating the extent of trimethylation of lysine 27 on histone 3 (H3K27me3) at ERα binding sites [18].

The key role of unliganded PR for the responsiveness of breast cancers to growth factors and endocrine therapy as well as the high correlation between the PR and ER levels in breast cancers [19] led us to explore whether and how unliganded PR regulates the *ESR1* expression in hormone-free breast cancer cells. Here we report that unliganded PR binds to the low-methylated *ESR1* promoter to maintain its basal expression and its low level of DNA methylation in hormone-deprived breast cancer cells. Consistent with these data, we show that DNA methylation hinders PR binding to hormone-responsive elements.

Methods

Cell culture

The T47D-MTVL (T47D) breast cancer cells used in this study have a stably integrated copy of the luciferase reporter gene driven by the MMTV promoter [20]. The T47D, T47D-Y [21], T47D-Y+PR [21], MCF7 and BT474 cells were routinely grown in medium (RPMI 1640 for T47D; DMEM for all the other cell lines) supplemented with 10% FBS and standard antibiotics. For the experiments, cells were grown 48 hours in medium without phenol red supplemented with 10% dextran-coated charcoal treated FBS (DCC/FBS). In contrast to normal FBS, the DCC/FBS does not contain hormone-similar compounds that can activate the PR activity. Moreover, since the cell cycle phase can influence the PR activity [22], cells were synchronized in G0/G1 by serum starvation for 16 hours to avoid the cell cycle phase variability. For 5-azacytidine treatment, T47D-Y+PR cells were grown for 96 hours (48 hours using DMEM medium supplemented with 10% FBS, and 48 hours with DMEM without phenol red supplemented with 10% DCC/FBS) with 5 μM of 5-azacytidine (A3656,

Sigma-Aldrich) or vehicle (1:1 acetic acid to water). Cells were finally synchronized in G0/G1 by 16 hours of serum starvation before performing chromatin immunoprecipitation assay.

Lentivirus preparation and infection

HEK-293 cells were transfected with pVSV-G [23] and pCMVΔR8.91 [24], together with the pLKO.1-puro non-targeting vector (SHC001; Sigma-Aldrich) or pLKO.1-shRNA against PR using $CaCl_2$ to permeabilize the cell membrane. Two different clones of PLKO.1-shRNA against PR have been used: clone trcn0000010776 and clone trcn0000003321 (SHCLND-NM_000926, Sigma-Aldrich). The viral particles containing the shRNA were collected 72 hours after the transfection and used to stably infect breast cancer cells. Cell populations were finally selected with puromycin (1 μg/ml) and processed to quantify mRNA and protein expression.

Reverse transcription and quantitative PCR

Total RNA was isolated with the RNeasy extraction kit (Qiagen). Complementary DNA (cDNA) was generated from 100ng of total RNA with the First Strand cDNA Superscript II Synthesis kit (Invitrogen; #11904018) and analyzed by quantitative PCR. Gene-specific expression was regularly normalized to *GAPDH* expression. Primers sequences are listed in table S1.

Western blotting

Cell lysates were resolved on SDS-polyacrylamide gels, and the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham). Membranes were blocked with TBS-0.1% Tween 20 (TBS-T) with 5% of skimmed milk, incubated for 1 hour at room temperature with a primary antibody (antibody against PR, sc-7208 from Santa Cruz Biotechnology; antibody against ERα, sc-543 from Santa Cruz Biotechnology; antibody against α-tubuline, T9026 from Sigma), and then diluted in TBS-T with 2.5% skimmed milk. After three washes with TBS-T, membranes were incubated for 45 min at room temperature with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). Antibody binding was detected by chemiluminescence on a LAS-3000 image analyzer (Fuji PhotoFilm).

Chromatin immunoprecipitation

Chromatin immunoprecipitation **(**ChIP) assays were performed as described previously [25], with minor modifications. Cells were cross-linked in medium containing 1% formaldehyde for 10 min at 37°C, and crosslinking was quenched with 125 mM glycine for 5 min at room temperature. After cells were lysed in hypotonic buffer, the nuclei were lysed with SDS-lysis buffer. Chromatin was sheared by sonication and incubated 16 hours with 5μg of antibody against progesterone receptor (PR, Santa Cruz Biotechnology, sc-7208) or rabbit IgG (Cell Signaling, #2729s). Immunocomplexes were recovered with protein A agarose bead slurry (Diagenode, #C03020002) for 2 hours with rotation and washed with ChIP buffer (Diagenode, #K0249001) and Tris-EDTA buffer. For reversing the crosslinking, samples were incubated with proteinase K (10 mg/ml) at 65ºC for 16 hours. DNA was purified and analyzed by quantitative PCR. Primer sequences are listed in table S1.

ChIP-sequencing analysis

For PR ChIP-seq, the reads of the previously published PR ChIP-seq [20] were trimmed using Trimmomatic (version 0.33) with the parameters values recommended by Bolger *et al*. [26]. The trimmed reads were aligned to the hg19 assembly version of the human genome using BWA (version: 0.7.12-r1039) [27]. The FASTA file containing the genome reference sequence of hg19 was downloaded from the UCSC Genome Browser discarding the random scaffolds and the alternative haplotypes from the reference sequence for the alignment [28]. BWA-MEM algorithm and SAMtools (version: 1.2, using htslib 1.2.1) [29] were used to convert SAM files to BAM files and to sort them to retain only uniquely aligned reads. The PR binding sites were identified with the MACS2 tool (version 2.1.0.20150420) [30]. Peaks were additionally filtered until those remaining had a false discovery rate (FDR) q-value $< 10^{-6}$ and a 4-fold enrichment over the control sample (input), leaving 476 peaks for subsequent analyses.

DNA methylation

The DNA methylation analyses were performed by methylated DNA immunoprecipitation assay coupled with quantitative-PCR (MeDIP-qPCR) or high-throughput sequencing (MeDIP-seq). For MeDIP-qPCR, genomic DNA was randomly sheared by sonication to generate fragments between 300 and 700 bp. Sonicated DNA was denatured and then immunoprecipitated as previously described [31] using an antibody against 5mC (Eurogentec; #BI-MECY-1000) or mouse IgG antibody. The immunocomplexes were

recovered using 8 μl Dynabeads (M-280; Life Technologies), and the pull-down products were detected by quantitative-PCR. Primers sequences are listed in table S1.

For MeDIP-seq, adaptors from the NEBNext Ultra DNA Library Prep Kit from Illumina were added to the fragmented DNA. Fragmented DNA was immunoprecipitated with antibody against 5mC as describe above, and the amplified library was prepared using NEBNext Ultra DNA Library Prep Kit for Illumina (E7370L) following manufacturer's instructions. Amplified libraries were sequenced, and reads were aligned with BowTie v1.1.2 using the reference human genome version 19 (hg19) [32]. The mapped reads were filtered for duplicates and used to compute genome-wide reads-per-million (RPM) normalized signal tracks. The 5mC and CpG heat maps were generated using DeepTools (version 2.2.0) [33] and BEDtools (version v2.24.0) [34] and the matrix underlying the heatmaps was used to generate the 5mC and CpG average profiles. To test the significance of the overall reduction of 5mC methylation observed in the progesterone-receptor binding sites (PRBs), we calculated the average 5mC normalized read counts signal over each PRBs and random regions resulting from shuffling the genomic coordinates of the PRBs, while keeping their sizes as in the true set of regions (this second step was repeated 1,000 times to generate an empirical null distribution of 5mC methylation averaged values). The Mann-Whitney U Test was applied using the stats module of the Python's SciPy library [35].

