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Progesterone induces progesterone receptor gene (*PGR*) expression via rapid activation of protein kinase pathways required for cooperative estrogen receptor alpha (ER) and progesterone receptor (PR) genomic action at ER/PR target genes

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Abstract

Progesterone Receptors (PRs) are critical effectors of estrogen receptor (ER) signaling required for mammary gland development and reproductive proficiency. In breast and reproductive tract malignancies, PR expression is a clinical prognostic marker of ER action. While estrogens primarily regulate PR expression, other factors likely contribute to a dynamic range of receptor expression across diverse tissues. In this study, we identified estrogen-independent but progestin (R5020)-dependent regulation of ER target genes including *PGR* in ER+/PR+ cancer cell lines. R5020 (10 nM–10 μ M range) induced dose-dependent PR mRNA and protein expression in the absence of estrogen but required both PR-B and ER α . Antagonists of either PR (RU486, onapristone) or ER α (ICI 182,780) attenuated R5020 induction of *TFF1*, *CTSD*, and *PGR*. Chromatin immunoprecipitation (ChIP) assays performed on ER+/PR+ cells demonstrated that both ER α and PR were recruited to the same ERE/Sp1 site-containing region of the *PGR* proximal promoter in response to high dose progestin (10 μ M). Recruitment of ER α and PR to chromatin and subsequent PR mRNA induction were dependent upon rapid activation of MAPK/ERK and AKT; inhibition of these kinase pathways via U0126 or LY294002 blocked these events. Overall, we have identified a novel mechanism of ER α activation initiated by rapid PR-dependent kinase pathway activation and associated with phosphorylation of ER α Ser118 for estrogen-independent but progestin-dependent ER/PR cross talk. These studies may provide insight into mechanisms of

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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persistent ER-target gene expression during periods of hormone (i.e. estrogen) ablation and suggest caution following prolonged treatment with aromatase or CYP17 inhibitors (i.e. contexts when progesterone levels may be abnormally elevated).

Keywords

progesterone; progesterone receptor; estrogen receptor; protein kinase; phosphorylation; transcription

1. Introduction

The coordinated actions of estrogen and progesterone drive complex genetic programs required for proliferation and differentiation during normal breast and uterine development and are essential for adult reproduction. Like other steroid hormone receptor (SR) family members, canonical progesterone receptor (PR) action involves progesterone binding to PR followed by receptor dimerization, nuclear localization or retention, and binding to specific progesterone response elements (PRE) in DNA located in the promoter or enhancer regions of PR target genes. In addition to direct binding to DNA, like other SRs, PR also cooperates with other DNA-bound transcription factors to alter gene transcription, including activator protein 1 (AP1) and specificity protein 1 (Sp1), to regulate gene promoters that lack complete canonical PRE binding motifs, such as *CDKN1A* (p21) and *CCND1* (cyclin D1) [1–4]. An essential feature of SR action includes their ability to rapidly activate numerous intracellular signaling pathways, including MAPKs [5–7]. These membrane-initiated events in turn direct phosphorylated SRs and their co-activators and other binding partners to highly specific genomic loci (reviewed in [8]). Indeed, context-dependent regulation of the cancer transcriptome is a hallmark of altered (i.e. phosphorylated) SR action in breast cancer models [8].

PR (the *PGR* gene) is considered to be a classical estrogen receptor (ER) target gene in normal mammary epithelial and breast cancer cells [9]. Two alternative transcriptional start sites (TSS) present in the *PGR* gene produce two protein isoforms, full-length PR-B (116 kDa) and N-terminal truncated PR-A (94 kDa). While ER- α (ER α) directly regulates *PGR* gene transcription in response to estrogen, there are no consensus estrogen response elements (EREs) for ER α binding near the TSS [9]. Early studies suggested that ER α is recruited to the *PGR* promoter region through association with AP1 or Sp1 and a nearby ERE motif half-site [10–12]. However, recent advances in genome-wide technologies and global analysis of ER α binding by chromatin immunoprecipitation (ChIP) combined with microarray (ChIP-on-chip) or sequencing (ChIP-seq) have led to the identification of several functional binding sites hundreds of kilobases upstream and downstream of the *PGR* TSS [13–16]. Chromatin conformation capture (C3) analyses demonstrated that these long-range binding sites associate with the TSS in an estrogen-dependent manner [17], suggesting that these distal sites create a chromatin loop to bind and tether ER-containing transcription complexes to the proximal promoter for estrogen-induced transcription of *PGR*. Indeed, although primarily developed in cancer cell models, this mechanism of regulation of target genes via ER- binding at distant sites has become a paradigm for all SR-driven genomic actions.

