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

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Abstract

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. 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. 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

Author summary

The tumor-specific expression of estrogen and progesterone receptors (ER and PR, respectively) strongly affects the prognosis and responsiveness of breast cancers to endocrine therapy. The double-negative ER–/PR– breast cancers, indeed, have a worse clinical outcome than ER+/PR+ breast cancers and do not respond to endocrine therapy. Although much is known about *ER* gene (*ESR1*) regulation after hormonal stimulation, how it is regulated in the absence of hormones is not fully understood. We have discovered here that PR maintains *ESR1* gene expression in hormone-free breast cancer cells by regulating DNA methylation at *ESR1* promoter. In addition, we also found that DNA methylation impedes PR binding to *ESR1* promoter. These findings clarify the molecular mechanism

that regulates the *ESR1* gene expression in hormone-free breast cancer cells and suggest that the analysis of DNA methylation of *ESR1* promoter in breast cancers can help to design the appropriate targeted therapies for different types of breast cancer patients.

Introduction

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 [2]. 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+ [3]. 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 [3]. Approximately one-third of breast cancers lacks both ER and PR (ER–/PR–) and generally shows poor histological differentiation with higher growth rates [3]. These cancers rarely respond to hormone therapies and exhibit a poor clinical outcome compared to ER+/PR+ breast cancers [3]. Therefore, 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* gene expression in up

to one-third of human breast cancers [1]. 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 [4-6]. CpG islands are cytosine/guanosine-rich genomic sequences located in the regulatory regions of genes [7]. They are generally unmethylated in normal somatic cells, with the exception of imprinted genes [7-9]. In cancer cells, however, a gain of CpG island methylation is associated with transcriptional silencing, either directly or through a change in chromatin conformation [10,11]. Initially, it was reported that there was no correlation between loss of *ESR1* gene expression and methylation of the coding region of *ESR1* in breast cancer [12-14]. However, a clear correlation between the lack of *ESR1* expression and gene promoter methylation has been shown [4-6]. Moreover, it has been reported that breast cancer patients with poor prognosis tend to have higher DNA methylation levels at the *ESR1* promoter [15]. The analysis of *ESR1* promoter methylation status, therefore, can be critical for stratifying breast cancers.

Much is known on how ER and PR regulate gene expression upon hormonal stimulation. Upon hormone exposure, ER and PR exhibit enhanced binding to specific DNA sequences called hormone-responsive elements, which are generally located within target gene enhancers or promoters [16,17]. The DNA-bound receptors orchestrate the assembly of large cofactor-containing protein complexes that can either positively or negatively affect gene transcription [2]. 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 [18]. In contrast, how the hormones regulate gene

expression in the absence of hormones is not well understood. It has been reported that in the absence of progesterone, the expression of PR in ER+/PR-low MCF-7 breast cancer cells enhances the response of a subset of estradiol target genes and cell proliferation [19]. On the other hand, depletion of ER in breast cancer cells resulted in estrogen/tamoxifen resistant cells and induces epithelial to mesenchymal transition phenotype [20]. Thus, the study of molecular mechanisms that regulate the levels and the interactions of PR and ER in hormone-free conditions may help to understand how epithelial breast cancer cells maintains cell homeostasis and how they respond to external stimuli. In hormone-deprived MCF7 breast cancer cells, ERα directly regulates the expression of hundreds of genes by modulating the extent of trimethylation of lysine 27 on histone 3 (H3K27me3) at ERα binding sites [21]. Unliganded PR can negatively regulate a subset of hormone-inducible genes by recruiting a repressive complex containing HP1γ (heterochromatin protein 1γ), LSD1 (lysine-specific demethylase 1), HDAC1/2 (histone deacetylases), CoREST (corepressor for REST), KDM5B (H3K4-specific histone demethylase), and the RNA SRA (steroid receptor RNA activator) [22]. However, whether and how PR positively regulates gene expression in the absence of hormone stimulation remains elusive. We show here that in hormone-deprived T47D breast cancer cells, unliganded PR binds to the low-methylated *ESR1* promoter to maintain its basal expression and its low level of DNA methylation. Consistent with these data, we show that DNA methylation hinders PR binding to hormone-responsive elements.