Electrophoresis mobility-shift assay

Recombinant human PR (isoform B; PRB) was expressed in baculovirus and purified as previously described [36]. Radioactive double-stranded oligonucleotides containing the

progesterone-responsive elements (PRE) were incubated with the indicated amounts of PR-B for 20 min at room temperature and analyzed in a 5% acrylamide-bisacrylamide electrophoresis gel. The radioactivity of the DNA-protein complex was then quantified by using the PhosphorImager and ImageQuant software (Molecular Dynamics). For the EMSA competition assay, a radioactive oligonucleotide without CpGs was first mixed with 100-fold of non-radioactive unmethylated or methylated probe containing two CpGs and then incubated with 2.4 μg of PR-B for 20 min at room temperature. DNA-protein complexes either in absence or presence of unlabelled oligonucleotides were then analyzed as described above. Oligonucleotides sequences are listed in table S1.

Results

PR is required to maintain *ESR1* gene basal expression in hormone-deprived breast cancer cells

Comparing the *ESR1* gene expression in T47D breast cancer cells and a derived clone selected for low PR expression (T47D-Y) [21], as previously observed [37], we confirmed that low PR expression is accompanied by a low expression of *ESR1* at both the transcript and protein levels (Fig. 1, A and B, *left panels*). Although the PR inhibits *ESR1* gene expression in T47D cells upon progestin exposure [38], the decrease of *ESR1* expression in T47D-Y cells led us to hypothesize that the unliganded PR could be involved in maintaining basal *ESR1* gene expression. To test this possibility, we knocked down PR in T47D breast cancer cells using a short-hairpin RNA (shRNA) approach and analyzed *PR* and *ESR1* gene expression by RT-qPCR and Western blotting assays. Concomitant with the decrease of PR

levels, RNA and protein amount of *ESR1* decreased in shPR cells compared to control cells (shC) (Fig 1, A and B, *right panels*; Fig S1 A and B). In addition, we analyzed the transcription of the estrogen-responsive *TFF1* gene in control cells, PR-depleted cells (shPR) as well as in PR-deficient cells (T47D-Y) and found that the estradiol (E2)-mediated induction of *TFF1* transcription in control cells was strongly reduced in shPR and T47D-Y cells (Fig 1C), consistent with the reduced ERα level upon PR loss. Finally, to test the role of PR in the maintenance of *ESR1* gene expression in different cellular backgrounds, we depleted the PR levels in two additional ER/PR positive cell lines, MCF7 and BT474, using the same short-hairpin RNA approach (shPR). Despite a moderate decrease of the PR levels in these cell lines (Fig S1 D and E), this slight decrease of PR was accompanied by a concomitant decrease of *ESR1* expression compared to control cells (Fig S1 D and E), confirming the importance of PR levels in maintaining *ESR1* gene expression in different breast cancer cell lines.

PR binds to the *ESR1* locus in hormone-deprived breast cancer cells

To test whether PR directly regulates *ESR1* gene expression prior to hormone stimulation, we analyzed ChIP-seq data obtained with an antibody to PR in serum-starved T47D cells [20]. In the absence of hormones, PR appears to bind to two genomic regions within the *ESR1* locus, one located within the gene promoter (chromosome 6: 152,128,500–152,129,000 hg19) and another within the third intron (chromosome 6: 152,222,250–152,222,650 hg19) (Fig 2A). The specificity of these two unliganded PR binding events was confirmed by ChIP-qPCR using T47D cells and PR-deficient cells (T47D-Y) or PR-depleted cells (shPR). PR

bound to the promoter and the intronic regions in T47D cells but not in T47D-Y cells (Fig 2B, *left panel*) or in shPR cells (Fig 2B, *right panel*). Strikingly, our analysis of previously published ChIP-seq experiments performed in the same conditions [8, 39] revealed that the intronic sequence bound by PR in hormone-deprived T47D cells exhibited marks of active enhancers, including histone H3 mono-methylation on lysine 4 (H3K4me1) and DNase hypersensitivity (Fig 2A) [40].

We also tested PR binding at *ESR1* locus in MCF7 cells by ChIP-qPCR and found that in this case unliganded PR also bound at *ESR1* promoter but not at the enhancer-like site in the third intron of *ESR1* gene (Fig S2).

Rescue of PR does not restore *ESR1* gene expression in PR-deficient cells

To explore whether stable expression of PR restores *ESR1* gene expression, we stably expressed PR in PR-deficient cells (T47D-Y+PR) and analyzed *ESR1* expression. Unexpectedly, *ESR1* expression remained significantly reduced at both transcript and protein levels after re-establishing PR levels (Fig 3, A and B). Similarly, the estrogen-mediated induction of the *TFF1* gene remained reduced after PR rescue (Fig 3C). Thus, PR expression alone is insufficient to restore *ESR1* gene expression to a level comparable to wild-type cells, suggesting that the *ESR1* gene is stably repressed through another mechanism once PR is absent in T47D breast cancer cells.

Lack of PR affects DNA methylation at the *ESR1* promoter

DNA methylation at the *ESR1* promoter represents one of the main epigenetic mechanisms for stably repressing *ESR1* expression in breast cancers [4, 7]. To explore whether PR loss

affects the DNA methylation profile of the *ESR1* locus, we compared the DNA methylation pattern at the *ESR1* promoter and intronic PR-binding sites between T47D control cells, PR deficient cells (T47D-Y) (Fig 4A), and PR-depleted cells (shPR) (Fig 4B) before and after PR rescue. Methylation of the *ESR1* promoter was strongly increased in PR-deficient and PR-depleted cells, and high DNA methylation amount of this genomic region persisted after PR rescue (T47D-Y+PR cells) (Fig 4A). In contrast, the DNA methylation profile of the intronic-PR binding site was not increased, neither in PR deficient nor PR depleted cells, and did not significantly change after PR rescue (Fig 4A).

Consistent with these data, we observed that analysis of the TGCA Breast Invasive carcinoma (BRCA) dataset showed a clear difference in *ESR1* gene methylation levels when the data was segregated based on the PR expression levels of the patients. PR negative breast carcinoma patients were more commonly also negative for ER and present an increased level of methylation of the *ESR1* gene than PR-positive breast carcinomas. The gain of *ESR1* methylation in PR-negative breast carcinomas was stronger at gene promoter rather than within gene body (Fig S3).

DNA methylation impedes PR binding to hormone responsive elements

DNA methylation can directly affect the affinity of transcription factors towards their binding sites [41]. To check whether higher *ESR1* promoter methylation levels affect PR binding to this genomic region, we compared PR binding levels at the *ESR1* locus between T47D control cells, PR-deficient cells (T47D-Y), and PR-rescue cells (T47D-Y+PR) by ChIP-qPCR assay. As described above, PR bound to the *ESR1* promoter and to an enhancer-like intronic sequence in control cells, whereas, this binding was completely impaired in PR-deficient

cells (Fig 2B, *left panel*). Rescue of PR in PR-deficient cells (T47D-Y+PR) completely restored PR binding at the low-methylated intronic sequence; however, it only partially restored PR binding at the highly-methylated promoter site, suggesting that hyper-methylation at the *ESR1* promoter impedes PR binding to this genomic region (Fig 5A). To test this hypothesis, we treated the PR-rescue cells (T47D-Y +PR) with the demethylating agent 5-azacytidine (5-azaC) or vehicle (control) and then compared the PR binding at the *ESR1* locus between control and 5-azaC-treated cells. The results showed that 5-azaC treatment (leading to DNA demethylation) did not affect PR binding at the *ESR1* intronic region but it had a tendency to increase PR binding levels at the *ESR1* promoter site (Fig 5B). The CpG island at the *ESR1* promoter contains a canonical progesterone-responsive elements (PRE) encompassing a CpG as well as several half palindromic PRE sites with one or two neighboring CpGs (Fig 6A). To determine whether the inhibition of PR binding was due directly to the CpG methylation at the PRE or rather to an altered chromatin state, we tested whether PR can bind to methylated or unmethylated forms of PRE oligonucleotides by electrophoresis mobility shift assay (EMSA). We observed that PR bound more efficiently to the unmethylated probes than to their methylated counterparts, especially when the PRE contained two CpGs rather than one (Fig 6, B and C). Further, an unmethylated PRE, but not a methylated PRE, was a high-affinity competitor in EMSA for an oligonucleotide probe without CpG, which was previously shown to be a strong PR binding site [8] (Fig 6D). Moreover, we observed that the CpG-containing PREs were bound by PR less efficiently than the PRE without CpGs (Fig 6, B and C), suggesting that the presence of CpG, even not methylated, reduces the PR binding affinity.