Concentrations of circulating progesterone and estrogen can vary dramatically between individuals and throughout the menstrual cycle in premenopausal adult individuals. Following ovulation, the physiological levels of progesterone within the microenvironment of the ovary and uterus are similar to those reported for estrogen and estimated to approach 100 μM [18, 19]. During pregnancy, estrogen and progesterone levels in blood rise to approximately 330 nM and 600 pM, respectively [20]. In addition to pregnancy, genetic conditions may confer heightened hormone levels. For example, human BRCA mutation carriers with either a BRCA1 or BRCA2 mutation [21] experience very high serum (circulating) levels of estrogen and in particular, progesterone. Specifically, estrogen levels were 33% higher while progesterone levels were 121% higher in women BRCA carriers relative to women with wild-type BRCA genes [21]. Studies in mice carrying a BRCA1 mutation in ovarian granulosa cells only confirmed these findings of greatly elevated circulating estrogen and progesterone levels, suggesting that a defect in ovarian steroid hormone biosynthesis contributes to breast and ovarian cancer risk in BRCA carriers [22–24]. Additionally, hormone-responsive tumors (more notably, breast and prostate cancer) readily generate significant local estrogen, androgen, progesterone and cortisol, from abundant circulating precursors, such as DHEA [25–28] and cholesterol [29–32]. In particular, hormone ablation strategies that include inhibitors of (local) steroid hormone biosynthesis (CYP17, aromatase) may abnormally elevate pathway precursors (i.e. upstream of the inhibited step) including cortisol or progesterone [33, 34]. While the levels of these steroid hormones are difficult to predict within the tumor microenvironment, they likely contribute to tumor progression during endocrine therapy [35, 36].

Notably, ER and PR are segregated in that these SRs reside in separate mammary epithelial cell compartments in the normal breast [37], but are most often co-expressed in malignant breast epithelial cells. Given that receptor co-expression is required for expression of target genes associated with metastatic behavior [37], it is essential to understand how these receptors may cooperate in cancer cells. Recent mechanistic studies by our group and others have revealed a direct molecular and genomic interaction between ER α and PR in breast cancer models [38–40]. PR enhanced estrogen-dependent ER α target gene regulation by increasing ER α phosphorylation and promoted recruitment of constitutively formed ER α , PR, and proline-, glutamine- and leucine-rich protein 1 (PELP1)-containing transcriptional complexes to target gene promoters associated with breast cancer cell proliferation and tamoxifen resistance [39]. Estrogen-induced global target gene expression required IGFR signaling and a scaffolding action mediated by the PR DNA-binding domain. Similarly, in response to progestins (but not estrogen alone), ER α cooperated with PR to induce the cancer relevant target genes, *CCND1* and *MYC*, and these events mediated breast cancer cell proliferation [38]. In more recent studies with combined hormone treatments, progesterone promoted the direct interaction between PR and ER α and dominantly redirected estrogen-induced ER α chromatin binding events and transcriptional activity while blocking the proliferative actions of estrogen alone [40]. The study of ER cross talk with PR is relevant to understanding how ER target genes may be regulated during conditions of hormone (i.e. estrogen) ablation, especially when key signaling pathways are also elevated in SR+ cancer cells. Herein, we hypothesized that hormone-induced rapid signaling events co-opt ER action at essential target genes, often in the absence of estrogen. To provide further insight

into this concept, we modeled ER α and PR cross-talk at the level of PR gene (*PGR*) expression, as a classical ER target gene. We conclude that when progestin is relatively abundant (i.e. 10 nM or higher), ER/PR transcriptional complexes regulate *PGR* expression by alternate enhancer(s) that require rapid signaling events.

2. Experimental

2.1. Cell culture

All cell lines were grown at 37°C under 5% CO₂ in water-jacketed incubators (Forma Scientific, Asheville, NC). MCF7 cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). ZR-75-1 cells were maintained in RPMI supplemented with 10% FBS and 1% P/S. MCF7 and ZR-75-1 cells were purchased from ATCC. Ishikawa cells were maintained in MEM supplemented with 1X GlutaMAX^{TM}, 1X MEM non-essential amino acids (NEAA) solution, 1% P/S, and were kindly provided by Dr. Jennifer K. Richer (University of Colorado). MCF7L cells were maintained in Modified IMEM supplemented with 5% FBS, 67.5 ng/mL insulin, 1% P/S, and were kindly provided by Dr. Deepali Sachdev (University of Minnesota). PEO4 cells were maintained in DMEM media supplemented with 10% FBS, 10 µg/mL insulin, 1:250 dilution NEAA, and 1% P/S, and were kindly provided by Dr. Scott Kaufmann (Mayo Clinic).

Cells were plated in dishes and allowed to adhere overnight. Afterwards, cells were washed with phosphate buffered saline (PBS) and serum starved in phenol red free Improved MEM (IMEM) for 24 hr. Afterwards, cells were washed with PBS prior to treatment with hormone or inhibitors in IMEM supplemented with 5% charcoal-stripped FBS (i.e. DCC) (Hyclone, #SH30068.03) at concentrations and times indicated.