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) [23], 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*) [24]. It is known that PR inhibits *ESR1* gene expression in T47D cells upon progestin exposure [25], but 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 cells) (Fig 1, A and B, *right panels*; S1 Fig A and B). In addition, the estradiol (E2)-mediated induction of *pS2* transcription in control cells was strongly reduced in PR-depleted cells (shPR cells) as well as in PR-deficient cells (T47Y) (Fig 1C), confirming a reduced ERα activity 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). Due to the low basal levels of PR in MCF-7 and BT474 compared to T47D (S1 Fig C), we only managed to get a moderate decrease of the PR levels in these cell lines. Nevertheless, this slight decrease of PR was accompanied by a concomitant decrease of

ESR1 expression compared to control cells (S1 Fig 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 [22]. 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) and another within the third intron (chromosome 6: 152,222,250–152,222,650) (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 [17,22] 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) [26-28].

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

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 *pS2* 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 [15]. 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), and PR-depleted cells (shPR) before and after PR rescue. Methylation of the *ESR1* promoter 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 4). 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 4).

Consistent with these data, analysis of the TGCA Breast Invasive carcinoma (BRCA) dataset shows a clear difference in *ESR1* gene methylation levels when the data is segregated based on the PR expression levels of the patients. PR negative breast carcinoma

patients are more commonly also negative for ER and present an increased level of methylation of the *ESR1* gene than PR-positive breast carcinomas. The increase of DNA methylation in PR-negative breast carcinomas is stronger at *ESR1* gene promoter than at gene body (S3 Fig).

DNA methylation impedes PR binding to hormone responsive elements

DNA methylation can directly affect the affinity of transcription factors towards their binding sites [29]. 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) for 5 days 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 [17] (Fig 6D).

Finally, we analyzed the methylation of 476 genomic regions bound by PR in the absence of hormones [22]. Despite having a higher CpG content than their flanking genomic regions, these PR binding sites had an overall lower level of methylation than their surrounding areas (S4 Fig), suggesting that not only the *ESR1* locus but also other PR binding sites require low levels of methylation for PR binding.

Discussion

The study of *ESR1* gene expression in breast cancer cells in the absence of hormones helps to clarify how epithelial breast cancer cells maintains cell homeostasis and how they respond to external stimuli, including estradiol and insulin-like growth factor 1 [19,20]. 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. 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). In line with this interpretation, demethylation of the *ESR1* promoter reactivates *ESR1* expression in ER-negative breast cancer cells [5].

Whether DNA methylation is the cause or the consequence of the altered gene expression is still unclear. Our findings that a loss of PR specifically affected the DNA methylation at the *ESR1* promoter but not at the *ESR1* intronic site that lacks a CpG island suggest that PR binding selectively prevents methylation around CpG islands and could simply be the consequence of a reduced *ESR1* expression upon PR loss.

In many cases, the transcriptional regulation of steroid target genes requires the action of

regulatory sequences located far away from the promoters [30-32]. A significant fraction of these distal sequences engage in physical interactions with promoters, suggesting that they act as enhancers [30]. 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 [26-28]. 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 hormone-deprived MCF7 breast cancer cells, ERα positively regulates gene expression, including that of the *PGR* gene (the PR-encoding gene), by modulating trimethylation of lysine 27 on histone 3 (H3K27me3) at ERα binding sites [21]. On the other hand, Mohammed *et al*. (2015) show 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 [33]. Unliganded PR-B induces robust expression of a subset of estradiol-responsive target genes, with a consequent increased cellular sensitivity to estradiol [19]. In contrast, in T47D breast cancer cells, progesterone-liganded PR can negatively regulate a subset of progesterone target genes, including *ESR1* [23,34].