Finally, we analyzed the methylation of genomic regions bound by PR [20] in the absence of

hormones by MeDIP-sequencing method. Despite having a higher CpG content than their flanking genomic regions, all the unliganded PR binding sites had an overall lower level of methylation than their surrounding areas (Fig S4), suggesting that not only the *ESR1* locus but also the other PR binding sites require low levels of methylation for PR binding.

Discussion

The study of the role of PR in hormone-free breast cancer cells helps to clarify how these cells respond to external stimuli, including growth factors that affect breast cancer progression and selective ER modulators used for the endocrine therapy. We show here that PR binds the *ESR1* locus and is required to maintain the *ESR1* gene expression in hormone-free breast cancer cells. When the levels of PR are reduced, *ESR1* gene expression decreases in parallel with an increase of the DNA methylation level at the *ESR1* promoter, suggesting that hormone-free PR maintains the *ESR1* expression by preserving a low DNA methylation profile at the *ESR1* promoter. Rescue of PR by stable expression of PR in PR-deficient cells did not affect the hyper-methylation found at the *ESR1* promoter, and was insufficient to reactivate *ESR1* gene expression. Moreover, rescue with PR completely restored the PR binding at low-methylated intronic sequence but only partially restored it at the highly methylated *ESR1* promoter site, suggesting that DNA methylation affects the PR binding to DNA. Consistently, treating PR-rescued cells with the demethylating agent 5-azaC had a tendency to increase PR binding at the *ESR1* promoter but not at the low-methylated intronic site. Moreover, in vitro, PR preferentially bound unmethylated PRE oligonucleotides rather than their methylated counterpart, demonstrating that PR is a methylation-sensitive DNA

binding protein. Consistent with these data, we also observed that all the PR binding sites in hormone-free breast cancer cells show low methylation levels. Taken together, these data demonstrate that the gain of DNA methylation at the *ESR1* promoter observed upon PR loss stably silences the *ESR1* gene expression and impedes PR binding to the *ESR1* promoter after PR rescue (Fig 7). Interestingly, the loss of PR specifically affected the *ESR1* promoter methylation but not at the *ESR1* intronic site that lacks a CpG island, suggesting that PR selectively prevents methylation around CpG islands. In line with our results, demethylation of the *ESR1* promoter reactivates *ESR1* expression in ER-negative breast cancer cells [4].

ER+/PR+ breast cancers are much more likely to respond to selective ER modulator (SERM) therapy than breast cancers that lack PR protein (ER+/PR−) [13]. Since the SERM treatments target ER, how the absence of PR affects this kind of therapy is not completely understood. So far, it was proposed that unliganded PR is a simple marker of ER activity [13]. Our finding that PR contributes to maintain the *ESR1* expression in the absence of hormones suggests that the ER+/PR+ breast cancers are more likely to respond to SERM therapy because they could maintain the ERα levels higher than the ER+/PR− breast cancers. In agreement with this hypothesis, it has been reported that the breast cancer response to endocrine therapy is directly related to ER levels [42].

In many cases, the transcriptional regulation of steroid target genes requires the action of regulatory sequences located far away from the promoters [43, 44]. A significant fraction of these distal sequences engages in physical interactions with promoters, suggesting that they

act as enhancers [43]. In this study, we showed that the PR binding site within the *ESR1* intronic sequence in T47D breast cancer cells exhibits the classical epigenetic marks found at active enhancer regions, including the monomethylation of lysine 4 of histone 3 (H3K4me1), low DNA methylation, and a DNase hypersensitive site [8, 39]. This suggests that PR through its binding to the *ESR1* promoter and the enhancer-like intronic sequence could facilitate the interaction between these two genomic regions to enhance the *ESR1* transcription in T47D breast cancer cells. However, further analyses are required to investigate the enhancer activity of the *ESR1* intronic PR binding site and its possible interaction with the gene promoter.

Several studies have shed light on the complex crosstalk between the PR and ER signaling pathways in the absence and presence of hormones. In hormone-deprived MCF7 breast cancer cells, unliganded-ERα positively regulates gene expression, including that of the *PGR* gene (the PR-encoding gene), by altering trimethylation of lysine 27 on histone 3 (H3K27me3) at ERα binding sites [18]. On the other hand, Mohammed *et al*. (2015) showed that, in the presence of agonist ligands, PR is not just an ERα-induced target gene but also interacts with ERα to direct its chromatin binding in breast cancer cells, resulting in a unique gene expression pattern that is associated with good clinical outcome [45]. Unliganded PR-B induces robust expression of a subset of estradiol-responsive target genes, with a consequent increased cellular sensitivity to estradiol [14]. In contrast, in T47D breast cancer cells, progesterone-liganded PR negatively regulates a subset of progesterone target genes, including *ESR1* [38]. Our finding that unliganded PR is required to maintain *ESR1* expression and its DNA methylation profile in the absence of hormones reveals a new molecular

mechanism of crosstalk between PR and ER and suggests that hormone binding to PR drastically affects its role in the *ESR1* gene regulation. In line with our data, Widschwendter *et al.* (2004) showed that PR-negative breast cancers have higher *ESR1* promoter methylation and lower *ESR1* gene expression than PR-positive tumors [46]. Moreover, we observed that PR negative breast carcinoma patients tend to be negative for ER and show higher *ESR1* methylation than PR-positive breast carcinomas. However, the copy number loss of *PGR* was previously observed to be a common feature in ERα-positive luminal B breast cancers [45], suggesting that different crosstalk between PR and ER could exist, depending on breast cancers subtypes.

Conclusions

In this study, we demonstrate that PR binds to the low-methylated *ESR1* promoter and maintains both gene expression and the DNA methylation profile of the *ESR1* locus in hormone-free breast cancer cells. These data expand our understanding of the complex crosstalk between PR and ER, and suggest that the analysis of DNA methylation of *ESR1* promoter in breast cancer cells can help to design the appropriate targeted therapies for different types of breast cancer patients.

List of abbreviations

BRCA: breast invasive carcinoma; cDNA: Complementary DNA; ChIP: Chromatin immunoprecipitation; DMEM: Dulbecco's modified Eagle's medium; EMSA: Electrophoresis mobility-shift assay; ER: Estrogen receptor; *ESR1: ER*α-encoding gene;

MeDIP: Methylated DNA immunoprecipitation; PR: Progesterone receptor; PGR: PR-encoding gene; PRE: Progesterone-responsive elements; RPM: Reads-per-million; RPMI: Roswell park memorial institute; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction; SERM: Selective ER modulator; shRNA: Short-hairpin RNA; 5-azaC: 5-azacytidine; 5mC: 5-methylcytosine.

Declarations

Ethics approval and consent to participate.

Not applicable

Consent for publication.

Not applicable

Availability of data and materials

The DNA methylation profile analyzed by high throughput sequencing during the current study are available in the GEO repository. For the reviewers: to access to this data, use the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107966 with the secure token: efwfueewdfizjov. All the others datasets obtained and/or analyzed during the current study are available from the corresponding author in reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Study conception and design: G.V., F.L.D., and M.B; acquisition of data: G.V., L.I.D.L, R.H.G.W.; analysis and interpretation of data: G.V., F.L.D.; bioinformatic analysis: J.Q. and G.V.; manuscript drafting: G.V. and F.L.D.; manuscript critical revision: G.V., M.B., F.L.D, L.I.D.L, R.H.G.W, S.P.