2.2. Reagents

Cells were treated with the following reagents (when applicable and at indicated doses): R5020 (Perkin Elmer, #NLP004005MG), β -estradiol (E2) (Sigma, #E8875), RU486 (Sigma, #M8046), ICI 182,780 (Tocris, #1047), U0126 (U0) (EMD Millipore, 19-147), and LY294002 (EMD Millipore, #440202). Onapristone was kindly provided by Arno Therapeutics.

2.3. Real-Time Quantitative-PCR (RT-qPCR)

Total RNA was extracted from cell samples using TriPure Isolation Reagent (Roche, #11667165001) and isopropanol precipitation. RNA (1.0 µg) was reverse transcribed to cDNA according to manufacturer's instructions using the qScript cDNA SuperMix (Quanta Biosciences, #95048-100). qPCR was performed using Light Cycler® FastStart DNA Master SYBR Green I (Roche, #12239264001) on a Light Cycler® 480 II Real-Time PCR System (Roche) with cycling conditions as described previously [41]. Primer sequences to human gene targets are available upon request.

2.4. Immunoblotting

Western blots were performed as previously described [41]. Western blots were probed using the following primary antibodies: PR (H-190, #sc-7208) and ER alpha (ER α) (F-10, #sc-8002) were purchased from Santa Cruz Biotechnology; phospho-ER α (Ser118, #2511), phospho-p44/p42 MAPK (ERK1/2, #9101), and MAPK (#9102) were purchased from Cell Signaling Technologies; and Actin (Sigma-Aldrich, #A4700). HRP-conjugated goat anti-rabbit and goat anti-mouse (Bio-Rad, #170-6515 and #170-6516) secondary antibodies were used to detect their respective primary antibodies, and immunoreactive proteins were visualized on Kodak X-OMAT LS film (Carestream Health, #864-6770) following ECL detection with Super Signal® West Pico Maximum Sensitivity Substrate (Pierce, #34087).

2.5. Chromatin Immunoprecipitation (ChIP)

Cells were treated for 1 hr either with R5020, E2, or ethanol vehicle in phenol red-free Modified IMEM supplemented with 5% DCC. Cell samples were fixed, harvested, and lysed according to optimized manufacturer's instructions using the ChIP-IT™ Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif, #53008). Samples were homogenized using a Bioruptor sonicator (Diagenode, Inc.). ChIP reactions were incubated overnight on an end-to-end rotator using 100 μ L of isolated chromatin and either 2 μ g of PR antibody (Ab-8, Fisher Scientific Lab Vision, #MS298P), ER α antibody (Santa Cruz Biotechnology Inc., #sc-8002) or 0.4 μ g of normal mouse IgG (Santa Cruz Biotechnology, Inc., #sc-2025). Samples were washed, eluted, reverse cross-linked, and treated with Proteinase K according to manufacturer's instructions (Active Motif). DNA was analyzed by RT-qPCR as described above.

2.6. Statistical Analysis

All reported values represent the mean $\pm$ the standard deviation (SD). Statistical analyses were performed using a Student's two-tailed *t*-test where significance was determined with 95% confidence (*p 0.05, **p 0.01, ◆*p* 0.05, ◆◆*p* 0.01).

3. Results

Human breast, uterine, and ovarian cancer cell lines basally express a broad range of ER α and PR mRNA (Figure 1A) and protein levels (Figure 1A inset), with PR mRNA and protein typically inducible 12–48 hrs following estrogen addition to ER+ models. Notably, PR is an important effector of ER α action and a growing body of literature supports extensive cooperation between these SRs, both in the presence and absence of either or both ligands (i.e. estrogen and progesterone or synthetic progestin R5020) [38–40]. Similarly, rapid (i.e. extranuclear) signaling to c-Src and MAPK in response to either estrogen or progesterone seems to require the presence of both SRs (i.e. ER- α and PR-B) [5, 6, 42]. ER and PR clearly interact directly [5, 43] and there is significant overlap in ER and PR target gene selection [39, 44]. We speculated that ER/PR cross talk extends to numerous key ER target genes, including *PGR*. To determine if ER+/PR+ cancer cells are capable of inducing endogenous PR expression when stimulated with progestin only, ER+ cells were maintained in estrogen-free conditions and treated with increasing concentrations of R5020 (R50); PR mRNA (24 hr) and protein (48 hr) expression levels were evaluated. As a positive control for

inducible PR expression, cells were treated in parallel (24 hr for mRNA, 48 hr for protein) with the ER α agonist, β -estradiol (E2, 1 nM).