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 [35]. 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 [33], suggesting that different crosstalk between PR and ER could exist, depending on breast cancers subtypes. Further studies are required to test this hypothesis.

In conclusion, our findings expand our understanding of the complex crosstalk between PR and ER, and 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.

Materials and 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 [22]. The T47D, T47D-Y (*23*), T47D-Y+PR [23] cells were routinely grown in medium (RPMI 1640 for T47D; DMEM for T47D-Y, T47D-Y+PR) supplemented with 10% FBS and standard antibiotics.

For the experiments, cells were grown 48 hours in RPMI medium without phenol red supplemented with 10% dextran-coated charcoal treated FBS (DCC/FBS) and synchronized in G0/G1 by 16 hours of serum starvation. 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 [36] and pCMVΔR8.91 [37], together with the pLKO.1-puro non-targeting vector (SHC001; Sigma-Aldrich) or pLKO.1-shRNA against progesterone-receptor (SHCLND-NM_000926, clones trcn0000010776 and trcn0000003321; Sigma-Aldrich) using $CaCl_2$ to permeabilize the cell membrane. The viral particles were collected 72 hours after the transfection and used to infect T47D cells. Cells were 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 chemiluminiscence on a LAS-3000 image analyzer (Fuji PhotoFilm).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [38], 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 5 µg 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*. [39]. The trimmed reads were aligned to the hg19 assembly version of the human genome [40] using BWA (version: 0.7.12-r1039) [41]. 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 [42]. BWA-MEM algorithm and SAMtools (version: 1.2, using htslib 1.2.1) [43] 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) [44]. 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.

Electrophoresis mobility-shift assay

Recombinant human PR (isoform B; PRB) was expressed in baculovirus and purified as previously described [45]. Radioactive double-stranded oligonucleotides containing the progesterone-responsive elements (PRE) were incubated with the indicate 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 PRB 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.

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 [46] using 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) [47]. 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) [48] and BEDtools (version v2.24.0) [49] 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 [50].