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Figures

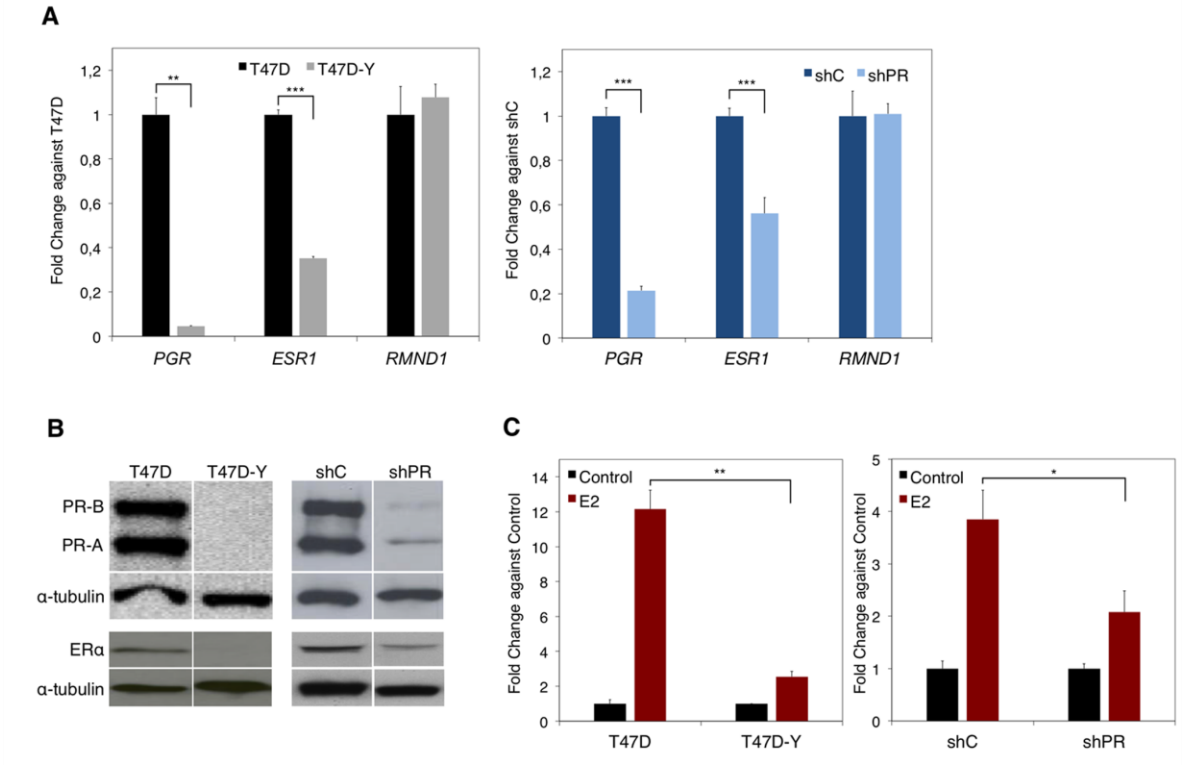

Fig 1. Loss of PR reduces the *ESR1* expression in hormone-deprived T47D breast cancer cells.

(A) Gene-specific mRNA expression measured by quantitative RT-PCR in hormone-deprived T47D or PR-deficient cells (T47D-Y) (left panel) and in hormone-deprived T47D cells transduced with shRNA against PR (shPR, clone trcn0000010776) or scramble shRNA (shC) (right panel). The gene-specific expression levels were normalized to *GAPDH* expression and are represented as relative values in the T47D cells. *RMND1* was used as a PR-independent control. *PGR*, PR-encoding gene; *ESR1*, ER-encoding gene. Error bars represent the SEM of three independent experiments. ***P* less or equal than 0.01, ****P* less or equal than 0.005, unpaired two-tailed Student's *t*-test.

(B) PR and ERα protein levels measured by Western blot in T47D and T47D-Y cells (left panel) and in T47D transduced with shRNA against PR (shPR; clone trcn0000010776) or scramble shRNA (shC) (right panel). α-tubulin protein was used as loading control. The vertical white line depicts a removed lane between the two samples. Blots are representative of three independent experiments.

(C) PR depletion impairs estrogen-mediated gene expression. T47D cells or PR-deficient (T47D-Y) cells (left panel), or short hairpin control (shC) and PR-depleted (shPR, clone trcn0000010776) cells (right panel), were treated with estradiol (E2) or ethanol (control) for 6 hours, at which point *TFF1* mRNA expression was measured by quantitative RT-PCR. The *TFF1* gene expression was normalized to *GAPDH* expression and is represented as fold change relative to the control. Error bars represent the SEM of three independent experiments. **P* less or equal than 0.05, ***P* less or equal than 0.01, unpaired two-tailed Student's *t*-test.

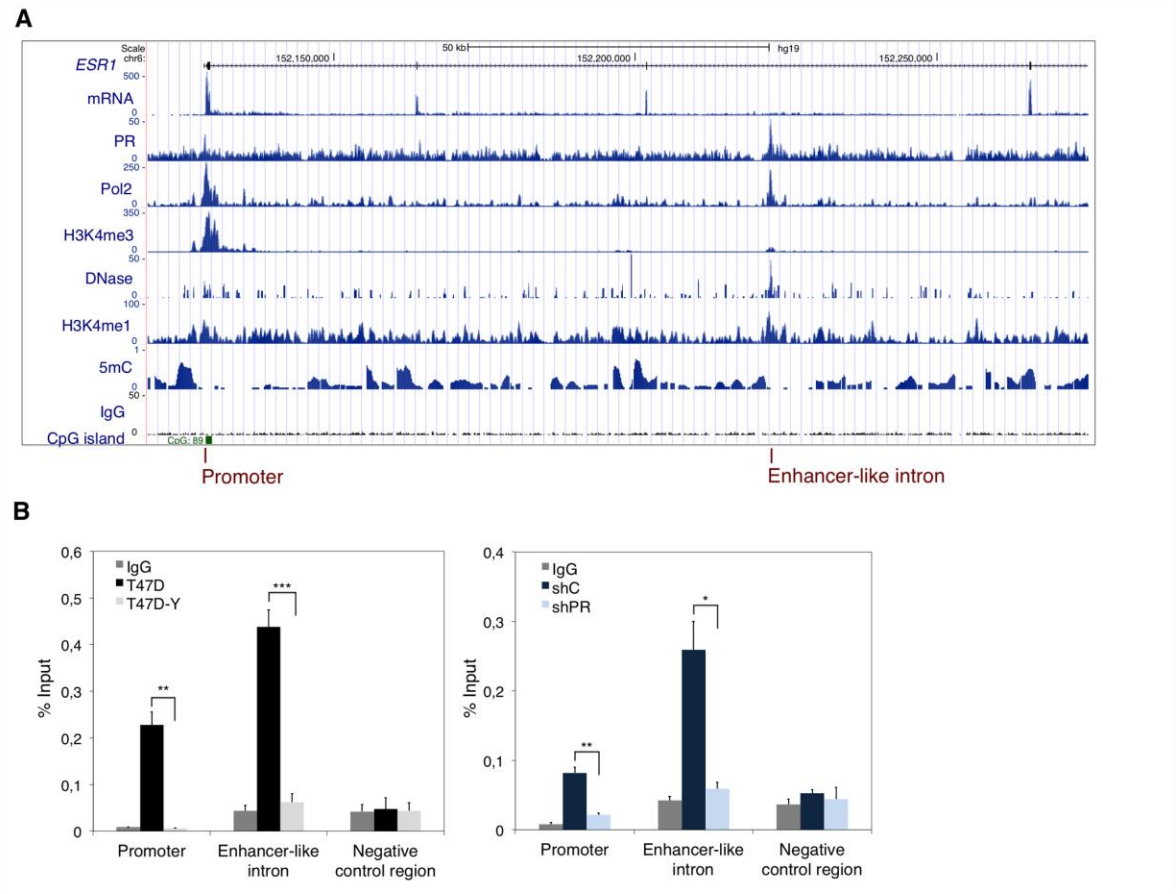

Fig 2. PR binds to the promoter and to an enhancer-like intron of the *ESR1* gene in hormone-deprived T47D breast cancer cells.