R50 treatment of Ishikawa cells significantly induced PR mRNA expression in a dose-dependent manner (10 nM to 10 μ M) compared to vehicle-treated controls (Figure 1B). The highest R50 concentration tested (10 μ M) induced PR mRNA levels that approached E2-induced levels. Changes in PR protein expression (inset) were modest or remained undetected at the same R5020 time point relative to estrogen (Figure 1B), perhaps because PRs undergo rapid ligand-dependent downregulation via the ubiquitin-proteasome [45]. Although there was considerable variation in the concentration range needed to induce PR mRNA expression, these results essentially repeated in numerous ER+/PR+ cell line models including PEO4 ovarian cancer cells [41] (Figure 1C; 10 μ M R5020), ZR-75-1 breast cancer cells (Figure 1D; 1 μ M R5020), and ER+ metastatic variants of MCF7 breast cancer cells known as MCF7L [46] cells (Figure 1E; 1 μ M R5020), as well as in parental MCF7 cells (Figure 1F; 10 μ M R5020). PR-B and PR-A protein expression was faintly increased only at high progestin concentration (10 μ M R5020), but generally correlated with observed mRNA levels (Figures 1B–F insets). Interestingly, R5020 treatment typically induced PR-B protein (as the predominant isoform; see ZR-75-1 and MCF-7L cell line Figure 1D–E insets) while estrogen reliably induced both PR-A and PR-B isoforms, with the exception of MCF-7 cells, in which both PR isoforms were clearly induced (~2.2-fold) following R5020 treatment (Figure 1F; inset). These data suggest that progestins weakly but reproducibly stimulate estrogen-independent PR expression at the transcription and translational levels in ER+ cancer cell models, especially at higher (100 nM) hormone concentrations. Similar results were observed with progesterone (100 nM – 10 μ M) (data not shown).

ER α and PR are members of the Type I nuclear receptor family that includes the glucocorticoid receptor (GR). While these receptors bind to their cognate ligands with high affinity, they are also capable of mediating signaling and biological effects when non-cognate ligands are present. High sequence homology within receptor ligand binding domains may permit promiscuous ligand binding. For example, the progestin medroxyprogesterone acetate (MPA) binds to GR with relatively high affinity [47–50]. A study by Courtin *et al* [50] reported that MPA activates GR in MCF7 cells to promote transactivation of GRE-luciferase reporters as well as endogenous GR target genes. As a specificity control for progestin-induced upregulation of PR expression, we evaluated the ability of GR to regulate PR expression by utilizing the GR agonist, Dexamethasone (Dex). While E2 treatment induced robust PR mRNA expression in MCF7 cells, treatment with high concentrations of Dex (1 and 10 μ M) failed to alter PR mRNA levels relative to vehicle controls (Supplementary Figure 1A). As expected, Dex treatment significantly regulated the classic GR (and PR) target gene, serum/glucocorticoid regulated kinase (SGK) [51, 52] in a dose-dependent manner (Supplementary Figure 1B). Regulation of SGK expression by E2 treatment, although significant, remained modest relative to Dex (Supplementary Figure 1B). PR levels were not altered following treatment of ER–/PR–/AR+/GR+ MDA-MB-231 cells with R50, E2, or Dex (data not shown). These data suggest that ER and/or PR but not GR are mediators of inducible PR expression in ER+/PR+ models.

Next, we evaluated if progestin-dependent upregulation of PR mRNA required PR transcriptional activity. To block PR activity, the competitive PR antagonist RU486 (RU) was utilized. As above in Figure 1F, R50 (10 μ M) treatment of ER+ MCF7 cells significantly induced PR mRNA expression, but this was blocked by co-treatment with RU (100 μ M) (Figure 2A). As RU is a known antagonist of both PR and GR [53] and high concentrations of RU may have off-targeting effects, we also utilized a clinically relevant, selective type II PR antagonist, Onapristone (Ona) [54]. Similar to our results with RU, Ona significantly blocked induction of PR mRNA in MCF7 cells co-treated with either R50 or E2 relative to treatment with R50-only or E2-only (Figure 2B). Similar results were observed for R50 or E2 induction of the PR target gene, SGK (Figure 2C). Together, these data suggest that regulation of PR expression by progestin is dependent on PR activity. Interestingly, the partial block of E2-induced PR expression by Ona also implicates PR in estrogen-dependent PR regulation.

Because expression of PR is driven by estrogen-dependent ER α , estrogen has been termed a *permissive hormone* for downstream PR actions [55]. We next examined whether progestin-induced expression of PR was dependent on upon ER α using the selective ER α antagonist, ICI 182,780 (ICI). Again, R50 (10 μ M) treatment of MCF7 cells for 24 hr significantly induced PR mRNA and protein expression (~3-fold) relative to vehicle controls (Figure 3A–B). Treatment with ICI (1 μ M) alone did not alter basal PR mRNA or protein expression, but ICI blocked R50-induced PR mRNA and protein expression (Figure 3A–B). Positive controls confirmed that E2 treatment significantly upregulated PR mRNA and protein levels, while ICI blocked E2-dependent upregulation of PR expression (Figure 3A–B). Similar results were observed for other known ER/PR target genes [39], *TFF1* and *CTSD* as measured by mRNA expression (Figure 3C–D). Our studies support similar studies where ICI inhibited progestin-dependent gene transcription [56, 57]. Taken together, these data implicate ER α as a required mediator of progestin-dependent regulation of PR expression in ER+/PR+ cells.