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Figure Legends

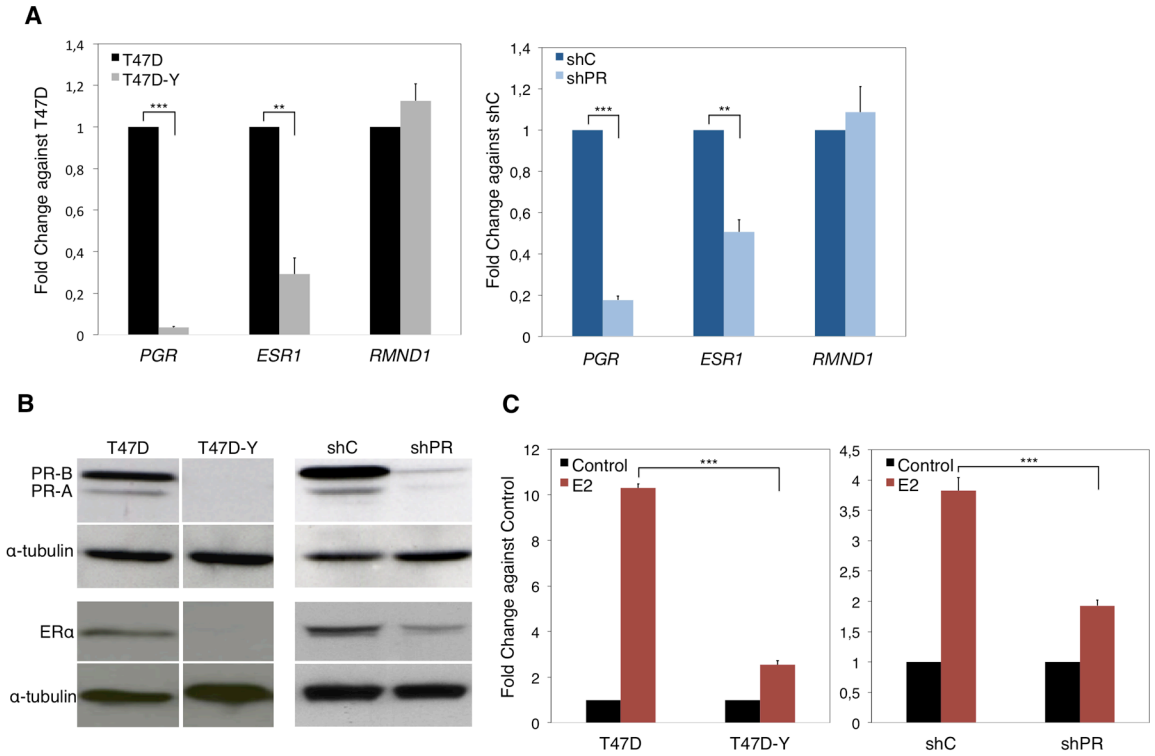

Fig 1. Loss of PR reduces the *ESR1* expression in hormone-deprived T47D breast cancer cells.

(A) Gene-specific mRNA expression measured by quantitative RT-PCR in T47D and PR-deficient cells (T47D-Y) (left panel) and in T47D cells transduced with shRNA against PR (shPR) 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 PR-independent control. *PGR*, PR gene; *ESR1*, ER 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 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) 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 reduces ERα activity. T47D cells or PR-deficient (T47D-Y) cells (left panel), or short hairpin control (shC) and PR-depleted (shPR) cells (right panel), were treated with estradiol (E2) or ethanol (control) for 6 hours, at which point *pS2* mRNA expression was measured by quantitative RT-PCR. The *pS2* 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 0.005, unpaired Student's t test.