(A) Screen shot from the UCSC genome browser showing the *ESR1* gene, the RNA reads, and the ChIP-seq results from PR binding, with a peak at the gene promoter marked by polymerase 2 binding (Pol2), histone 3 trimethylated at lysine 4 (H3K4me3), low DNA methylation (5mC), and a CpG island at the bottom. Another PR peak is found in an intronic region containing the classical enhancer epigenetic marks of DNase hypersensitive site (DNase), histone 3 monomethylated at lysine 4 (H3K4me1), and low DNA methylation signal (5mC). The negative control immunoprecipitation is indicated by the IgG antibody.

(B) ChIP assay was performed with a specific antibody against PR or total rabbit IgG. Specific binding was assessed by qPCR amplification of the *ESR1* gene promoter, an enhancer-like intronic sequence, and a genomic region localized at 3′-end of the enhancer-like intron (negative control region). Left panel, results in T47D cells and T47D-Y cells; right panel, T47D cells depleted of PR with shPR (clone trcn0000010776), or expressing a scrambled shRNA (shC). Error bars represent the SEM of three independent experiments. **P* less or equal than 0.05, ***P* less or equal than 0.01, ****P* less or equal than 0.005, unpaired two-tailed Student's *t*-test.

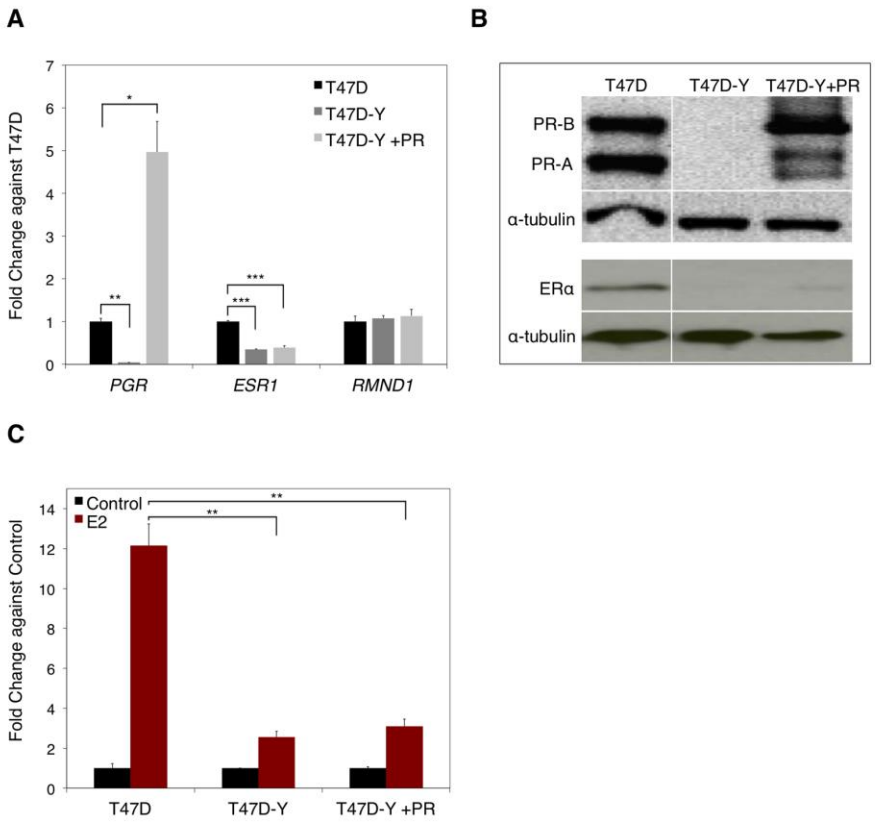

Fig 3. PR rescue of PR-deficient cells does not restore *ESR1* gene expression.

Gene-specific mRNA (A) and protein (B) expression measured by quantitative RT-PCR and Western blot, respectively, in T47D control cells, PR-deficient cells (T47D-Y) and PR-rescue cells (T47D-Y+PR). The gene-specific mRNA expression levels were normalized to *GAPDH* expression and are represented as values relative to the T47D cells. *PGR*, PR-encoding gene; *ESR1*, ER-encoding gene. Error bars represent the SEM of three independent experiments. **P* less or equal than 0.05, ***P* less or equal than 0.01, ****P* less or equal than 0.005, unpaired two-tailed Student's *t*-test. Blots are representative of three independent experiments.

(C) PR rescue of PR-depleted cells does not restore the estrogen-mediated gene expression.

T47D, PR-deficient cells (T47D-Y), and PR-rescue (T47D-Y+PR) cells were treated with estradiol (E2, 10 nM) or ethanol (control) for 6 hours, at which point TFF1 mRNA expression levels were measured by quantitative RT-PCR. Gene-specific expression levels were normalized to *GAPDH* expression and are represented as values relative to the control. Error bars represent the SEM of three independent experiments. ***P* less or equal than 0.01, unpaired two-tailed Student's *t*-test.

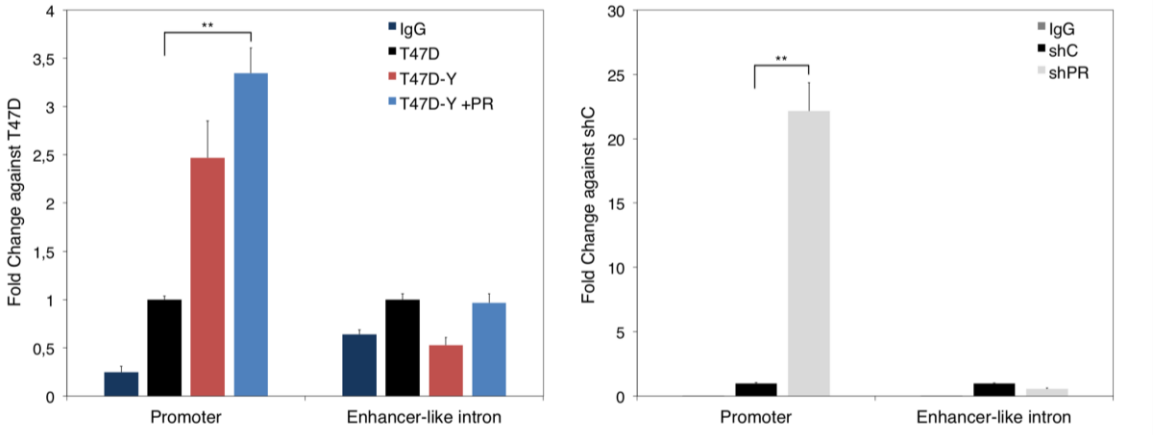

Fig 4. The loss of PR increases the DNA methylation level at the *ESR1* gene promoter. DNA methylation of the *ESR1* promoter and the enhancer-like intron was assessed by MeDIP-qPCR in T47D cells, T47D-Y cells and T47D-Y cells with stable PR transfection (T47D-Y+PR) (left panel), or in T47D cells transduced with scrambled shRNA (shC) or shRNA against *PR* (shPR; clone trcn0000010776) (right panel). The results are represented as values relative to the T47D cells (left panel) or the shC cells (right panel). IgG, negative control for immunoprecipitation. Error bars represent the SEM of three independent experiments. ***P* less or equal than 0.01, unpaired two-tailed Student's *t*-test.