Our studies utilizing antagonists to either PR or ER α suggested transcriptional cross-talk between ER α and PR on numerous classical ER-target genes (i.e. *SGK*, *TFF1*, *CTSD*, and *PGR*). Both receptors appear to be required for progestin-regulated *PGR* transcription. As both PR and ER α are known to directly interact in transcriptional complexes [38–40], we performed chromatin immunoprecipitation (ChIP) assays to determine if PR and ER α bind to the same region within the endogenous *PGR* promoter in response to progestin. After 1 hr of R50 (10 μ M) treatment, ER α was significantly recruited (9-fold) to a region containing an ERE half site and two adjacent Sp1 (ERE/Sp1) sites within the *PGR* promoter previously identified by ChIP studies conducted in ER+/PR+ cell models [11, 58] (Figure 4A). Similar results were observed for PR recruitment to the same promoter region of the *PGR* gene (Figure 4B). Although ER α has been shown to recruit to multiple sites in the proximal and enhancer regions of the *PGR* gene with 1 hr E2 treatment [14, 17, 59], both ER α and PR were not significantly detected on the ERE/Sp1 site with 1 hr E2 treatment in our assays. (It was previously demonstrated that ER α was recruited to the same ERE/Sp1 site with 24 hr E2 treatment [11]; perhaps E2 treatment for 24 hr allows for induction of sufficient PR levels and recruitment of ER/PR complexes to this region.) Overall, these data are consistent with the occupancy of both PR and ER α within a selected region of the *PGR* gene and suggest

some degree of specificity for regulation of *PGR* by estrogen/ER α -only relative to both receptors when progestin is present.

Progesterone, via PR-dependent rapid signaling, transiently activates numerous protein kinases, including the MAPK pathway, in diverse cell types [4–6]. To confirm this in our models, MCF7 cells were plated in media devoid of growth factors (see Methods) and treated with a time course (0–60 min) of R5020 (10 μ M). R50 induced a subtle up-shift in PR gel mobility in MCF7 cells as early as 30 min and peaked at 60 min as measured by Western blotting (Figure 5A). Ligand-dependent PR gel mobility upshift has been shown to be due in large part to phosphorylation events within the PR N-terminus (reviewed in [60]). Consistent with other reports [4–6], MAPK/ERK was transiently activated by R50 treatment as measured by Thr202/Tyr204 phosphorylation with peak levels observed as early as 5 min; MAPK/ERK activity subsided and returned to near basal levels by 15 min (Figure 5A). Rapid activation of the PI3K/AKT pathway as measured by AKT Ser473 phosphorylation remained undetectable over a similar time course of R50 treatment in MCF7 cells (data not shown).

The rapid induction of the MAPK pathway by progesterone is known to activate a variety of transcription factors via phosphorylation [4, 6, 38]. The MAPK-dependent phosphorylation of ER α Ser118 is required for full activity of the ER α activation function 1 (AF1) [61]. Therefore, we examined the levels of Ser118 phosphorylation levels in MCF7 and MCF7L cells in response to R50 treatment. Densitometry analysis of Western blots revealed a modest increase (1–1.6-fold) in phosphorylated ERK with either 10 nM or 10 μ M R50 treatment in MCF7 cells at 5 min relative to vehicle-treated controls (Figure 5B). At 15 min, increased levels of phosphorylated ERK (1.3–2.3-fold) were detected at 10 nM, 1 μ M, and 10 μ M concentrations of R50 relative to vehicle controls, but only the 1 μ M and 10 μ M R50 doses induced appreciable ER α Ser118 phosphorylation, as detected by Western blotting (Figure 5B). EGF treatment (100 ng/mL for 15 min) was included as a positive control for ER α Ser118 and ERK activation (i.e. as measured by phosphorylation). Similar results were observed in MCF7L cells; phosphorylation levels of ER Ser118 (1.5–2.7-fold) and ERK (1.7–2.4-fold) increased in a R50 dose-dependent manner at 15 min (Figure 5C). Overall, rapid MAPK activation by R50 suggests the formation of progestin-dependent ER α -PR complexes with protein kinases that in turn coordinate the phosphorylation and transcriptional activation of ER α at novel ER/PR target genes [38, 39].

Lastly, we tested if MAPK/ERK activity was required for R50-dependent chromatin binding of ER/PR transcriptional complexes and induction of PR mRNA by utilizing the small molecule MEK inhibitor, U0126 (U0). ER α and PR recruitment to the same ERE/Sp1-containing promoter region of the *PGR* promoter was reduced in MCF7L cells when cotreated with U0 and R50 relative to R50-only (10 μ M) treatment (Figure 6A). Interestingly, we detected significant recruitment of ER α with U0-only treatment relative to vehicle controls. It is possible that U0126 at 10 μ M mimics estrogen or one or more of its metabolites has sufficient estrogenic properties to allow recruitment of ER α to regulatory elements in DNA as measured in highly sensitive ChIP assays; estrogenic activities have been reported to occur with MAPK inhibitors [62].