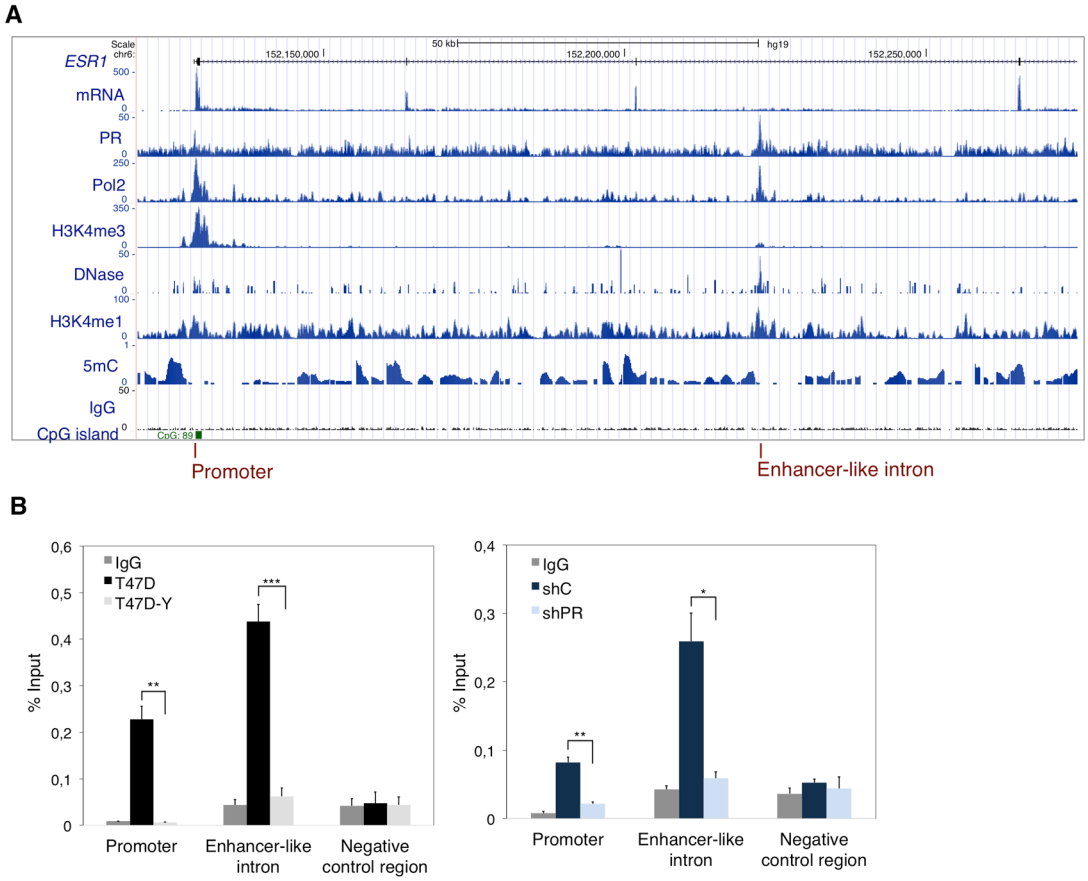

Fig 2. PR binds to the promoter and to an enhancer-like intron of the *ESR1* gene in hormone-deprived T47D breast cancer cells.

(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 an shPR, 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 Student's *t* test.

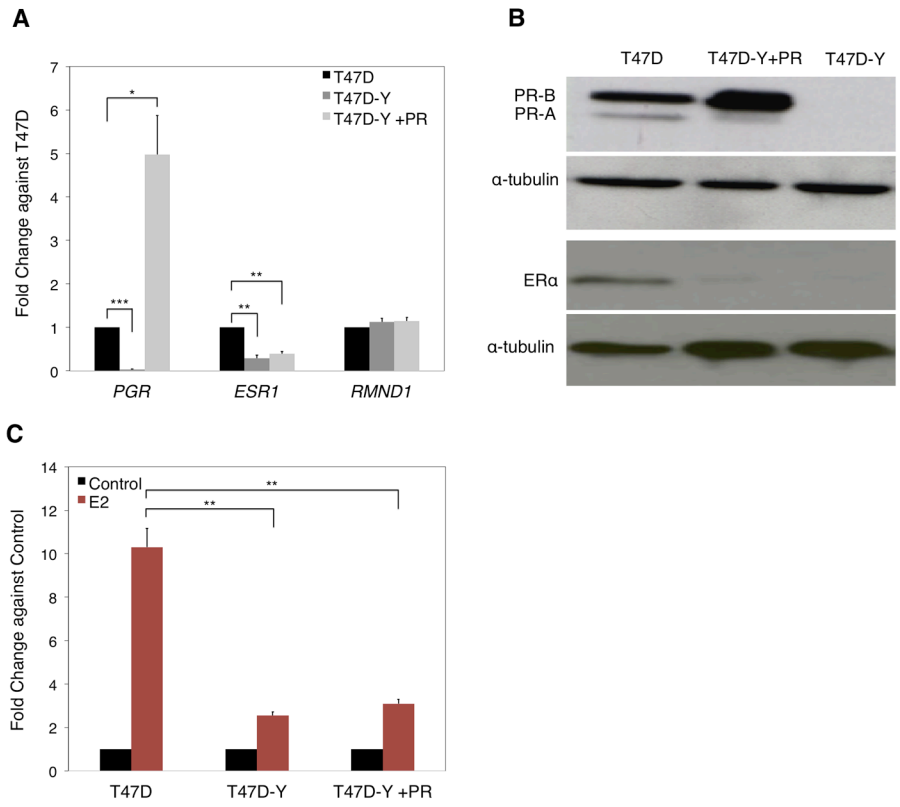

Fig 3. PR rescue of PR-deficient cells does not restore *ESR1* gene expression.