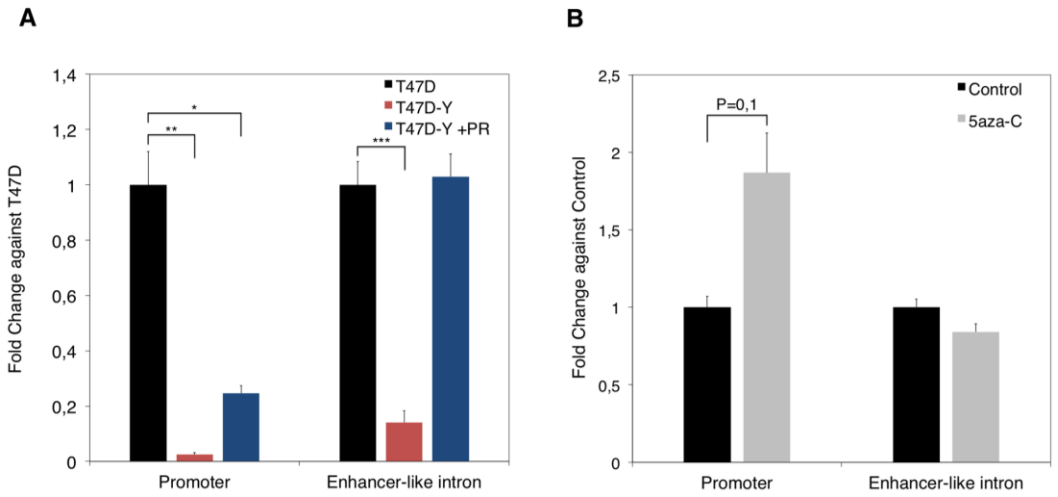

Fig 5. DNA methylation affects PR binding to the *ESR1* promoter.

(A) The high-methylated *ESR1* promoter, in contrast to the low-methylated intronic sequence, was only partially bound by PR in PR-rescued cells (T47D-Y+PR). ChIP assays were performed with a specific antibody against PR. Specific binding was assessed by qPCR amplification of the *ESR1* gene promoter and an enhancer-like intronic sequence in T47D, T47D-Y PR-deficient cells, and T47D-Y cells with re-expressed PR (T47D-Y+PR). Error bars represent the SEM of three independent experiments. **P* less or equal than 0.05, ***P* less or equal than 0.01, ****P* less or equal than 0.005, unpaired two-tailed Student's *t*-test.

(B) The demethylating agent 5-azaC increases PR binding at the *ESR1* promoter in PR-rescued cells (T47D-Y+PR). ChIP was performed as in Fig 5A using T47D-Y+PR cells treated for 112 hours with the demethylating agent 5-azaC (5 µM) or vehicle (Control). Error bars represent the SEM of three independent experiments. *P* =0.1, unpaired two-tailed Student's *t*-test.

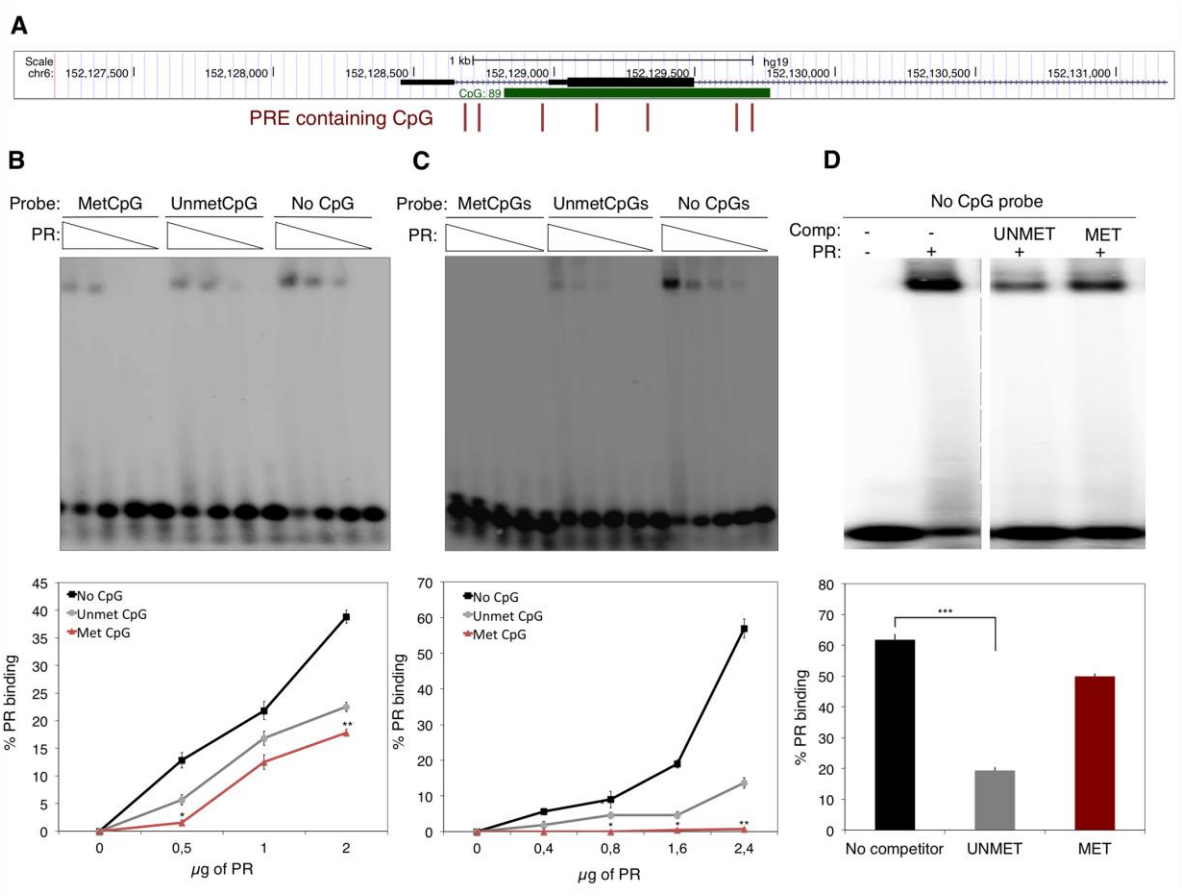

Fig 6. DNA methylation impedes PR binding to progesterone-responsive elements (PREs).

(A) Screen shot from the UCSC genome browser showing the CpG island (CpG 89) at the *ESR1* promoter and the positions of PREs containing one or two CpG dinucleotides.

(B, C) Electrophoretic-mobility shift assay using the indicated amount of purified human PR to capture the PRE with no CpG (ACAGTTTGT; No CpG), one methylated (MetCpG) or unmethylated CpG (UnmetCpG) (ACGGTTTGT) (B), two methylated (MetCpGs) or two unmethylated CpGs (UnmetCpGs) (ACGGTTCGT); (C). Blots are representative of three independent experiments. Quantification of the percentage of PR binding to different probes

is shown in the lower part of the gel images. **P* less or equal than 0.05, ***P* less or equal than 0.01, unpaired two-tailed Student's *t*-test.

(D) A double-stranded oligonucleotide probe with no CpG (ACAGTTTGT) was incubated with 2.4 μg of purified human PR and analyzed by PAGE either in the absence (–) or presence (+) of 100-fold excess of unlabelled oligonucleotides containing two unmethylated (UNMET) or two methylated (MET) CpGs (ACGGTTCGT). Error bars represent the SEM of three independent experiments. ****P* less or equal than 0.005, unpaired two-tailed Student's *t*-test. The dashed grey line indicates that a lane between the two samples was removed.