Consistent with our above results using ChIP assays (Figure 6A), R50 treatment of MCF7 cells for 24 hr again significantly induced PR mRNA expression relative to vehicle controls at 1 and 10 μ M (Figure 6B). Treatment with U0 (10 μ M) alone did not affect basal PR mRNA expression, although U0 significantly blocked R50-induced PR mRNA induction at the R50 10 μ M dose, but not at the lower dose of 1 μ M (Figure 6B). E2-dependent regulation of PR mRNA levels remained insensitive to U0 cotreatment relative to E2-only treatment (Figure 6B) consistent with the regulation of *PGR* expression by E2 relative to R50 via different binding sites (i.e. enhancer elements) in chromatin (discussed above). Notably, Daniel *et al* [39] reported that estrogen-dependent regulation of a novel class of ER α /PR-B/PELP1/IGFR1 target genes required signaling from the IGFR1-AKT pathway. We therefore included study of the small molecule inhibitor, LY294002 (LY) herein as a specificity control. LY treatment (10 μ M) alone did not affect basal PR mRNA expression, but significantly blocked R50-induced PR mRNA induction at both the 1 μ M and 10 μ M doses (Figure 6B). Similar to U0 treatment, E2-dependent regulation of PR mRNA levels remained insensitive to LY cotreatment relative to E2-only treatment (Figure 6B). In sum, our data suggest that membrane-initiated ER/PR/c-Src signaling complexes form in response to high dose progestins [43] and persist as nuclear transcriptional complexes capable of regulating kinase-driven ER/PR target genes that include *PGR*. Our studies reveal an alternate (i.e. ER/PR-driven) mechanism of *PGR* gene expression that is functionally distinct (i.e. regulated at a separate enhancer(s) that requires kinase pathway activation) from that induced by estrogen/ER alone (Figure 6C); this pathway may be most relevant to conditions of abnormally high progesterone levels (discussed above and see Discussion).

4. Discussion

PRs are key downstream effectors of estrogen signaling required for normal mammary gland (stem cell self-renewal) and uterine development. Studies over the last 20 years have identified factors that regulate PR levels, with primary focus on estrogen-dependent regulation. This study is consistent with a model in which ER+/PR+ cells induce endogenous PR expression in the presence of either estrogen or progestins, albeit at high concentrations (discussed below). Progestin-dependent rapid activation of MAPK/ERK is permissive for phosphorylation of ER α Ser118 and induction of PR expression in hormone-sensitive ER+/PR+ cancer cells. Overall, our study underscores the critical link between rapid PR signaling and non-classical modes of ER α activation in progestin-treated cancer cells.

While it is known that progestins (R5020) bind to PR within the nanomolar range, this study suggests that PR induction in response to high concentrations of progesterone may be most relevant to the hormone-rich environment of the reproductive tract (relative to cells in the breast). Physiologic conditions, such as pregnancy and ovulation, significantly raise circulating progesterone levels [63]. Additionally, in non-pregnant adults, local progesterone levels are much higher in tissues of the reproductive tract and ovaries relative to those in the breast [18, 19]. While BRCA1/BRCA2 mutations clearly disrupt DNA repair signaling pathways (subsequently increasing the risk of cancer development), the presence of these mutations in all tissues does not explain the organ specific penetrance for breast and ovarian cancers among carriers. Notably, recent studies have demonstrated that BRCA1 or BRCA2

mutation carriers have higher serum (circulating) levels of estrogen and, in particular, progesterone [21–24]. This finding fully recapitulated in mice carrying a BRCA1 mutation expressed only in ovarian granulosa (i.e. hormone producing) cells, suggesting that BRCA1/BRCA2 mutations confer heightened ovarian steroid hormone biosynthesis or decreased turnover [22–24]. The removal of both ovaries and fallopian tubes reduces the risk of ovarian and breast cancer, supporting a causal or contributing role for the dysregulation of ovarian hormone production in carriers.

Moreover, as ovarian secretion of estrogen and progesterone ceases in women at menopause, it is known that hormone-responsive tumors (more notably, breast and prostate cancer) generate these steroid hormones locally from circulating precursors. Many studies have focused on DHEA and its metabolism toward estrogen and androgen. Recently, two studies reported that breast cancer cells intratumorally metabolize the precursor 27-hydroxycholesterol via the upregulation of CYP27A1 to induce cell proliferation and metastasis in ER+ uterine and breast tumors [29, 30]. In addition, P450 side-chain cleavage enzyme (P450_{scc}) may produce progesterone *in situ* from circulating cholesterol while 5 α -reductase converts progesterone to cancer-promoting metabolites in breast tumors [31, 32]. Inhibition of aromatase or CYP17 as part of hormone ablation therapies may inadvertently raise the levels of upstream intermediates, including cortisol and progesterone. While the intratumor progesterone levels of breast tumors are not known, it is reasonable to infer that progesterone levels can be at least as high as those reported for estrogen (863–3050 fmol/g of tissue in premenopausal adults; 160–450 fmol/g of tissue in postmenopausal adults) [64]. Overall, we speculate that a dormant “sensing” system may be biologically relevant to high levels of progesterone produced either at the site of hormone release, experienced during pregnancy or upon BRCA loss, and/or synthesized locally within the tumor microenvironment. We postulate that lifetime elevated exposure to both hormones increases the activity and expression of their cognate receptors in breast and ovarian tissues, thereby driving chronically elevated expression of ER/PR target genes (i.e. namely *PGR* itself as a feed-forward loop) and augmenting proliferative signaling, in part by enabling novel transcriptional programs regulated by ER/PR complexes [38, 39].