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 gene; *ESR1*, ER 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 Student's *t* test. Blots are representative of three independent experiments.

(C) PR rescue of PR-depleted cells does not restore the ERα activity. 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 pS2 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 Student's *t* test.

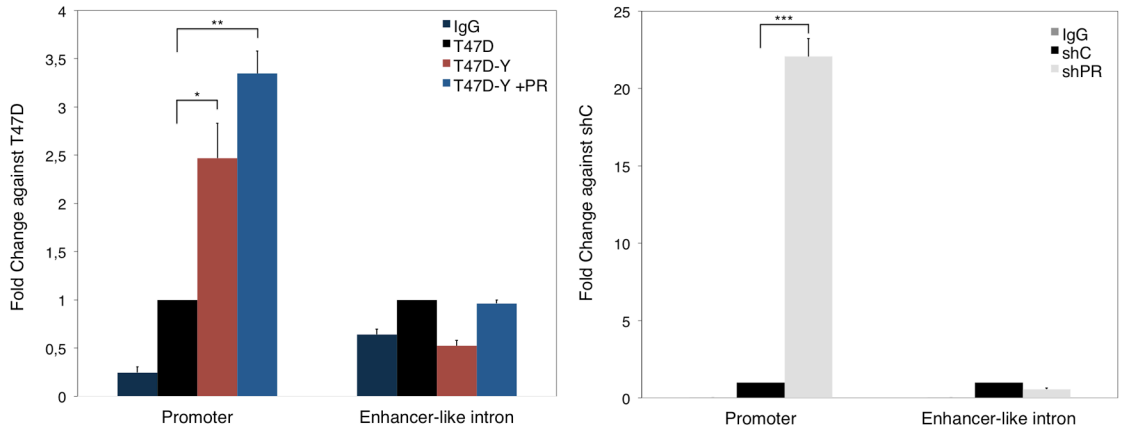

Fig 4. The loss of PR increases the DNA methylation level at the *ESR1* gene promoter. DNA methylation of the *ESR1* promoter and enhancer-like intron was assessed by MeDIP-qPCR in T47D cells, T47D-Y cells and T47D-Y cells with stable PR transfection (T47D-Y+PR) (left panel), or in T47D cells transduced with scrambled shRNA (shC) or shRNA against *PR* (shPR) (right panel). The results are represented as values relative to the T47D cells (left panel) or shC cells (right panel). IgG, negative control for immunoprecipitation. 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 Student's *t* test.

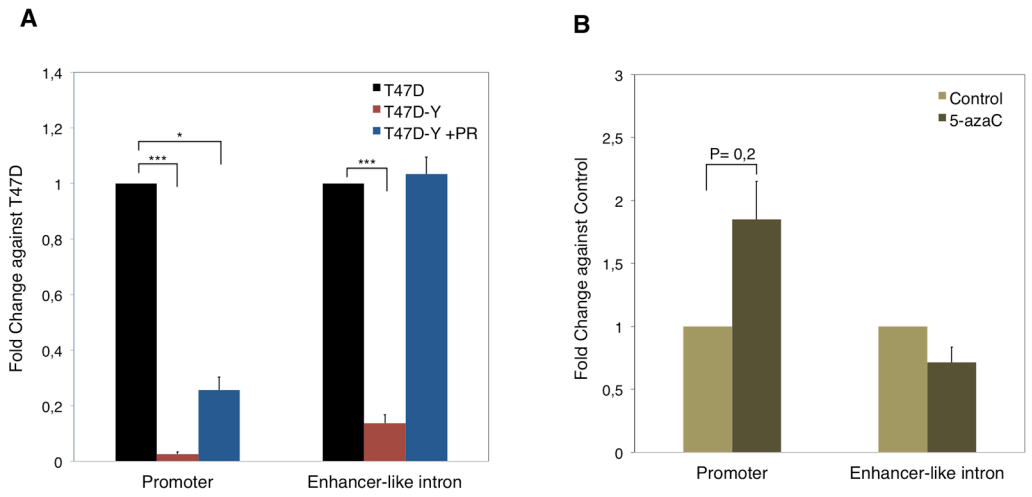

Fig 5. DNA methylation affects PR binding to the *ESR1* promoter.