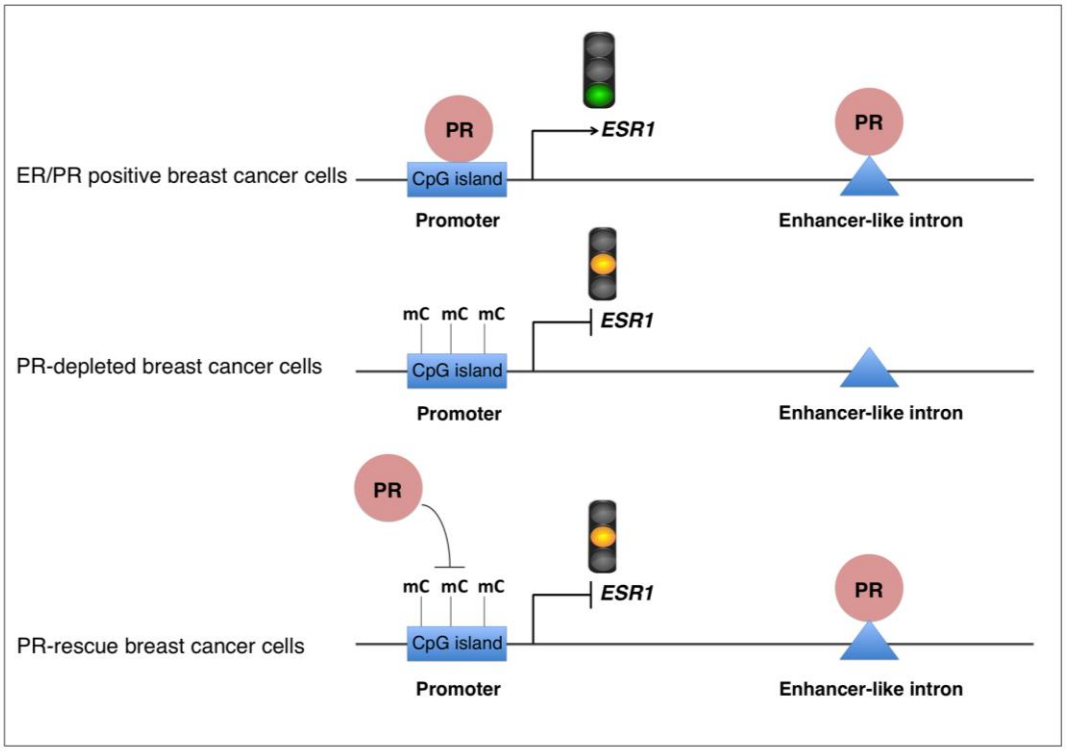

Fig 7. Model of regulation of the *ESR1* gene expression and DNA methylation by PR in hormone-deprived breast cancer cells.

In ER+/PR+ breast cancer cells, PR binds to low-methylated gene promoter as well as to an enhancer-like intronic sequence of *ESR1*. PR binding at gene promoter is required for maintaining *ESR1* transcription. In the absence of PR, DNA methylation (mC) increases at the *ESR1* promoter, and *ESR1* transcription is reduced. Re-expression of PR in PR-depleted cells leads to PR binding to the low-methylated enhancer-like intronic sequence, but the high level of DNA methylation (mC) at the *ESR1* promoter impedes PR binding to this genomic region. Consequently, re-expression of PR in PR-depleted cells is insufficient to restore *ESR1* expression.

Supplementary Figures

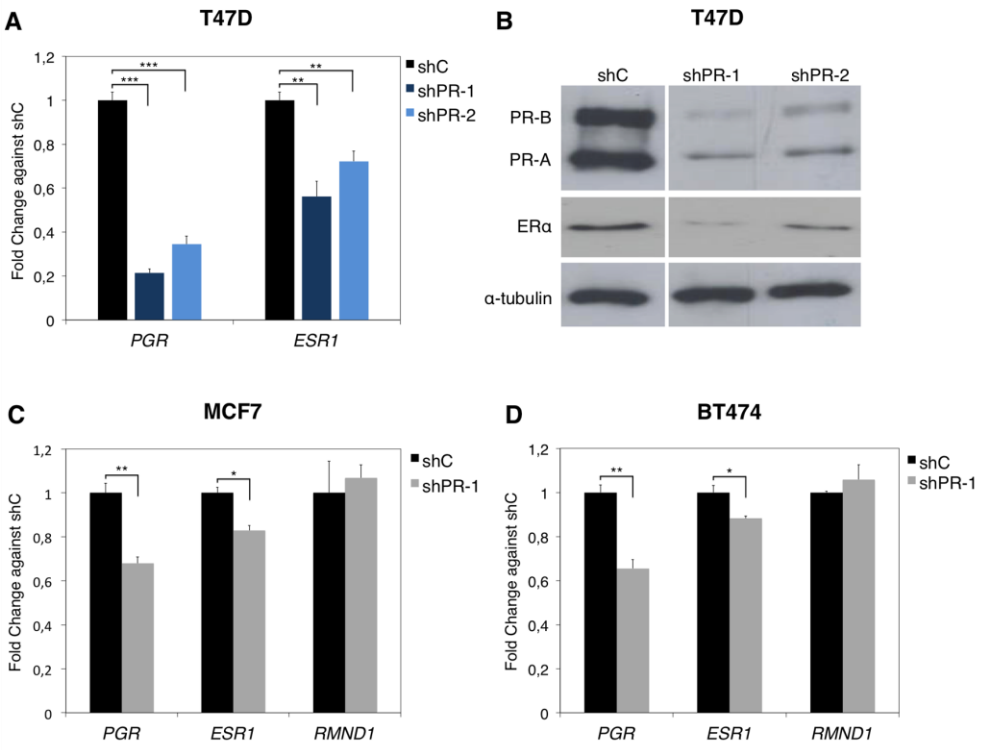

Fig S1. Depletion of PR reduces the *ESR1* expression in hormone-deprived breast cancer cells.

(A) Gene-specific mRNA expression measured by quantitative RT-PCR in T47D cells transduced with shRNA against PR (shPR-1, clone trcn0000010776; shPR-2, clone trcn0000003321) or scrambled shRNA (shC). The gene-specific expression levels were normalized to *GAPDH* expression and are represented as relative values in the T47D cells. *PGR*, PR-encoding gene; *ESR1*, ER-encoding gene. Error bars represent the SEM of three independent experiments. ***P* less or equal than 0.01, ****P* less or equal than 0.005, unpaired two-tailed Student's *t*-test

(B) PR and ERα protein levels measured by Western blot in T47D cells transduced with

shRNA against PR (shPR-1, clone trcn0000010776; shPR-2, clone trcn0000003321) or scramble shRNA (shC). α-tubulin protein was used as loading control. The vertical white line depicts a removed lane between the two samples. Blots are representative of three independent experiments

(C and D) Gene-specific mRNA expression measured by quantitative RT-PCR in MCF7 cells (C) and BT474 (D) transduced with shRNA against PR (shPR-1, clone trcn0000010776) or scrambled shRNA (shC). The gene-specific expression levels were normalized to *GAPDH* expression and are represented as relative values in the MCF7 (D) or BT474 (E) cells. Error bars represent the SEM of three independent experiments. **P* less or equal than 0.05, ***P* less or equal than 0.01, unpaired two-tailed Student's *t*-test

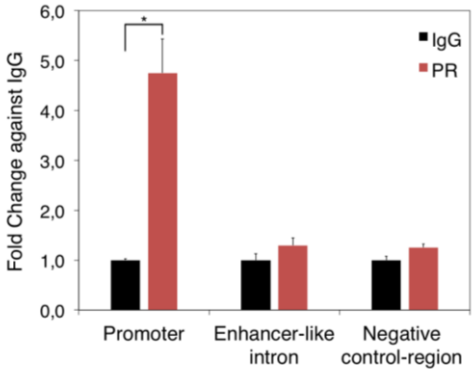

Fig S2. PR binds at *ESR1* gene promoter in MCF7 breast cancer cells.

ChIP assay was performed with a specific antibody against PR or total rabbit IgG. Specific binding was assessed by qPCR amplification of the *ESR1* gene promoter, an enhancer-like intronic sequence, and a genomic region localized at 3′-end of the enhancer-like intron (negative control region). Error bars represent the SEM of three independent experiments. **P* less or equal than 0.05, unpaired two-tailed Student's *t*-test.