While most studies have used human breast cell models to characterize estrogen-dependent regulation of PR, estrogens do not regulate PR levels in all tissues. In rat granulosa cells, estrogens fail to induce PR expression, while PR levels are regulated by cAMP and gonadotropins [65]. Similarly, FSH, LH and forskolin (all factors that also increase cAMP) induce PR in rat preovulatory granulosa cells [66, 67]. Even in human breast cancer cells, other factors, such as cAMP and growth factors, can regulate PR expression [68, 69]. In MCF7 cells, treatment with the growth factor IGF-1 increased PR protein levels but not transcript, whereas estrogen and activators of protein kinase A (cholera toxin plus 3-isobutyl-1-methylxanthine) stimulated intracellular cAMP and increased both PR mRNA and protein levels [69]. In addition, growth factors present in serum may be permissive for PR regulation, as PR induction by estrogen is greatest in cell culture media containing at least 5% DCC but not in media containing serum concentrations below 1% [68]. In general, exquisite cooperation between steroid hormone receptors and growth factors appears to be required for optimal regulation of PR expression.

While the factors listed above regulate overall PR protein expression, post-translational modifications including phosphorylation, SUMOylation, acetylation, and ubiquitination also dictate PR protein activity. T47D breast cancer cells treated with R50 rapidly activated c-Src and ERK2 within 5 min [5]; we have confirmed the rapid activation of ERK1/2 in MCF7 cells in the presence R50. Notably, progestin-induced MAPK activation was blocked following treatment with ICI, thus implicating ER in the progestin-dependent activation of MAPK [5]. We previously demonstrated a requirement for MAPK-dependent PR Ser345 phosphorylation in PR/SP1 binding [4], suggesting that SP1 may participate in ER/PR transcriptional complexes (Figures 4 and 6). While not a focus in this study, we suspect that the function of rapid membrane-proximal signaling events may be required to induce highly specific PR phosphorylation events on either MAPK-dependent Ser294 [45, 70] or Ser345 [4] sites to mediate interactions with SP1 and/or ER α that serve to facilitate subsequent non-canonical target gene transcription.

The rapid and extranuclear actions of PR signaling have significant nuclear consequences. Upregulation of PR target genes, including *CCND1*, *CDKN1A*, *DUSP1*, *EGFR*, and now *PGR*, are dependent on MAPK activity. Rapid signaling is proposed to regulate PR nuclear localization and targeting to selected PRE motifs. Notably, a subset of PR target genes including the aforementioned lack canonical PREs in their proximal promoters [1, 71, 72]. In this study, we observed progestin-bound ER/PR rapid signaling pathway regulation of *PGR*, an example of a non-classical PR target gene. We report the recruitment of PR and ER α to the *PGR* promoter and further implicate the importance of the MAPK signaling input in this process at sites distinct from those regulated by estrogen alone (Figure 6A–B). Notably, progestin-dependent (but not estrogen-dependent) up-regulation of PR mRNA levels was sensitive to both MAPK and AKT inhibition. Our data support a model in which progestin binding to cytosolic and membrane-proximal PR initiates a kinase-dependent interaction with ER α (Figure 6C). The rapid, direct phosphorylation of ER α in response to activation of PR-induced cytoplasmic protein kinase pathways suggests a positive feedback loop for hormone-responsive cells to sense and further bolster hormone-sensing pathways in order to rapidly adapt to hormonal flux over a broad range of concentrations. In the context of breast or ovarian cancer progression, as basal (i.e. constitutive) kinase pathway activation increases, the requirement for ligand may be greatly reduced, allowing for induction of ER/PR target genes in relatively low ligand (i.e. if sustained phosphorylation events are already in place). Regulation of ER/PR target genes including *PGR* by abnormally elevated signaling pathways (i.e. in diminishing hormone levels or during hormone ablation) is relevant to mechanisms of endocrine resistance and a topic for further study.