(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 0.005, unpaired 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 5 days with the demethylating agent 5-azaC (5 µM) or vehicle (control). Error bars represent the SEM of three independent experiments. $P = 0.20$, unpaired Student's *t* test.

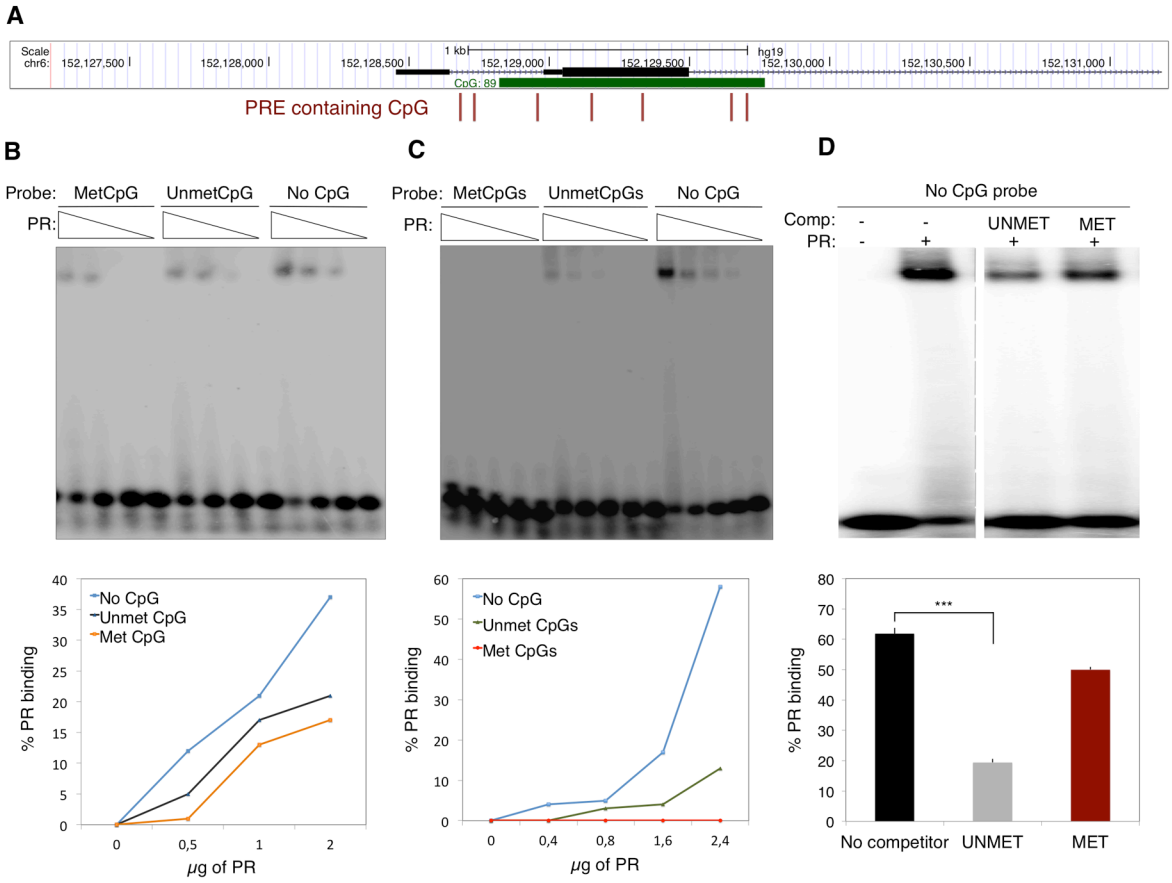

Fig 6. DNA methylation impedes PR binding to progesterone-responsive elements (PREs).