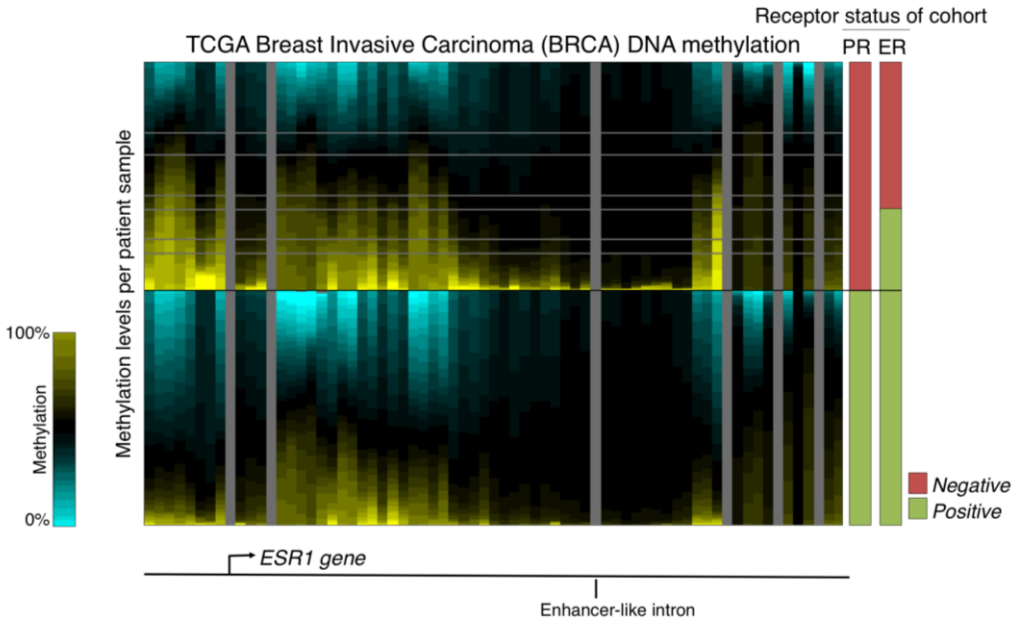

Fig S3. DNA methylation at the *ESR1* locus is higher in PR-negative than PR-positive breast invasive cancers.

The TCGA database was used to assess the percentage level of methylation across the *ESR1* gene using the breast invasive carcinoma (BRCA) dataset; methylation levels had been assayed previously using human methylation 450 arrays (n = 837). The *ESR1* percentage methylation levels across all patient samples were plotted. Methylation levels are presented for both patient data and are stratified based on PR and ER status (right).

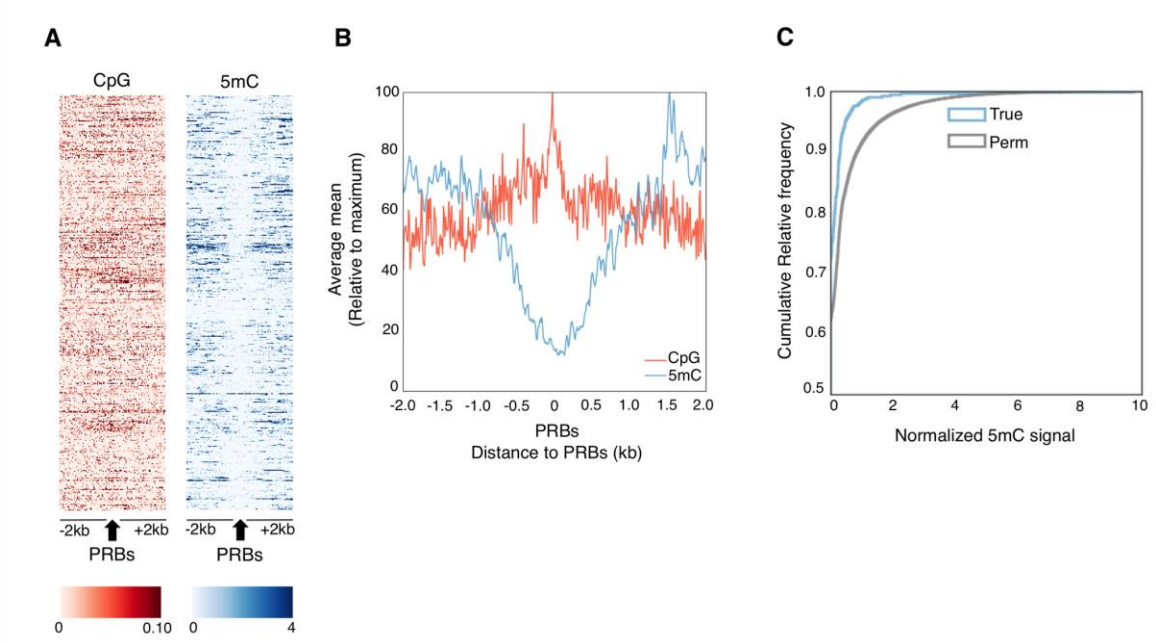

Fig S4. The progesterone-receptor binding sites are methylated at low levels in hormone-deprived breast cancer cells.

(A) Heatmaps of the normalized CpG content and DNA methylation (5mC) analyzed by MeDIP-seq around all PR binding sites (476 PRBs) in hormone-deprived T47D cells.

(B) Average signal intensity of the normalized CpG content and DNA methylation (5mC) signal centered around all PRBs (476) in hormone-deprived T47D cells. DNA methylation was significantly lower at PRB sites than their flanking region (-/+ 2000 bp). (Wilcoxon test: $P = 5.07 \times 10^{-16}$)

(C) Normalized DNA methylation (5mC) signal in PRB sites (True) or in random genomic regions of the same size as PRBs (Perm; data points were pooled from 1,000 permutations). DNA methylation was significantly lower at PRB sites than at random genomic regions (Mann-Whitney U test: $P = 3.85 \times 10^{-9}$

Table S1. Primer and oligonucleotide sequences used for RT-qPCR, ChIP-qPCR, MeDIP-qPCR and EMSA.

Primers RT-qPCR	**Sequence: 5′-3′**
ESR1 forward	CAGACGAGACCAATCATCAG
ESR1 reverse	TCTATTCCGAGTATGATCCTACCA
PR forward	GGCGAGAGGCAACTTCTTTC
PR reverse	CATCTGCCCACTGACGTGTT
RMND1 forward	ATCTTGAGACAGATTTCCCAG
RMND1 reverse	ACCTTCCAACCATGAAATCC
pS2 forward	TTGTGGTTTTCCTGGTGTCA
pS2 reverse	CCGAGCTCTGGGACTAATCA
GAPDH forward	TTGATTTTGGAGGGATCTCG
GAPDH reverse	GAGTCAACGGATTTGGTCGT
Primers ChIP-qPCR and MeDIP-qPCR	**Sequence: 5′-3′**
Promoter forward	CAGCAGCGACGACAAGTAAA
Promoter reverse	ACTGGTCTCCCGAGCTCATA
Enhancer-like intron forward	GGAAAGCCGGCAGTTACA
Enhancer-like intron reverse	GGAGGCATGAAAGCCCTAC
Negative control region forward	CATGGACTGGCAGAGCATAA
Negative control region reverse	CCCAACATATTGAAGGTGTGC
Oligonucleotides EMSA	**Sequence: 5′-3′**
No CpG probe forward	GTTGAGTCAGAACAGTTTGTTCTCTAGGCAC
No CpG probe reverse	GTGCCTAGAGAACAAACTGTTCTGACTCAAC
One CpG probe forward	GTTGAGTCAGAACGGTTTGTTCTCTAGGCAC
One CpG probe reverse Unmethylated	GTGCCTAGAGAACAAACCGTTCTGACTCAAC
One CpG probe reverse Methylated	GTGCCTAGAGAACAAACC*GTTCTGACTCAAC
Two CpGs probe forward	GTTGAGTCAGAACGGTTCGTTCTCTAGGCAC
Two CpGs probe reverse Unmethylated	GTGCCTAGAGAACGAACCGTTCTGACTCAAC
Two CpGs probe reverse Methylated	GTGCCTAGAGAAC*GAACC*GTTCTGACTCAAC

*Methylated cytosine