Supplementary Material

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Acknowledgments

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Fig. 1. Progestin upregulates PR expression in ER+/PR+ cells

(A) RT-qPCR analysis and (*Insert*) Western blot analysis of ER α and PR expression in a panel of breast cancer (MCF7L, MCF7, ZR-75-1), endometrial cancer (Ishikawa), and ovarian cancer (PEO4) cell lines. RT-qPCR analysis of PR mRNA expression in (B) Ishikawa, (C) PEO4, (D) ZR-75-1, (E) MCF7L, and (F) MCF7 cells treated with vehicle (ethanol), R5020 (R50), or β -estradiol (E2) for 24 hr ($n=3$, ** $p < 0.01$). *Insert*, Western blot analysis of PR protein expression in (B) Ishikawa, (C) PEO4, (D) ZR-75-1, (E) MCF7L, and (F) MCF7 cells treated with vehicle control, R50, or E2 for 48 hr. In Western blot experiments, actin was used as a loading control. Western blots (insets) are representative blots of at least two or more independent experiments. Densitometry was performed and results are indicated below each representative blot.

Fig. 2. Progestin upregulation of PR expression is dependent on PR

(A) RT-qPCR analysis of PR mRNA expression in MCF7 cells treated with vehicle, RU486 (RU), R50, or the combination of R50 and RU for 24 hr at concentrations indicated (n=3, $\blacklozenge\blacklozenge$ p 0.01, $\ast\ast$ p 0.01). RT-qPCR analysis of (B) PR and (C) SGK mRNA expression in MCF7 cells treated with vehicle, Onapristone (Ona), R50, the combination of Ona and R50, E2, or the combination of Ona and E2 for 24 hr at concentrations indicated (n=3, $\blacklozenge\blacklozenge$ p 0.01, $\ast$ p 0.05, $\ast\ast$ p 0.01).

Fig. 3. ER α is required for progestin upregulation of PR expression

(A) RT-qPCR analysis of PR mRNA expression in MCF7 cells treated with vehicle, ICI 182,780 (ICI), R50, combination of ICI and R50, E2, or the combination of ICI and E2 for 24 hr at concentrations indicated (n=3, $\blacklozenge$ p 0.01, $\ast\ast$ p 0.01). (B) A representative Western blot analysis (100 μ g/lane) of PR and ER α protein expression in MCF7 cells treated with vehicle, ICI (1 μ M), R50 (10 μ M), combination of ICI and R50, E2 (1 nM), or the combination of ICI and E2 for 48 hr (n=2). Actin was used as a loading control. Densitometry was performed and results are indicated below each blot. RT-qPCR analysis of (C) TFF1 and (D) CTSD mRNA expression in MCF7 cells treated with vehicle, ICI, R50, combination of ICI and R50, E2, or the combination of ICI and E2 for 24 hr at concentrations indicated (n=3, $\blacklozenge$ p 0.01, $\ast\ast$ p 0.01).

Fig. 4. Progesterone recruits ER α and PR to the PR promoter

RT-qPCR analysis of (A) ER α and (B) PR recruitment to PR. MCF7L cells were stimulated with vehicle, R50 (10 μ M) or E2 (1 nM) for 1 hr. Fixed lysates were chromatin immunoprecipitated with antibodies to ER α or PR and qRT-PCR analysis was performed on isolated DNA (n=3, *p 0.05).

Fig. 5. Progestins rapidly activate ERα Ser118 phosphorylation via the MAPK/ERK pathway
 (A) Representative Western blot analysis (100 μg/lane) of PR, phospho-ERK (p42/44), and total ERK in MCF7 cells stimulated with R50 (10 μM) at the indicated time points. MCF7 cells were stimulated with EGF (30 ng/mL) for 15 min as a positive control for phospho-ERK (n=2). Actin was used as a loading control. (B) Representative Western blot analysis (100 μg/lane) of PR, phospho-ERα (Ser118), total ERα, phospho-ERK, and total ERK in MCF7 cells at concentrations and time points indicated (n=2). (C) Representative Western blot analysis (50 μg/lane) of PR, phospho-ERα (Ser118), total ERα, phospho-ERK, and total ERK in MCF7L cells stimulated with R50 or E2 at indicated concentrations for 15 min (n=2). Densitometry was performed and results are indicated below each blot.

Fig. 6. Progestin upregulation of PR expression is dependent on activated ERK

(A) RT-qPCR analysis of ERα and PR recruitment to *PGR* promoter. MCF7L cells were pretreated with U0 (10 μM) for 30 min prior to treatment with either R50 (10 μM) or E2 (1 nM) for 1 hr. Fixed lysates were chromatin immunoprecipitated with antibodies to ERα or PR and qRT-PCR analysis was performed on isolated DNA (n=2, *p 0.05). (B) RT-qPCR analysis of PR mRNA expression in MCF7 cells treated with vehicle, U0126 (U0, 10 μM), LY294002 (LY, 10 μM), R50, combination of R50 and U0 or R50 and LY, E2, or the combination of E2 and U0 or E2 and LY for 24 hr at concentrations indicated (n=3, ♦♦p 0.01, *p 0.05). (C) Proposed model of the rapid activation of ERα. Ser118 phosphorylation by progestin and crosstalk between PR and ERα transcriptional complexes to upregulate PR expression.