(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 (No CpG), one methylated CpG (MetCpG), one unmethylated CpG (UnmetCpG) (B), two methylated CpGs (MetCpGs) or two unmethylated CpGs (UnmetCpGs) (C). Quantification of the percentage of PR binding to different probes is shown in the lower part of the gel images. Blots are representative of three independent experiments.

(D) A double-stranded oligonucleotide probe with no CpGs 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. Error bars represent the SEM of three independent experiments. ***P less or equal than 0.005, unpaired Student's t test. The dashed grey line indicates that a lane between the two samples was removed.

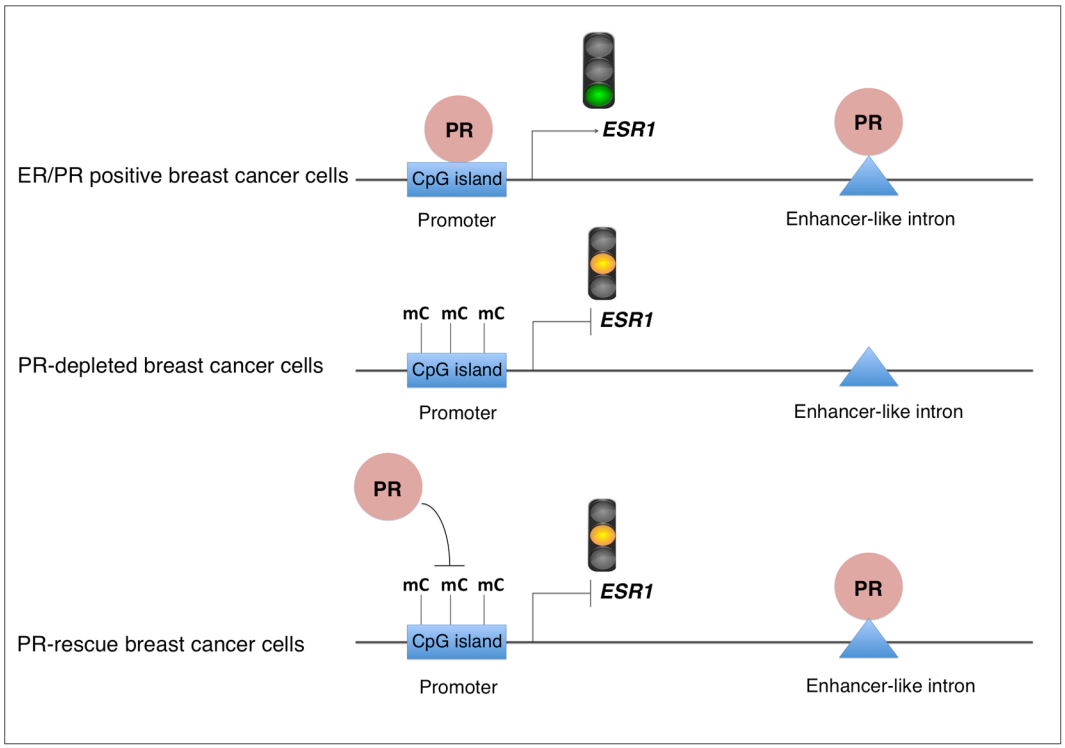

Fig 7. Model of regulation of the *ESR1* gene expression and DNA methylation by PR in hormone-deprived breast cancer cells.

In ER+/PR+ breast cancer cells, PR binds to low-methylated gene promoters as well as to an enhancer-like intronic sequence of *ESR1*. PR bound at the promoter binding is required for maintaining *ESR1* gene transcription. In the absence of PR, DNA methylation (mC) increases at the *ESR1* promoter, and *ESR1* gene 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.